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**The effect of a milk lipid fraction on bone properties of  
growing female rats and the growth and function of bone  
cells**

A thesis presented in partial fulfilment of the requirements for

the degree of

Master of Science

in

Human Nutrition

at Massey University, Manawatu, Palmerston North,

New Zealand

Zhongling Cao

2014

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## Abstract

**Objective:** To investigate the effects of a milk lipid fraction (MLF) on the accrual of bone mass in growing rats by evaluating the effects of MLF on growth and bone parameters such as bone mineral content (BMC), bone mineral density (BMD) and biomechanical properties in growing rats and the effects of MLF on the development and activity of bone cells, including osteoblasts and osteoclasts.

**Methods:** There were one hundred and eight 3-month old female Sprague-Dawley rats were randomly allocated into three groups: a control group (n=48), a low-dose MLF group (n=30) fed with 250 mg/rat/day of MLF, and a high-dose MLF group (n=30) fed with 500 mg/rat/day of MLF. Forty-five rats (n=15 for each group) were selected to terminate at month 3 for biomechanical testing while sixty-three continued into a second arm of the trial after ovariectomy. Body composition and bone parameters of animals (n=108) were measured *in vivo* by Dual Energy X-Ray Absorptiometry (DEXA) at baseline and week 12 of the study. Length and diaphyseal width and thickness of the left femur were measured. The three-point bending test was used to evaluate the biomechanical characteristics of the left femur of rat. The effect of MLF on proliferation, differentiation and mineralization of murine osteoblasts were investigated using the osteoblastic cell line MC3T3-E1. The cells were cultured with 0-1,000 µg/ml or 0-100 µg/ml MLF for 5, 9 and 24 days, respectively for proliferation, differentiation and mineralization. Cell proliferation was determined using the methyl-thiazolyl tetrazolium (MTT) assay. The differentiation of osteoblasts was detected using the alkaline phosphatase (ALP) activity assay. Mineralized nodules were examined using an Alizarin red histochemistry assay. The effect of MLF on RANKL-induced osteoclastogenesis was evaluated in the murine monocytic cell line RAW264.7. The cells were cultured with 0-100 µg/ml MLF for five days. Osteoclastogenesis was determined using the tartrate-resistant acid phosphatase (TRAP) staining assay and counting numbers of TRAP-positive multinucleated cells.

**Results:** Rats fed with the high-dose MLF diet had a significant increase in body lean mass compared with those fed with the control and low-dose MLF diets. The high-dose MLF group also had significant gains in BMC at the femur and in BMD at

the femur and lumbar spine compared with the control group. There were no significant differences in dimensional and biomechanical results among groups. The MLF significantly increased the proliferation of MC3T3-E1 at 0.1, 1.0 and 100 µg/ml. There was a dose-dependent, but not significant increase in the differentiation of osteoblasts cultured with MLF for 9 days. After 24-days of cell culture, the MLF at the low concentrations of 0.1 and 1.0 µg/ml led to non-significant increase in calcium deposition by the differentiated osteoblasts. MLF at 10 and 100 µg/ml significantly inhibited calcium nodule formation. RANKL-stimulated osteoclastogenesis was significantly increased in RAW 264.7 cells cultured with the MLF at concentrations up to 10 µg/ml.

**Conclusion:** These results indicated that oral administration of MLF to the growing rats improved bone accrual and has a favourable effect on achievement of peak bone mass. The MLF increased the proliferation of MC3T3-E1 pre-osteoblast cell line, but there was no effect on osteoblast differentiation and the higher concentration of MLF may have inhibited the function of mature osteoblast. Additionally, the MLF stimulated osteoclastogenesis from RAW264.7 cells. Further studies are required to investigate some of the contradictory findings presented in this report.

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## Abbreviations

1,25(OH) <sub>2</sub> D <sub>3</sub>	1,25-dihydroxyvitamin D <sub>3</sub>
AA	Arachidonic acid
ALA	α-linolenic acid
ALP	Alkaline phosphatase
BA	Bone area
BMC	Bone mineral content
BMD	Bone mineral density
BMI	Body mass index
BMP	Bone morphogenic protein
BMU	Basic multicellular unit
BSA	Bovine serum albumin
Cbfa1	Core-binding factor α1
CLA	Conjugated linoleic acid
COX	Cyclooxygenases
CV	Coefficients of variation
DEXA	Dual energy X-ray absorptiometry
DHA	Docosahexaenoic acid
DMSO	Dimethyl sulfoxide
DPA	Docosapentaenoic acid
DPA	Docosapentaenoic acid
Dpyd	Deoxy pyridinoline
EFA	Essential fatty acid
EPA	Eicosapentaenoic acid
ER	Estrogen receptor
ERK	Extracellular regulated kinase
FBS	Fetal bovine serum
FOS	Framingham Osteoporosis Study
GPR	G protein-coupled receptor
HDAC	Histone deacetylase
Hyp	Hydroxyproline
IGF-1	Insulin-like growth factor-1

Ihh	Indian hedgehog
IL	Interleukin
iNOS	inducible nitric oxide synthase
LA	Linoleic acid
LOX	Lipoxygenase
LPS	Lipopolysaccharide
LT	Leukotriene
MAPK	Mitogen-activated protein kinases
MBP	Milk basic protein
M-CSF	Macrophage-colony stimulating factor
MLF	Milk lipid fraction
MSC	Mesenchymal stem cell
MTT	Methyl-thiazolyl tetrazolium
MUFA	Monounsaturated fatty acid
MyD88	Myeloid differentiation factor 88
NF- $\kappa$ B	Nuclear factor - $\kappa$ B
NO	Nitric oxide
NTx	N-telopeptides
OPG	Osteoprotegerin
OSX	Osterix
PBS	Phosphate-buffered saline
PGE <sub>2</sub>	Prostaglandin E <sub>2</sub>
p-NPP	p-nitrophenyl phosphate
PPAR <sub>s</sub>	Peroxisome proliferator-activated receptors
PTH	Parathyroid hormone
Pyd	Pyridinoline
QC	Quality control
RANK	Receptor activator of nuclear factor - $\kappa$ B
RANKL	Receptor activator of nuclear factor - $\kappa$ B ligand
RCT	Randomised controlled trial
SFA	Saturated fatty acid
STAT3	Signal transducer and activator of transcription 3
T <sub>3</sub>	Triiodothyronine

T <sub>4</sub>	Thyroxine
TBS	Tris buffered saline
TFA	Trans-fatty acid
TGF-β	Transforming growth factor-β
TLR4	Toll-like receptor 4
TNF	Tumour necrosis factor
VA	Vaccenic acid
WHO	World Health Organization

## Introduction

Historically, osteoporosis was diagnosed as a disease involving back pain, osteopenia on medical images and vertebral fractures (Gooren, 2007). Over past few decades, however, osteoporosis has become well-recognized as a systemic skeletal disorder with characteristics of low bone mass and deterioration in microarchitecture of bone tissue, which consequently induces increased risk for skeletal fragility and fracture (Bonnick, 2006). Nowadays, age-related osteoporosis is a major focus (Gooren, 2007). It has been considered as a cause of morbidity and mortality in older human, affecting approximately 25% of aging women and 5-10% of men in the Western world, and the prevalence is increasing (Mundy, 2000; Marcus, 2013). The incidence of osteoporosis is higher in women than in men due to less bone size, bone mass, longer life span and hormone disturbance after menopause (Bonnick, 2006).

Bone mass dramatically increases in childhood and adolescence, reaching a peak in young adulthood. Bone mass then plateaus and remains constantly for a few years and finally declines (Heaney et al., 2000). Failure to reach optimal peak bone mass and bone strength during growth is one of the important causes of osteoporosis in later life (Raisz, 2005; Becker, 2008). Peak bone mass is primarily affected by genetic factors and is also dependent on dietary nutrient intake and physical activity (Mundy, 2000). Genetic factors are estimated to contribute approximately 70% of the variance to bone mass (Högström et al., 2007). Other factors such as nutritional intake also affect peak bone mass. Identifying and optimizing nutritional factors that affect peak bone mass are therefore critical to protecting against late-life bone fragility and fractures.

Dairy products are a major source of protein, lipids and several essential minerals such as calcium, phosphorus and magnesium in the human diet (Cashman, 2006; Cornish et al., 2008). Bovine milk is an important biological fluid that provides various nutrients during the period of rapid skeletal development and growth in human life. Previous observational studies have indicated that female subjects with low milk consumption during childhood and adolescence have lower bone mass and higher fracture risk in adulthood (Kalkwarf et al., 2003). Milk consumption in childhood and adolescence is related with greater bone mineral acquisition during

development of peak bone mass (Cadogan et al., 1997; Teegarden et al., 1999), and favoured bone mass in middle-aged and older females (Murphy et al., 1994). To date, limited studies have been performed to assess the effects of milk fats on peak bone mass and bone cells.

# Chapter 1. Literature Review

## Section 1. Bone

### 1.1 Bone function, structure and composition

#### 1.1.1 Skeletal function

The skeleton has many functions. It supports the body structurally; permits body movement and locomotion along with the muscles; protects vital internal organs and soft body parts from trauma (e.g. heart, lungs and central nervous system); produces blood cells for hematopoiesis in the bone marrow; stores minerals for homeostasis (e.g.  $\text{Ca}^{2+}$ ,  $\text{Mg}^{2+}$ ,  $\text{PO}_4^{3-}$ ) and acid-base balance; and stores cytokines and growth factors (Rodan, 2003; Mader & Galliart, 2005; Boskey, 2006; Clarke, 2008).

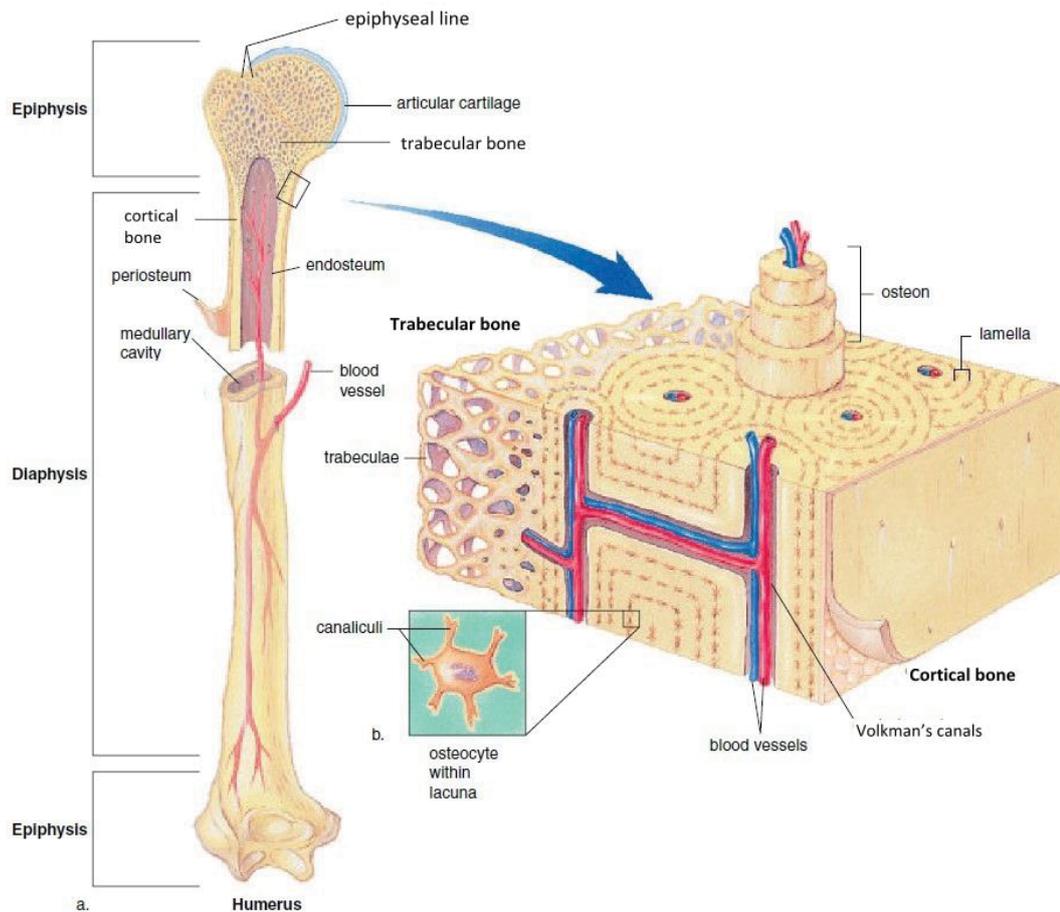
#### 1.1.2 Skeletal structure

Bones are generally classified into four categories; long bones (e.g. femurs, tibiae and humeri), short bones (e.g. carpal and tarsal bones), flat bones (e.g. skull, ribs and sternum) and irregular bones (e.g. vertebrae and sacrum). Long bones are produced by both intramembranous bone formation, in which bone develops between sheets of fibrous connective tissue, and endochondral bone formation, in which bone begins as hyaline cartilage that is replaced by bone tissue subsequently, while flat bones are only formed by membranous bone formation (Mader & Galliart, 2005; Clarke, 2008).

The structure of a long bone consists of the diaphysis, the cone-shaped metaphysis below the epiphyseal line (growth plate) and the rounded epiphysis above the epiphyseal line (Clarke, 2008). The anatomical features of the long bone are shown in Figure 1.1.

The diaphysis is mainly composed of cortical bone (also called compact bone), which is solid and dense and surrounds the medullary cavity. The metaphysis and the epiphysis consist primarily of trabecular bone (also called spongy or cancellous bone), which is meshwork-like with interconnecting trabecular rods and plates in the marrow space and surrounded by a thin layer of cortical bone. At the joints, the cortical bone is lined with a layer of articular cartilage outside. The periosteum is a connective fibrous tissue layer and is tightly adhered to the outer surface of cortical

bone by the thick collagenous Sharpey's fibres. The endosteum covers the inner cortical surface, trabecular bone and Volkman's blood vessel canals. Both the periosteum and the endosteum contain blood vessels and bone cells called osteoclasts and osteoblasts, while the periosteum also contains nerve fibres (Clarke, 2008).



**Figure 1.1** The anatomical features of a long bone, derived from Mader & Gallart (2005) with permission from McGraw-Hill Higher Education.

- a. The structure of lone bone.
- b. The detailed structure of trabecular and cortical bone together with an osteocyte within lacuna shown in the enlargement.

In the skeleton of an adult human, the overall ratio of cortical to trabecular bone by mass is 80:20. This ratio varies within different skeletal sites and for different bones, for example 25:75 in vertebrae and 50:50 in the femoral head (Clarke, 2008).

### 1.1.3 Composition of bone

The major composition of bone includes inorganic components such as minerals (50-70% of bone weight), water (5-10%), organic components (20-40%) such as

collagenous and non-collagenous proteins, vascular tissues, cells (2-5%) and lipids (<3%). The amounts of these substances vary according to age, sex, ethnicity, tissue site, state of development and dietary status (Sommerfeldt & Rubin, 2001; Clarke, 2008).

The inorganic minerals of bone exist in crystals and mostly contain hydroxyapatite [ $\text{Ca}_{10}(\text{PO}_4)_6(\text{OH})_2$ ]. The hydroxyapatite crystals of bone are nanometer sized and smaller than the apatite crystals found in tooth enamel or natural geologic apatites. The small, carbonate-substituted and poorly crystalline features of bone mineral crystals facilitate their dissolution in acidic environments during bone remodelling, enable the absorption and incorporation of foreign ions, and produce the flexible mineral-collagen composite fibrils which give bone its structural rigidity (Boskey, 2006; Clarke, 2008).

The organic matrix of bone is mainly composed of collagenous proteins (85-90% of total bone protein), predominantly type I collagen. Collagen fibres are formed in sheets in periosteal bone or circumferentially in osteonal bone. They provide elasticity and flexibility for bone and modulate the organization of bone matrix (Boskey, 2006). Non-collagenous proteins make up 10-15% of total protein content. They are a series of molecules that derive from either exogenous or local sources, and have been divided into several categories such as phosphoproteins (i.e. bone sialoprotein and osteonectin), proteoglycans (i.e. versican and syndecan), glycoproteins (i.e. alkaline phosphatase), serum proteins (i.e. albumin and fetuin),  $\gamma$ -carboxylated proteins (i.e. osteocalcin and matrix Gla protein) and other proteins (i.e. proteolipids) (Boskey, 2006; Clarke, 2008). These non-collagenous proteins have various functions in bone. For example, the exogenously derived serum albumin, fetuin ( $\alpha$ 2-HS-glycoprotein) and growth factors may regulate mineralization of the bone matrix, bone cell proliferation and bone cell activity. Alkaline phosphatase is the major glycosylated protein in bone and has a potential role in bone mineralization (Clarke, 2008). Lipids account for less than 3% of bone, while they are essential component of cell membranes, cell function and initial mineralization of bone (Boskey, 2006; Clarke, 2008).

Water in bone tissue is vital for the mechanical function of the mineral-collagen composite and for the nutrition and function of cells. It is present within the fibrils,

between fibrils, between fibres and between triple-helical molecules (Weiner & Wagner, 1998). There are hydrogen bonds between the collagen and water, which are needed for the stabilization of the collagen fibril. It is suggested that the dehydration of bone collagen through the replacement of water by mineral may occur during the process of bone mineralization (Boskey, 2006).

## **1.2 Bone cells**

### **1.2.1 The osteoblast**

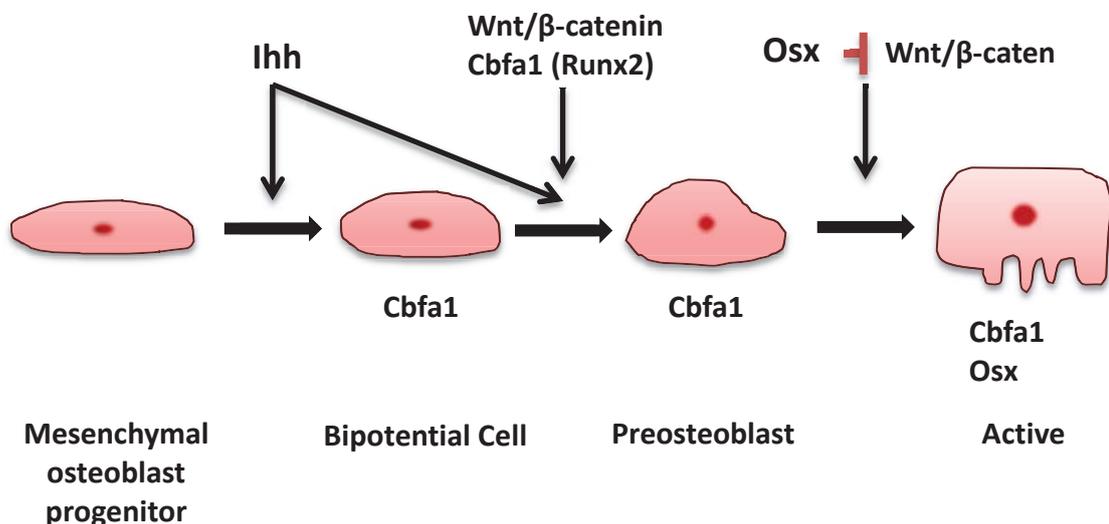
#### **1.2.1.1 Origin of the osteoblast**

Embryologically, osteoblasts derive from self-renewing, pluripotent mesenchymal stem cells, which can also generate osteocytes, myocytes, chondrocytes (cartilage cells), adipocytes, tendon cells and fibroblasts (Sommerfeldt & Rubin, 2001; Rodan, 2003). Morphologically, osteoblasts have a cuboidal shape and are located at the surface of the bone together with their precursors in a tight cell layer (Sommerfeldt & Rubin, 2001).

There are two different osteoblast differentiation pathways during embryonic development. During the process of endochondral ossification, mesenchymal cells primarily differentiate into chondrocytes and produce a cartilaginous template. Most of the skeletal bones, excluding the mandibles, clavicles and certain bones of the skull develop from the cartilage template, which is encircled by the bone collar. During the invasion of blood vessels, the chondrocytes begin apoptosis and are substituted by osteoblasts from the bone collar. During the development of the mandibles, clavicles and certain skull bones, however, osteoblasts differentiate directly from the mesenchymal progenitor cells without any cartilage template. This pathway is called intramembranous ossification (Ducy et al., 2000; Katagiri & Takahashi, 2002).

Mechanistically, the differentiation from the mesenchymal progenitor cells to the osteoblast lineage cells is determined by transcriptional factors and growth factors (Figure 1.2). For example, the expressions of transcriptional factors such as core-binding factor  $\alpha 1$  (Cbfa1), also known as Runx2, which is an osteoblast-specific transcript, and the downstream factor osterix (OSX) stimulate osteoblast differentiation and limit mesenchymal cell differentiation to osteoblasts, while a

homeobox protein (Hoxa2) inhibits this progress (Ducy et al., 2000; Rodan, 2003). Indian hedgehog (Ihh) as a growth factor has the greatest influence in the differentiation of the osteoblast *in vivo*. Bone morphogenic proteins (BMPs) can stimulate the expression of Cbfa1 *in vitro* (Ducy et al., 2000). Additionally, the Wnt/ $\beta$ -catenin pathway plays multiple roles in osteoblastogenesis. It promotes the differentiation of osteoblast lineage during early development and is involved in proliferation, survival and lifespan of mature osteoblasts. Binding of Wnt to the transmembrane receptor of Frizzled and the co-receptors of low-density lipoprotein receptor related protein 5/6 activates this signalling pathway, which allows increased levels of  $\beta$ -catenin and promoted target gene transcription by Lymphoid enhancer factor/T cell factor (Caetano-Lopes et al., 2007).



**Figure 1.2** The proposed progress of osteoblast differentiation and proliferation regulated by Wnt/ $\beta$ -catenin, Cbfa1 (Runx2) and Osx signalling.

Ihh can inhibit the endochondral ossification. Bipotential cells express Cbfa1 and potentially differentiate into either chondrocytes or osteoblasts. Cbfa1 and Wnt/ $\beta$ -catenin pathway play essential roles in differentiation from bipotential cells to preosteoblasts. Then Osx stimulates the preosteoblasts to differentiate into mature osteoblast. Osx also can inhibit the activity of the Wnt/ $\beta$ -catenin pathway and consequently inhibit osteoblast proliferation. Cbfa1: core-binding factor  $\alpha$ 1; Osx: Osterix; Ihh: Indian hedgehog.

### 1.2.1.2 Function of the Osteoblast

Functionally, mature osteoblasts lay down the new bone matrix, type I collagen, on the bone-forming surfaces and modulate its mineralization. In addition, osteoblasts synthesize and secrete several non-collagenous proteins. For example, osteoblasts synthesize osteocalcin that is only expressed after complete differentiation of

osteoblasts and acts as a biochemical marker of bone turnover (Clarke, 2008). Except for two osteoblast-specific transcripts, which can encode Cbfa1 and osteocalcin, all genes identified in fibroblasts are also expressed in osteoblasts. Therefore the osteoblast is regarded as a sophisticated fibroblast (Ducy et al., 2000).

### **1.2.1.3 Modulation of osteoblast activity**

The lifespan of the osteoblast varies from 8 weeks in humans to 3 days in young rabbits (Sommerfeldt & Rubin, 2001). The function, proliferation and apoptosis of osteoblasts are strongly affected by hormones, including growth hormone, steroid hormones (e.g. glucocorticoids) and sex hormones (estrogen and androgen), and vitamin D<sub>3</sub>. All factors have separate regulatory effects in the specific stages of osteogenesis. Growth hormone mainly stimulates the proliferation of the osteoblasts and their apoptosis is primarily limited by vitamin D<sub>3</sub>. Thus, the combination of growth hormone and vitamin D<sub>3</sub> increases the number of osteoblasts (Morales et al., 2004). The metabolites of vitamin D<sub>3</sub>, including 25(OH)<sub>2</sub>D<sub>3</sub> and 1,25(OH)<sub>2</sub>D<sub>3</sub>, have anti-proliferative effects, which reduce the replication of osteoblastic lineage cells, promote the differentiation of cells and positively regulate the mineralization of extracellular matrix (Atkins et al., 2007). Glucocorticoids can inhibit the differentiation, proliferation and function of osteoblasts, as well as lead to the apoptosis of mature osteoblasts. These effects suppress bone formation (Canalis et al., 2007). Androgens enhance apoptosis of both proliferating and mature osteoblasts (Wiren et al., 2006). However, estrogens efficiently inhibit the activation of caspase-3/7, a marker for apoptosis and decrease the expression of several important apoptotic genes in osteoblasts (Bradford et al., 2010).

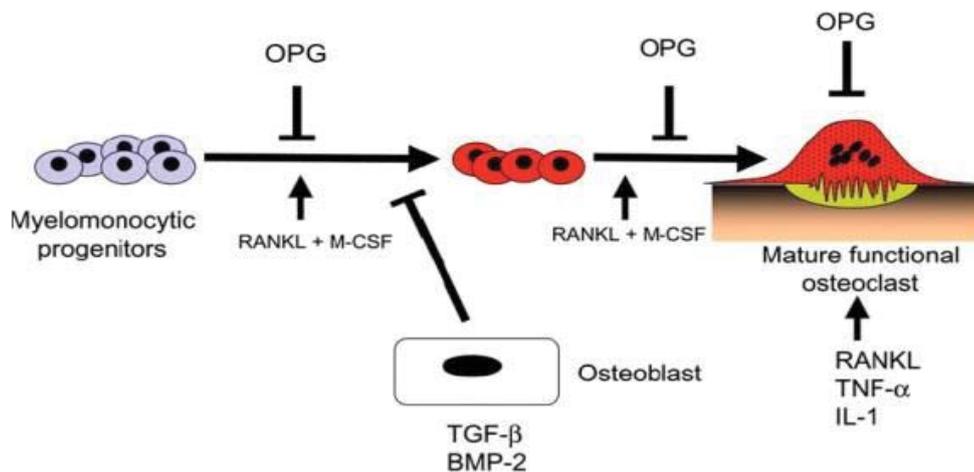
## **1.2.2 The osteoclast**

### **1.2.2.1 Origin of the Osteoclast**

Osteoclasts develop from hematopoietic stem cells, which also generate macrophages. Therefore, in some respects, osteoclasts act as the tissue macrophages of bone (Teitelbaum, 2000; Matsuo & Irie, 2008). Osteoclasts are multinucleated (usually 4-10 nuclei in human osteoclast), highly migratory and polarized cells, containing lysosomal enzymes (Sommerfeldt & Rubin, 2001). They lie in a small cavity named Howship's lacunae.

The osteoclastic precursors firstly differentiate into mononuclear osteoclasts, which contain all osteoclastic proteins and have the abilities of resorption, at least *in vitro*. On the surface of bone, mononuclear osteoclasts fuse to develop into multinucleated osteoclasts, which have large cell membranes and are able to resorb bone most efficiently (Rodan, 2003).

The progression of osteoclast differentiation is regulated by cytokines (e.g. nuclear factor  $\kappa$ B ligand, macrophage-colony stimulating factor, transforming growth factor- $\beta$  and interleukins), transcriptional factors (e.g. nuclear factor  $\kappa$ B), hormones (e.g. parathyroid hormone and 1,25-dihydroxyvitamin D<sub>3</sub>) and intra-extracellular signalling factors (e.g. integrins) (Figure 1.3).



**Figure 1.3** The proposed progress of osteoclastogenesis and the regulatory factors for osteoclast differentiation and activation, adapted from Quinn (2005) with permission from Elsevier.

RANKL and M-CSF are essential for osteoclast differentiation and maturation. OPG can neutralise RANKL and inhibit osteoclastogenesis and activation of mature cells. Shown centrally is the progression of osteoclast differentiation and maturation. Shown above are the direct inhibitors for this progress, while below are the indirect inhibitors. RANKL: receptor activator for nuclear factor- $\kappa$ B ligand; M-CSF: macrophage colony-stimulating factor; OPG: osteoprotegerin; IL-1: interleukin-1; TGF- $\beta$ : transforming factor- $\beta$ ; BMP-2: bone morphogenic protein-2; TNF- $\alpha$ : tumor necrosis factor- $\alpha$ .

Receptor activator of nuclear factor  $\kappa$ B (NF- $\kappa$ B) (RANK) ligand (RANKL), also known as TRANCE, is of the tumour necrosis factor (TNF) superfamily and can regulate several biological processes. In the bone, osteoblasts express RANKL that

binds to its receptor on osteoclasts, RANK, together with the macrophage-colony stimulating factor (M-CSF), on osteoclastic precursors, induces differentiation, maturation and activation of osteoclasts (Caetano-Lopes et al., 2007).

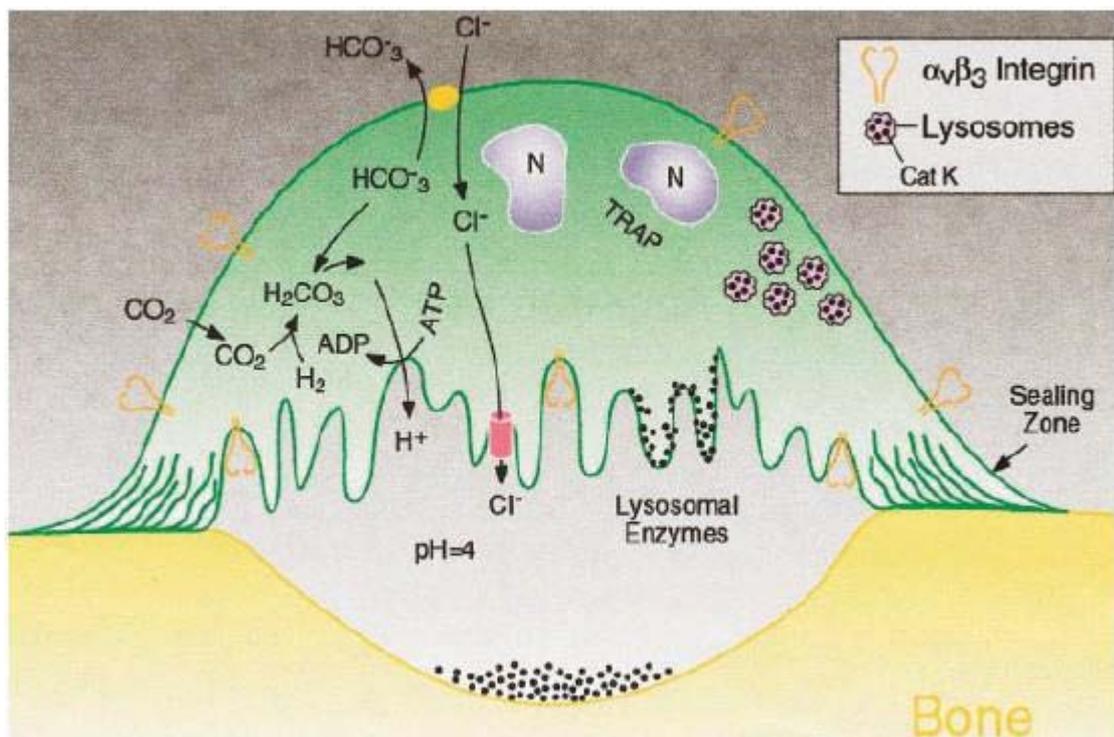
Osteoprotegerin (OPG) is a soluble protein and a member of the TNF receptors secreted by the osteoblast. It mainly functions as a decoy receptor, which competes with RANK for RANKL and thus inhibits the signalling pathway between osteoclastic precursors and osteoblasts to block osteoclast formation and survival of mature osteoclasts (Teitelbaum, 2000; Caetano-Lopes et al., 2007).

RANKL and OPG are both secreted by the osteoblast and have antagonistic effects on osteoclastogenesis and the consequent bone mass. Several factors can regulate osteoclastogenesis by controlling the ratio of RANKL/OPG. For example, transforming growth factor- $\beta$  (TGF- $\beta$ ) and BMP-2, TNF- $\alpha$ , TNF- $\beta$ , interleukin-1 $\alpha$  (IL-1 $\alpha$ ), IL-1 $\beta$  and 1,25-dihydroxyvitamin D<sub>3</sub> (1,25(OH)<sub>2</sub>D<sub>3</sub>) can increase the levels of OPG, while parathyroid hormone (PTH), insulin-like growth factor-1 (IGF-1), prostaglandin E<sub>2</sub> (PGE<sub>2</sub>) and glucocorticoids can decrease them. Additionally, the expression of RANKL is stimulated by PTH, PGE<sub>2</sub>, 1,25(OH)<sub>2</sub>D<sub>3</sub> and inflammatory cytokines, whereas it is attenuated by TGF- $\beta$  and estrogen (Caetano-Lopes et al., 2007). The T cells also generate RANKL in bone and their activation *in vitro* and *in vivo* results in increased osteoclastogenesis and bone resorption (Boyle et al., 2003; Gillespie, 2007).

#### **1.2.2.2 Function of the Osteoclast**

Osteoclasts have the ability to completely resorb mineralized bone at the Howship's lacunae (Sommerfeldt & Rubin, 2001). After activation and polarization mediated by  $\alpha_v\beta_3$  integrin, the multinucleated osteoclast firmly attaches to the bone surface forming a sealed zone resulting in a closed compartment where bone resorption occurs (Figure 1.4). The membrane of the osteoclast facing the compartment forms a ruffled border by the insertion of lysosome-type vesicles. Then the osteoclast releases H<sup>+</sup>, which are produced by the action of carbonic anhydrase ( $\text{H}_2\text{O} + \text{CO}_2 \rightarrow \text{HCO}_3^- + \text{H}^+$ ), and Cl<sup>-</sup> into the resorption lacuna with the aid of ATPase through the ruffled border. The pH value inside the compartment is maintained at approximately 4.0 and the mineralized bone matrix is acidified and dissolved into Ca<sup>2+</sup>, H<sub>2</sub>CO<sub>3</sub>, H<sub>3</sub>PO<sub>4</sub>, water and other substances. At the same time, lysosomal enzymes are

secreted into the compartment to digest proteins in bone matrix. Cathepsin K, a collagenase, is the most abundant enzyme in the resorption space and capable of digesting collagen, while metalloproteinase-9 digests both collagenous and non-collagenous proteins in the matrix. All products of resorption are transported by transcytosis through the basolateral membrane to the outside of osteoclast. During the process of bone resorption, osteoclasts move along the bone surface from site to site (Rodan, 2003).



**Figure 1.4** Bone resorption by osteoclasts, derived from Rodan (2003) with permission from John Wiley and Sons.

Osteoclast is firmly attached to bone surface by sealing zone forming a closed compartment. Hydrogen ions ( $H^+$ ) are generated by carbonic anhydrase and released into the resorption space through ruffled border by proton pumps of vacuolar ATPase together with chloride against the high concentration gradient. The pH in resorption compartment is maintained at 4.0 that help lysosomal Cat K to digest proteins of bone matrix. The black dots represent bisphosphonates that can inhibit the activity of osteoclasts and lead them to apoptosis. Cat K: cathepsin K; N: nuclei; TRAP: tartrate-resistant acid phosphatase.

### 1.2.2.3 Modulation of osteoclast activity

The activated osteoclasts cannot proliferate and they become apoptotic after an average of 15-20 days (Sommerfeldt & Rubin, 2001; Rodan, 2003). Each activated osteoclast can resorb  $2 \times 10^5 \mu m^3$  of bone per day, which is the amount of bone produced by 7-10 generations of osteoblasts (Sommerfeldt & Rubin, 2001). The

local concentrations of RANKL and OPG are also important determinants for osteoclast survival. Several factors positively or negatively regulate osteoclast life span, including cytokines, hormones and growth factors. PTH and  $1,25(\text{OH})_2\text{D}_3$  increase bone resorption and inhibit osteoclast apoptosis by the increased expression of RANKL and the decreased expression of OPG by stromal cells (Xing & Boyce, 2005). The cytokines such as RANKL, IL-1 and TNF- $\alpha$  prolong the activity of osteoclasts for bone resorption, while estrogen and TGF- $\beta$  negatively affect the survival time of osteoclasts (Lian & Stein, 2006).

### **1.2.3 The osteocytes**

Osteoblast lineage cells have two fates: a) some of them form the worker cells, active osteoblasts, which lay down the bone matrix on bone formation surfaces and finally become apoptotic, and b) the remaining cells further develop into the regulatory cells, including osteocytes and lining cells (Rodan, 2003). Osteocytes, the star-shaped cells, are the most abundant cells within the lacunae of mineralized bone matrix. They contain extensive filopodia that form canaliculi channels that provide interconnections with each other and connections with the surface-lining cells through gap junctions, forming a syncytial network that maintains bone structure and metabolism (Sommerfeldt & Rubin, 2001; Lian & Stein, 2006; Clarke, 2008). Gap junctions are communication junctions that allow nutrients and waste to pass between two adjacent cells (Lian & Stein, 2006; Clarke, 2008). They are required for maturation, activity and survival of osteocytes. The activated osteocytes also contain lysosomes and work as phagocytic cells. Osteocytes generate bone matrix proteins, which can support intercellular adhesion and modulate mineral exchange in the fluid of the canaliculi and bone lacunae (Clarke, 2008). Osteocytes primarily function as the mechanosensor cells by transiting signals across gap junctions to regulate the activity of osteoclasts and osteoblasts in the basic multicellular unit (BMU) (Lian & Stein, 2006; Clarke, 2008).

Osteocytes have a long lifespan lasting for several decades in the human skeleton. Disruption of cell-cell interaction through gap junctions and cell-matrix interaction lead to osteocyte apoptosis, which has been observed in empty lacunae within the aging bone. Sex steroids (e.g. estrogen) and bisphosphonates have anti-apoptotic effects and may help to mediate osteocyte survival (Xing & Boyce, 2005).

## **1.3 Bone development and metabolism**

### **1.3.1 Bone modelling and growth**

Bone modelling, or ossification, refers to the formation of new bone. As mentioned above, there are two distinctive ways for bone to form during embryonic development—intramembranous ossification in flat bone and endochondral ossification in long bone. In intramembranous ossification, bone directly develops between fibrous sheets of connective tissue, while endochondral ossification also includes cartilage deposition. During childhood and adolescence, bones constantly grow and develop in longitudinal and radial directions. After birth, the epiphyses continuously develop in the long bone, and then secondary ossification centres occur within these regions. The epiphyseal plate, a band of cartilage, remains between the primary and secondary ossification centres. The diaphysis of bone continues to grow in width and length in the presence of epiphyseal plates. Finally, the growth of bone stops when the epiphyseal plates become ossified (Mader & Gallart, 2005).

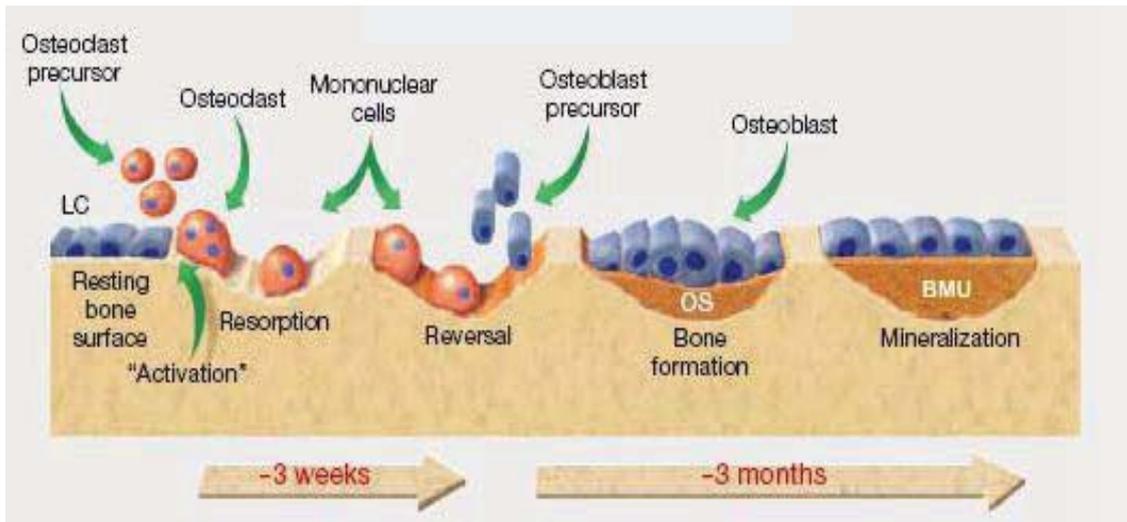
### **1.3.2 Peak bone mass**

Peak bone mass is a major determinant of bone density and osteoporotic fractures in later life. Accumulation of bone mass begins in utero and reaches a maximum by the age of 40 years. More than 90% of peak bone mass gain is achieved by the age of 18 years, little can be accumulated after this age (Gunnes & Lehmann, 1996; Lane, 2006). By the age of 80-90 years, bone mass decreases to half of the peak value. Peak bone mass is primarily affected by genetic factors and is also dependent on physical activity and dietary calcium intake during infancy and adolescence (Mundy, 2000). Females have 10-12% less peak bone mass and a smaller bone size than males do and have accelerated bone loss after menopause (Mundy, 2000; Bonnick, 2006). Low peak bone mass in early adult life together with the increased bone loss during aging increase the risk of osteoporotic fractures (Mundy, 2000; Lane, 2006).

### **1.3.3 Bone remodelling**

Bone modelling constantly occurs in BMUs before birth and until death. It is composed of two tightly coupled processes, including the resorption of old bone on a particular surface, followed by the new bone formation phase. It is useful to replace old or damaged bone tissue (Sommerfeldt & Rubin, 2001; Clarke, 2008). In adults, the coupling processes ensure that the amounts of bone resorption by osteoclasts and

bone formation by osteoblasts are maintained in balance. Approximately 20% of the cancellous bone surface experiences remodelling at any one time. Bone modelling includes four cyclic stages: activation-resorption-reversal-formation (Figure 1.5), and 3-6 months are required to complete one cycle in humans. In the activation phase, the lining cells and osteocytes located on the resting bone surface receive signals (e.g. hormones or cytokines) which trigger the remodelling process. The precursors of the osteoclasts attach to the bone surface and become activated multinucleated osteoclasts that resorb mineralized bone matrix in the BMU, a process lasting 2-4 weeks during the phase of resorption. In the reversal phase, the resorbed bone surface is cleaned up by mononuclear cells during the transition from resorption to formation. Additionally, osteoblast precursors proliferate and differentiate locally into mature osteoblasts, which migrate into the resorption lacuna and terminate osteoclastic activity. The formative phase lasts for about 3 months, which involves osteoblasts laying down the new bone matrix and filling the resorption lacuna with unmineralized osteoid, which subsequently mineralize the newly formed BMU. Finally, the remaining osteoblasts either terminally differentiate into osteocytes or flatten and form quiescent lining cells on the resting surface of bone until activated (Hill, 1998; Rodan, 2003; Clarke, 2008).



**Figure 1.5** Bone remodelling cycle (from the left to right), adapted from Rodan (2003) with permission from John Wiley and Sons.

Lining cells receive the signals of hormones or cytokines to initiate the activation of remodelling process. Osteoclast precursors attach to the bone surface and become multinucleated osteoclasts that can resorb mineralized bone matrix for approximately 3 weeks. Mononuclear cells clean up the resorbed bone surface during the transition from resorption to formation. Osteoblast precursors proliferate and differentiate locally into mature osteoblasts, which migrate into the resorption lacuna and terminate osteoclastic activity. Osteoblasts lay down the new bone matrix and fill the resorption lacuna with unmineralized osteoid, which subsequently mineralize the newly formed basic multicellular unit. Finally, the remaining osteoblasts either terminally differentiate into osteocytes or flatten and form quiescent lining cells on the resting surface of bone until activated. LC: lining cell; OS: osteoid; BMU: basic multicellular unit.

## 1.4 Regulation of bone metabolism

In adults, bone mass is maintained by the balance between osteoblastic bone formation and osteoclastic resorption, each of which is affected by lifestyle factors (e.g. physical activity, dietary nutrition), hormones and local factors (e.g. growth hormones, cytokines, prostaglandins and leukotrienes) (Table 1.1 and 1.2), which affect both osteoblasts and osteoclasts, the proliferation of undifferentiated cells and the function of differentiated cells.

### 1.4.1 Lifestyle factors

Normal bone development and its maintenance after growth are associated with a variety of dietary nutrients such as protein, calcium, phosphorus, manganese, zinc, copper and vitamin D, K and C. During childhood and adolescence, adequate nutritional intake is needed for bone growth to achieve peak bone mass and to compensate for any daily bone loss. In adults, nutrients are used to preserve the bone mass and to help with the recovery from skeletal injury and illness. The dietary intake of nutrients also has an important beneficial effect on the susceptibility to

fragility fractures and recovery in the elderly (Gaffney-Stomberg et al., 2009; Jarvinen et al., 2012; Fan et al., 2013).

Apart from diet, smoking, alcohol consumption and physical activity, also affect bone mass. Smoking is associated with a significant reduction in hip bone mineral density (BMD) and an increased risk of hip fracture in the elderly. Smoking cessation can decrease bone loss induced by smoking (Hollenbach et al., 1993). Smoking may have direct cellular effects on bone cells and lead to changes in secretion and metabolism of calciotropic hormones, sex hormones and adrenal cortical hormones, dysregulation in intestinal absorption of calcium, and alterations in the RANK-RANKL-OPG system (Yoon et al., 2012). Excess alcohol consumption is likely to have both direct and indirect irreversibly adverse effects on the skeleton, especially during adolescence and the young adult years (Sampson, 2002). Alcohol affects the formation and activity of bone cells in both *in vivo* and *in vitro* studies (Cheung et al., 1995; Dyer et al., 1998). Alcohol abuse may also limit dairy food intake and lead to hypercalciuria (Heaney, 1996; Sampson, 2002). Physical activity is positively associated with bone development and bone mass maintenance. It has been shown that increased levels of weight-bearing physical activity and calcium intake improved bone mass gains in childhood and adolescence (French et al., 2000), and these skeletal benefits can be maintained into young adulthood (Baxter-Jones et al., 2008). In the later decades of life, adequate calcium intake and physical activity potentially limit bone loss and reduce the risk of osteoporosis in the elderly (Nguyen et al., 2000).

### 1.4.2 Effects of hormones

Bone metabolism is regulated by various hormones including polypeptide hormones, steroid hormones and thyroid hormones, shown in Table 1.1.

**Table 1.1** Hormones involved in bone metabolism

Polypeptide hormones	Parathyroid hormone (PTH) Calcitonin Insulin Growth hormone
Steroid hormones	1,25-dihydroxyvitamin D <sub>3</sub> (1,25(OH) <sub>2</sub> D <sub>3</sub> ) Glucocorticoids Sex steroids (estrogen and androgen)
Thyroid hormones	

#### 1.4.2.1 Parathyroid hormone

The endogenous PTH in the human body is a polypeptide hormone containing 84 amino acids (PTH 1-84) and is secreted by the parathyroid glands. PTH 1-84 and its bioactive fragments (e.g. PTH 1-34) act to regulate serum calcium status through contrasting effects on both bone resorption and formation. Increased extracellular calcium concentration inhibits PTH secretion, while a decreased level of extracellular calcium promotes the secretion of PTH and then PTH enhances calcium release from the bone reservoir. PTH is a key regulator that promotes proliferation and differentiation of osteoblasts and prevents them from apoptosis (Pettway et al., 2008), but it indirectly stimulates the activity of osteoclasts (Poole & Reeve, 2005). In the kidney, PTH enhances calcium and magnesium reabsorption from the proximal tubules, phosphate excretion and active vitamin D formation through increasing the activity of the 1- $\alpha$ -hydroxylase enzyme. In the intestine, PTH indirectly increases absorption of calcium, which is modulated by the increased production of 1,25(OH)<sub>2</sub>D<sub>3</sub> (Poole & Reeve, 2005). Intermittent administration of PTH is an effective therapy that stimulates bone formation in patients with osteoporosis (Thomas, 2006).

#### **1.4.2.2 Calcitonin**

Calcitonin is a hormone produced by thyroid C-cells and neuroendocrine cells, in response to high blood calcium level. Calcitonin functions to decrease blood calcium levels, opposing the effects of PTH. Calcitonin leads to the immediate inhibition of bone resorption by decreasing osteoclast activity through binding to the G protein-coupled-type calcitonin receptor (Lian & Stein, 2006).

#### **1.4.2.3 Insulin**

Insulin that centrally regulates energy homeostasis in the body is produced by  $\beta$ -cells of the pancreas in response to elevated levels of blood glucose after food consumption. In bone, insulin signalling is associated with osteoblastic function and thus is needed for normal bone acquisition. Osteoblasts express insulin receptors that bind insulin to increase the anabolic activity of bone, including the production of alkaline phosphatase, collagen synthesis and glucose uptake (Fulzele & Clemens, 2012). Insulin signalling also mediates the production and bioavailability of osteocalcin, which is solely secreted by osteoblasts and a high serum level of insulin is used as biomarker for bone formation (Fulzele & Clemens, 2012).

#### **1.4.2.4 Growth hormone**

Growth hormone is a single chain polypeptide and is secreted by the pituitary gland. The synthesis and secretion of growth hormone are stimulated by growth hormone-releasing hormone, sex steroids and thyroid hormones, and is inhibited by somatostatin and glucocorticoids. Growth hormone controls IGF-1 secretion from the liver, which directly limits growth hormone secretion in somatotrophs and indirectly inhibits this by stimulating somatostatin secretion (Giustina et al., 2008). Growth hormone directly promotes the proliferation of the osteoblastic lineage cells and influences the differentiation fate of mesenchymal precursors, including stimulating osteoblastogenesis, chondrogenesis and limiting adipogenesis. It also promotes the activity of the mature osteoblasts through IGF-1 and increases bone formation. Additionally, growth hormone indirectly stimulates the differentiation and activity of osteoclasts (Giustina et al., 2008).

#### **1.4.2.5 Vitamin D**

The endocrine vitamin D system has the principal effect of maintaining the homeostasis of calcium and phosphate in the body. The active vitamin D,  $1,25(\text{OH})_2\text{D}_3$ , plays an essential role in mineralization of bone and cartilage.

1,25(OH)<sub>2</sub>D<sub>3</sub> together with growth hormone increases the number of osteoblasts through inhibiting apoptosis by 1,25(OH)<sub>2</sub>D<sub>3</sub> and stimulating proliferation by growth hormone (Morales et al., 2004). 1,25(OH)<sub>2</sub>D<sub>3</sub> also indirectly stimulates osteoclastic bone resorption through a primary effect on osteoblast expression of RANKL (Lian & Stein, 2006).

#### **1.4.2.6 Glucocorticoids**

Glucocorticoids is a class of steroid hormones that are commonly used to treat immune and inflammatory disorders such as periodontal disease and rheumatoid arthritis. Although glucocorticoids suppress inflammation efficiently, they also induce a development of osteoporosis. Long-term administration of glucocorticoids results in an average of about 12% bone loss in the first year and approximately 3% less per annum thereafter (Kim et al., 2007). Glucocorticoids impair intestinal calcium absorption, inhibit osteoblastic bone formation and stimulate apoptosis of osteocytes. They also increase the activity and/or life span of osteoclasts through inhibiting OPG synthesis and promoting RANKL expression (Xing & Boyce, 2005).

#### **1.4.2.7 Sex steroids**

Estrogen deficiency induced by menopause or ovariectomy leads to increased osteoclast formation and subsequent bone loss resulting from an excess of bone resorption over formation, and treatment with estrogen can reverse this. Estrogen can prevent osteoblast apoptosis by increasing the expression of the 1,25(OH)<sub>2</sub>D<sub>3</sub> receptor. In addition, estrogen and testosterone increase the apoptosis of both mature osteoclasts and their precursors. This pro-apoptotic effect is due to the increased expression of OPG, TGF-β and IL-1 decoy receptors and the decreased expression of IL-1 receptor 1 mRNA (Xing & Boyce, 2005).

#### **1.4.2.8 Thyroid hormones**

Thyroid gland synthesis and secrete the inactive thyroid hormone thyroxine (T<sub>4</sub>) and a small amount of the active form of triiodothyronine (T<sub>3</sub>). T<sub>3</sub> modulates skeletal development, peak bone mass acquisition and bone maintenance in adulthood. T<sub>3</sub> deficiency in childhood leads to growth arrest, delayed bone age, epiphyseal dysgenesis and short stature, while excess T<sub>3</sub> results in accelerated bone formation, growth and potentially the severe condition of premature closure of the cranial sutures and growth plates. Thyrotoxicosis in adult causes T<sub>3</sub> excess, which increases

bone remodelling, thus resulting in net bone loss and an elevated risk for osteoporotic fracture.  $T_3$  stimulates osteoblast proliferation, differentiation and apoptosis as well as osteoclastic formation and activity through cytokines and growth factors (Duncan-Bassett & Williams, 2003).

### 1.4.3 Local factors

Local factors are synthesized and secreted by bone cells, including cytokines, growth factors, PGs and leukotrienes (LTs) (Table 1.2). For example, IGF-1 plays a role in bone matrix maintenance by directly stimulating the synthesis of type I collagen and reducing the enzyme activity of collagenase 3 (Lian & Stein, 2006). TGF- $\beta$  promotes progenitor cell replication and directly increases the synthesis of collagen. IGF-1 and 2 released by osteoblasts are essential regulators for anabolic activity of osteoblasts (Lian & Stein, 2006). TNF- $\alpha$  and IL-1 directly increase osteoclastogenesis and osteoclastic activity.

**Table 1.2** Local factors involved in bone metabolism.

Growth factors	Insulin-like growth factors (IGF) 1 and 2  Transforming growth factor- $\beta$ (TGF- $\beta$ ) superfamily, including the bone morphogenetic proteins (BMPs)  Fibroblast growth factors (FGF)  Platelet-derived growth factors (PDGF)
Cytokines	Interleukin (IL), such as IL-1, IL-6 and IL-7  Tumour necrosis factor (TNF)  Colony-stimulating factor (CSF) families
Prostaglandins	PGE <sub>2</sub> , PGI <sub>2</sub> , PGI <sub>3</sub>
Leukotrienes	LTC <sub>4</sub> , LTD <sub>4</sub> , LTC <sub>5</sub>

## **Section 2. Mis-regulation of bone metabolism**

### **1.5 Definition of osteoporosis**

In 1994, osteoporosis was described by the World Health Organization (WHO) as a skeletal disorder in which decreased bone mass and bone strength predispose an individual to increased bone fragility and consequently an increase in fracture risk (WHO, 1994). It is diagnosed as BMD value of  $\pm 2.5$  standard deviation or more below the average value in young adults (WHO, 1994). Using this criteria in the Western world, approximately 25% of aging women and 5-10% of men would be diagnosed as being osteoporotic (Mundy, 2000; Marcus, 2013). Worldwide, osteoporosis results in about 9 million fractures annually (Kanis, 2007). The most common osteoporotic fractures occur in the vertebrae, hip and rib (Lane, 2006; Brown et al., 2011). These fractures also cause serious complications that are related to an increased incidence of morbidity such as disability, back pain and lung disease, as well as mortality (Lane, 2006). In New Zealand, osteoporosis and osteoporotic fractures lead to an important burden on the health system. In 2007, there were over 84,000 fractures resulting from osteoporosis in New Zealand. Osteoporotic fractures directly cost the New Zealand health care system approximately NZ\$330 million in 2007. It is expected that the cost for treatment and management of osteoporosis will increase to over NZ\$458 million in 2020 (Brown et al., 2011).

### **1.6 Pathogenesis of osteoporosis**

#### **1.6.1 Basic pathogenetic mechanisms**

Osteoporosis can develop from: (i) failure to reach optimal peak bone mass and bone strength during growth; (ii) excessive bone resorption inducing bone loss and micro-architectural damage; and (iii) inadequate bone formation in response to enhanced bone resorption during bone remodelling (Raisz, 2005; Becker, 2008). The incidence of osteoporotic fractures is further affected by the direction and frequency of falls (Raisz, 2005).

#### **1.6.2 Risk factors for osteoporosis and osteoporotic fractures**

Several interacting variables contribute to the risk of osteoporosis and osteoporotic fractures, such as clinical (e.g. peak bone mass and sex hormone status), medical (e.g.

medications and medical diseases), lifestyle (e.g. behaviours and nutrition) and genetic (sex, race and body composition) factors.

#### **1.6.2.1 Hormonal factors**

Sex hormone deficiency is the main cause of bone loss after middle age. Females usually have an abrupt estrogen withdrawal after loss of ovarian function (menopause), resulting in rapid postmenopausal bone loss, while bone loss in males is slow due to the more gradual decrease in the secretion of sex hormones. Bone loss following menopause shows greater effects on cancellous bone than on cortical bone (Mundy, 2000). Additionally, a low body weight, low body mass index (BMI) or low fat mass are independent contributors to postmenopausal osteoporosis, which result in increased risk of low bone mass and accelerated bone loss (Lane, 2006).

#### **1.6.2.2 Medical factors**

Exposure to certain medications and several medical disorders may contribute to secondary osteoporosis. For example, glucocorticoid excess causes rapid bone loss through increased bone resorption and reduced bone formation rate. The increase in bone resorption is associated with both the direct anti-apoptotic effects of glucocorticoid on osteoclasts and the elevated osteoclast activity and survival via increased RANKL production and inhibited OPG synthesis. Glucocorticoid excess leads to a decrease in bone formation rate through the reduction of osteoblast number, which is partially caused by the increased apoptosis of osteoblasts and their progenitors. Moreover, glucocorticoids are likely to increase apoptosis and decrease the function of osteocytes (Jilka, 2003; Canalis et al., 2007). Glucocorticoids also have catabolic effects on skeletal muscle and result in muscular weakness, which may contribute to the risk of fracture due to the increased incidence of falls (Canalis et al., 2007). Additionally, certain diseases can cause osteoporosis such as malabsorption syndromes, thalassemia and amenorrhea (Lane, 2006).

#### **1.6.2.3 Lifestyle factors**

Behavioural risk factors including smoking, a low level of physical activity, alcohol consumption and caffeine intake increase the development of osteoporosis and the risk of osteoporotic fracture (Lane, 2006). They are associated with the decreased intestinal absorption of calcium and accelerated bone loss, especially in the elderly.

#### **1.6.2.4 Genetic factors**

Race, sex and body composition are important determinants of BMD and the fracture risk. For example, black males have greater BMD and bone mineral content (BMC) compared with White or Hispanic males. Age-related decreases in BMD and BMC are significantly steeper within Hispanic than White or Black males (Araujo et al., 2007). Males have greater regional and whole body BMD than females. Muscle strength is higher in men than women and in Blacks than Whites (Taaffe et al., 2001).

### **1.7 Effects of estrogen imbalance on bone metabolism**

Women with menopause show a rapid phase of bone loss following the cessation of ovarian function. In the 10 years following menopausal, women are expected to lose approximately 50% of cancellous bone and 35% of cortical bone, and about 2/3 of these amounts in men. The loss of cancellous bone mass occurs earlier than the loss of cortical bone mass. In women, about 50% of bone loss in cancellous bone results from menopause, and about 50% from the aging process (Mundy, 2000). Natural menopause or ovariectomy is associated with a rapid decrease in circulating estrogen.

#### **1.7.1 Effects of estrogen on bone modelling**

Estrogen causes the pubertal growth spurt in both sexes through the mediation of estrogen receptors (ER- $\alpha$  and ER- $\beta$ ) within the growth plate. These receptors are expressed in osteoclasts, osteoblasts, osteocytes and their precursors (Pfeilschifter et al., 2002; Pacifici, 2008) and act as transcription factors to activate specific genes (Riggs et al., 2008). Estrogen has a biphasic dose-response effect on bone growth. Low levels of estrogen stimulate bone growth directly at the growth plate by stimulating growth hormone and insulin-like growth factor axis without influence in sexual maturation, while high levels of estrogen reduce or inhibit linear growth and initiate the bone remodelling cycle and the development of secondary sexual characteristics (Cutler Jr, 1997; Juul, 2001). Estrogen promotes epiphyseal fusion and terminates linear growth especially in pubertal girls, while androgens do not have these effects (Cutler Jr, 1997). Additionally, prepubertal girls show approximately 20% faster skeletal maturation than prepubertal boys due to 8-fold higher estradiol levels compared with boys (Cutler Jr, 1997).

Estrogen deficiency inhibits epiphyseal closure and bone growth in young adults. For example, the genetic mutations in the CYP19 gene (aromatase deficiency) or in the *ER* gene (estrogen resistance) in men induced an insufficient secretion of estrogen or insensitivity to estrogen, which results in a tall stature by the failure of normal pubertal growth spurt and the continuous growth throughout adulthood due to lack of epiphyseal closure (Juul, 2001). However, girls with idiopathic precocious puberty experience elevated estrogen levels resulting in a marked increase in the rate of growth but resulting in very short stature due to the early closure of the epiphyses (Juul, 2001).

### **1.7.2 Effects of estrogen on bone remodelling**

Bone resorption and formation do not randomly occur in the skeleton but are coupled together at BMUs. Estrogen deficiency in humans increases the rates of bone remodelling with a remarkable enhancement of bone resorption coupled with an increase in bone formation. However, with resorption exceeding formation this results in a net bone loss (Jilka et al., 1998; Syed & Khosla, 2005).

#### **1.7.2.1 The role of estrogen in osteoclastogenesis**

Estrogen deficiency results in an increase in the number of BMUs via an increase in activation frequency, which enlarges the remodelling space, elevates cortical porosity and expands the resorption area on trabecular surfaces. This is primarily caused by an increased proliferation of osteoclasts and their precursors on the bone surface and in bone marrow, suggesting that estrogen may limit recruitment of osteoclasts (Väänänen & Härkönen, 1996; Pacifici, 2008). Estrogen deficiency also increases osteoclast erosion depth through the prolonged resorption phase of the remodelling cycle by increasing osteoclast life span (Pacifici, 2008). Estrogen deficiency increases the number of osteoclasts due to delayed apoptosis of osteoclasts through a defect in the programmed pathway of cell death (Väänänen & Härkönen, 1996).

Estrogen deficiency increases osteoclastogenesis by stimulating the expression and secretion of pro-inflammatory cytokines (e.g. IL-1, IL-6, TNF- $\alpha$  and M-CSF) from osteoblasts, peripheral blood monocytes and bone marrow stromal cells (Pfeilschifter et al., 2002; Syed & Khosla, 2005) as well as by inhibiting TGF- $\beta$  expression in bone (Syed & Khosla, 2005). TGF- $\beta$  acts to induce apoptosis of osteoclasts.

At the cellular level, the major effects of estrogen are to inhibit activation frequency of BMUs as well as to maintain the balance of bone remodelling through suppressing osteoclast function and promoting osteoblast function. Thus the balance between bone resorption and formation is maintained at the tissue level and bone mass is conserved at the organ level.

Estrogens have direct effects on osteoclasts by inhibiting their differentiation and the bone-resorbing activity of mature cells (Väänänen & Härkönen, 1996). This may be partially caused by an increase in estrogen-induced apoptosis of osteoclasts through the estrogen-receptor mediated mechanism (Kameda et al., 1997; Syed & Khosla, 2005). Estrogen is capable of blocking RANKL production by osteoblasts, T- and B-cells, and promoting OPG production (Syed & Khosla, 2005). The reduced ratio of RANKL/OPG inhibits osteoclastogenesis.

Additionally, estrogens also indirectly regulate osteoclast-mediated bone resorption via local cytokines. Some of them have “upstream actions” that affect the proliferation of osteoclastic precursors (e.g. IL-1, IL-6, IL-7, TNF- $\alpha$ , M-CSF and PGE<sub>2</sub>), while others have “downstream actions” that regulate the differentiation, activity and apoptosis of osteoclasts (e.g. TGF- $\beta$ ). Some “upstream” cytokines such as TNF- $\alpha$  and M-CSF also have “downstream” effects (Riggs et al., 2008).

Free radicals are considered potent stimulators for NF- $\kappa$ B-mediated expression of pro-inflammatory cytokines. The decrease in estrogen levels may increase the production of free radicals, thus enhancing cytokine production. Estrogen has direct anti-oxidative effects either on the production and/or scavenging of reactive oxygen species (ROS), or on maintaining the concentration of endogenous antioxidants (Pfeilschifter et al., 2002; Raisz, 2005; Pacifici, 2008). This anti-oxidative effect of estrogen has more implications in maintaining vascular function (Pfeilschifter et al., 2002).

#### **1.7.2.2 The roles of estrogen in osteoblastogenesis**

In addition to enhanced bone resorption, estrogen deficiency is associated with an impairment in the compensatory bone formation response. This phenomenon is caused by increased osteoblastogenesis fuelled by the replication of early mesenchymal progenitors and by enhanced differentiation of these pluripotent

precursors into the osteoblastic lineage. However, the net augmentation of bone formation is insufficient to compensate for the rate of bone resorption due to an increase in osteoblast apoptosis (Syed & Khosla, 2005; Pacifici, 2008). Moreover, the activity of mature osteoblasts is inhibited by an increase in production of inflammatory cytokines (e.g. TNF and IL-7), which is induced by estrogen deficiency (Väänänen & Härkönen, 1996; Pacifici, 2008). Estrogen deficiency may also induce osteocyte apoptosis, which may reduce the ability of the osteocyte/canalicular mechanosensor network to repair microdamage, and therefore resulting in further fragility of bone (Jilka, 2003). This impaired survival of osteocytes can be reversed by estrogen treatment (Syed & Khosla, 2005).

Estrogens seem to restrict osteoblastogenesis as it has an inhibitory effect on osteoblastic proliferation, stimulate the differentiation of osteoblastic progenitors into osteoblasts rather than the formation of the adipocytes, and prolong the lifespan of osteoblasts by suppressing their apoptosis, thus enhancing the functional activity of osteoblasts in bone formation (Syed & Khosla, 2005).

The regulatory roles of estrogen in osteoblastogenesis are associated with the modulation of specific gene transcriptions that determine ER isoform expression, cell system heterogeneity, differentiation stage and receptor concentration (Väänänen & Härkönen, 1996; Syed & Khosla, 2005).

### **1.7.2.3 Effect of estrogen on calcium metabolism**

Besides the primary effects on bone turnover, estrogen has secondary actions on peripheral calcium metabolism by directly affecting the intestine and kidney (Riggs et al., 2008). Estrogen not only increases intestinal calcium absorption through intestinal ER but also increases renal calcium conservation through enhancing tubular calcium resorption (Riggs et al., 2008).

In addition, estrogen indirectly acts on calcium homeostasis by modulating serum levels of  $1,25(\text{OH})_2\text{D}_3$  and the secretion of PTH (Pfeilschifter et al., 2002; Riggs et al., 2008). Estrogen treatment is capable of increasing the estimated levels of free  $1,25(\text{OH})_2\text{D}_3$ , while the serum levels of PTH are decreased (Riggs et al., 2008).

## **Section 3. Dairy and bone health**

### **1.8 Composition of bovine milk**

Bovine milk is composed of about 87% water, 5% lactose, 4% lipid, 3.2% protein, 0.7% mineral salts and 0.1% vitamins, which provide critical nutrients, biologically active substances and immunological protection to both neonates and adults (Séverin & Xia, 2005; Månsson, 2008). The exact composition of milk varies depending on several factors such as breeding, animal nutrition, management of the cow, lactation stage and season (Månsson, 2008). It has been shown that the components of bovine milk have important effects on bone and dental health (Mills et al., 2011).

Milk and dairy products are considered as the major source of lauric acid (C12:0) and myristic acid (C14:0) in the human diet and also markedly contribute to the dietary intake of palmitic acid (C16:0) and trans-fatty acids (TFAs) (Bosze, 2007). Numerous human studies have indicated that a high intake of saturated fatty acids (SFAs) and TFAs are associated with an increased risk for cardiovascular disease as well as the metabolic syndrome, therefore promoting a decreased consumption of total fat, SFAs and TFAs by reducing intake of full fat dairy products. However, those recommendations ignore that these foods also provide various nutrients such as protein, lipids, minerals and bioactive compounds, which may have beneficial effects, for example to reduce the risk of osteoporosis (Lötters et al., 2013; Rizzoli, 2014), atherosclerosis (Sofi et al., 2010) or cancer (Lampe, 2011; Aune et al., 2012).

#### **1.8.1 Lipids**

In bovine milk, the lipids (about 3-5% of total weight) are emulsified as globules within the water phase (87%). The composition of lipids mainly consists of triacylglycerols (98% of the total fat). There are small amounts of phospholipids (0.8%), sterols (e.g. cholesterol) (0.3%), diacylglycerols (0.3%), monoacylglycerols (0.03%) and free (unesterified) fatty acids (0.1%). In addition, bovine milk also contains trace amounts of carotenoids, fat-soluble vitamins and flavour compounds (Jensen, 2002; MacGibbon & Taylor, 2006).

### **1.8.1.1 Saturated fatty acids**

#### **a. The composition of saturated fatty acids in bovine milk**

The SFAs account for a significant proportion, approximately 70-75%, of total fatty acids in bovine milk. The molecules of these fatty acids have un-branched hydrocarbon chains with various backbone lengths from 4 to 18 carbon atoms. Palmitic acid (C16:0) is present in the highest concentration, about 25-30% of the total fatty acids. Myristic acid (C14:0) and stearic acid (C18:0) have reasonably high concentrations in the range of 9-14%. The short-chain SFAs, for example butyric acid (C4:0) and caproic acid (C6:0), are typically present at approximately 4% and 2.5%, respectively. Medium-chain SFAs (C8:0-C12:0) are also present in appreciable quantities in bovine milk fat (MacGibbon & Taylor, 2006; Månsson, 2008).

#### **b. The origin of saturated fatty acids in bovine milk**

The fatty acids in bovine milk are mainly acquired from feeding or produced by microbial activity in bovine rumen. The fatty acids with even numbers (4-16) of carbons are produced in the mammary glands of cows. All C4:0-C14:0 fatty acids and half of C16:0 fatty acids are synthesised from acetate and 3-hydroxybutyrate that are generated by fermentation of feed components in the rumen (Månsson, 2008). Butyric acid (C4:0) is generated from fermentation of carbohydrates by bacteria in the rumen. During absorption by the rumen epithelium butyric acid is converted to 3-hydroxybutyrate and then transported to the mammary gland via blood vessels, where it is finally reduced to butyric acid. In humans, butyrate is also synthesised by microbial degradation of non-digestible carbohydrates and prebiotic substances in the intestine (Månsson, 2008; Mills et al., 2011).

Bovine milk also contains certain SFAs with an odd number of carbons including pentadecanoic acid (C15:0) and heptadecanoic acid (C17:0), which are produced by the bacterial flora in the rumen. The long-chain SFAs (e.g. C18:0) and the remaining C16:0 are derived from dietary fats and the lipolysis of triacylglycerols in adipose tissue (Månsson, 2008).

### **c. The digestion of saturated fatty acids in the human body**

After consumption by humans, triacylglycerols of milk are lipolysed by lingual lipase enzymes in the mouth as well as by both lingual and gastric lipase in the stomach. These lipases primarily hydrolyse fatty acids at the *sn*-3 position, and selectively produce shorter chains fatty acids. Consequently, C4:0-C10:0 acids pass through the intestinal wall, enter the portal vein and are transported to the liver for oxidation. Approximately 25-40% of the triacylglycerols are digested in the stomach (Månsson, 2008).

#### **1.8.1.2 The composition and origin of unsaturated fatty acids**

About 25% of the total fatty acids in bovine milk are mono-unsaturated, for example palmitoleic acid (C16:1) and oleic acid (C18:1), while 3% are poly-unsaturated, such as linoleic acid (C18:2 n-6, LA),  $\alpha$ -linolenic acid (C18:3 n-3, ALA) with a n6/n3 ratio of approximately 2-3:1, as well as conjugated linoleic acid (C18:2 conj, CLA) (Bosze, 2007; Månsson, 2008). The mono-unsaturated fatty acids originate from the desaturation of corresponding medium- and long-chain SFAs, mainly C18:0, in the mammary gland (Månsson, 2008).

LA and ALA cannot to be synthesised by the human body. However, they are needed for survival of humans and other mammals, and therefore have to be acquired from the diet and are called essential fatty acids (EFAs) (Månsson, 2008). LA has a critical effect on the structure of cell membranes and lipoproteins through their incorporation into phospholipids. In addition, LA and ALA are important precursors for the synthesis of arachidonic acid (AA, C20:4 n-6) and eicosapentaenoic acid (EPA, C20:5 n-3), respectively, through  $\Delta$ 6-desaturation, elongation and  $\Delta$ 5-desaturation. EPA can be further metabolised to synthesise docosahexaenoic acid (DHA, C22:6 n-3), which is extremely critical for the development of the central nervous system and is distributed in both tissues and phospholipids (Mills et al., 2011).

#### **1.8.1.3 Trans-fatty acids**

TFAs that have one or more trans double bonds, account for about 3% of the total fatty acids and vary with season (Bosze, 2007; Månsson, 2008). The main isomer of TFA is vaccenic acid (VA, C18:1, 11t), however milk fat also contains low concentrations of the trans double bonds in a 4-16 position (Månsson, 2008). VA

plays a double role in fat metabolism as it acting as both a TFA and a precursor of 9*cis*, 11*trans*-CLA (rumenic acid) (Månsson, 2008).

### **1.8.2 Proteins**

In bovine milk, the major protein fractions include caseins (approximately 80% of bovine milk proteins) and whey proteins (about 20%). Caseins are a group of phosphate-containing proteins with the names of  $\alpha_{s1}$ -,  $\alpha_{s2}$ -,  $\beta$ - and  $\kappa$ -casein. They are produced by the mammary secretory epithelium. Whey proteins of bovine milk have globular 3-dimensional structures and mainly consist of  $\beta$ -lactoglobulin (about 50% of total whey protein),  $\alpha$ -lactalbumin (20%), immunoglobulins (13%), bovine serum albumin (7-8%), lactoferrin, transferrin as well as proteose-peptone fractions (Fox, 2003). Whey proteins are resistant to the chymosin action and cannot be coagulated by acid. Therefore, these proteins can exist in rennet whey and acid (Séverin & Xia, 2005). Whey proteins are the by-product of cheese or casein produced from cow's milk and are widely utilised in food products.

Lactoferrin is a whey protein in bovine milk and has an important effect on iron absorption within the intestine. Bovine colostrum and milk contain 1.5-5 mg/ml and 0.1  $\mu$ g/ml lactoferrin, respectively. The high level of lactoferrin in bovine colostrum is required for the primary defence system against pathogenic bacteria (Séverin & Xia, 2005).

Bovine milk also contains small amounts of other proteins and peptides that have several physiological activities, such as involvement with the endogenous antibacterial system and hormone releasing factors (Séverin & Xia, 2005; Mills et al., 2011). Milk basic proteins (MBPs) that have basic isoelectric points seems to contain growth factors such as IGF and TGF- $\beta$ , which have biological activities (K. Kato et al., 2000; Toba et al., 2000). Milk proteins are metabolised in the gastrointestinal tract and form several bioactive peptides, which exert numerous physiological effects in the human body. Bioactive proteins include antihypertensive, antithrombotic, opioid, casein phosphopeptides, antimicrobial, cytomodulatory, immunomodulatory and miscellaneous peptides (Séverin & Xia, 2005; Mills et al., 2011).

### **1.8.3 Other components in bovine milk**

#### **1.8.3.1 Oligosaccharides**

Bovine milk contains very low levels of oligosaccharides (1g/L) when compared with human milk (7-20 g/L) (Séverin & Xia, 2005; Mills et al., 2011). The monomers of oligosaccharides in milk include D-glucose, D-galactose, N-acetylglucosamine, L-fucose and sialic acid (Mills et al., 2011).

Lactose can increase calcium absorption and is used to produce lacto-oligosaccharides and lactulose, which play a role in promoting growth of probiotic bacteria in the intestine and have been widely used in infant formulas (Séverin & Xia, 2005).

#### **1.8.3.2 Calcium**

On the average, the total calcium content in an adult is approximately 1 kg. More than 99% of it is in bone and teeth. In the bone, calcium exists in mineral form as hydroxyapatite and affects bone strength by its effect on bone mass (Zhu & Prince, 2012). Dietary intake of calcium can play a significant role in the maintenance of skeletal health, management of body weight, maintenance of blood pressure and decrease risk of colon cancer (Séverin & Xia, 2005; Huncharek et al., 2008; Mills et al., 2011). Calcium intake influences bone mineral accretion during bone growth and thus affects the attainment of peak bone mass in early adulthood. In older age, calcium intake plays a role in preventing bone loss and fracture (Zhu & Prince, 2012).

Dairy products are good calcium sources. Other foods such as bony fish, legumes, breakfast cereals and certain nuts also contain calcium. The bioavailability of calcium is a more important factor compared with the simple calcium content of food. It refers to calcium absorbability from the intestine and the incorporation of absorbed calcium into bone (Zhu & Prince, 2012). Various food components, including protein, lactose, fibre, vitamin D, tannins in tea and sodium, can affect calcium bioavailability (Zhu & Prince, 2012).

#### **1.8.3.3 Phosphorus**

The adult human body contains approximately 1% phosphorus in total weight, 85% of which exists in bone and teeth mostly as hydroxyapatite and 15% is in soft tissues and the extracellular space (Lamberg-Allardt et al., 2010). Phosphate,  $\text{PO}_4^{2-}$ , is the

most common form of phosphorus in the body, and plays several roles in biological processes such as in bone mineralization, membrane function, energy metabolism and in cellular metabolism (Lamberg-Allardt et al., 2010). Phosphorus homeostasis in the body mainly depends on the dietary intake, intestinal absorption and reabsorption of phosphorus from the renal tubule. The regulation of phosphorus homeostasis mainly occurs in the kidney (Lamberg-Allardt et al., 2010). The active form of vitamin D, 1,25(OH)<sub>2</sub>D<sub>3</sub>, facilitates phosphorus absorption, while the consumption of high dose of calcium carbonate and aluminium-containing antacids decrease its absorption (Thompson et al., 2007). Serum phosphorus level directly affects PTH secretion. Phosphorus also has direct effects on bone cells (Lamberg-Allardt et al., 2010).

Milk products, meat and grain products are high in phosphorus and contribute to most of the total dietary phosphorus intake. The bioavailability of phosphorus is different among food sources. For example, organic forms of phosphorus such as phytin are incompletely hydrolysed and absorbed. However, inorganic phosphorus such as phosphate is readily hydrolysed and absorbed from the intestine (Lamberg-Allardt et al., 2010).

## **1.9 The relationship between dairy lipids and bone**

### **1.9.1 The effect of saturated fatty acids on bone**

Over the past several years, there has been growing evidence indicating that dietary fat intake has important effects on skeletal health (Watkins et al., 2001; Maggio et al., 2011; Mangano et al., 2013). Different fat compositions ingested in the diet, including total fat, SFA, USFA play various roles in bone metabolism (Kruger & Horrobin, 1997; Watkins et al., 2001; Reid, 2008; Kruger et al., 2010; Maggio et al., 2011).

#### **1.9.1.1 Epidemiological studies**

Paakkunainen et al. (2002) found that dietary fat intake was positively associated with bone properties measured by calcaneal ultrasound in prepubertal children. An observational study including 85 prepubertal children aged 8 years old showed that the serum level of stearic acid (C18:0) was weakly but inversely associated with total body BMD, while palmitic (C16:0) and arachidic (C20:0) acids were positively correlated with bone parameters of the lumbar spine (Eriksson et al., 2009). In a

cross-sectional study with one year of follow-up, Gunnes et al. (1996) found that total dietary SFAs intake was positively associated with cortical BMD gain at the forearm within girls, but not in boys.

High dietary intake of total fat is also associated with increased risk of fractures in postmenopausal women (I. Kato et al., 2000). Orchard et al. (2010) examined the relationship between dietary fatty acid intake and fracture risk among postmenopausal women with a mean age of 63 years. They reported that women with higher SFA intakes had an increased hip fracture risk compared to those with lower intakes ( $p$ -trend = 0.001).

Lloyd et al. (1991) observed a positive relationship between dietary fat consumption and serum estrogen levels among premenopausal women, suggesting a positive indirect effect of a high-fat diet on skeletal health. Macdonald et al. (2004) indicated that total fat and SFA intakes were not associated with either BMD or its change after a long-term follow-up of 5-7 years among women during the menopausal transition. Similarly, Corwin et al. (2006) reported that SFA intake was inversely associated with BMD in several hip regions, and the strongest effects were observed within men <50 years old in the National Health and Nutrition Examination Survey III. However, there was no relationship between total fat and SFA intakes and hip BMD in females. Total fat and SFA intakes did not induce changes of either BMD or BMC at the lumbar spine and femoral neck in the premenopausal women (Macdonald et al., 2004).

A human study showed that there was an inverse relationship between the bone marrow fat content and bone density among postmenopausal women, indicating that as the content of marrow fat increased there was a corresponding decrease in cortical and trabecular bone density. Moreover, this fat content in marrow was relatively higher in saturated than unsaturated lipids (Yeung et al., 2005).

#### **1.9.1.2 Intervention studies**

Wohl et al. (1998) investigated the roles of a high-SFA diet (8% palmitic acid, noncholesterol) in the structure and mechanics of mature bone in mature roosters. They found that there were no significant effects of a high-SFA diet on geometric structure, mechanical properties and mineral content of cortical bone, but dietary fat

content did influence the composition and mechanical properties of cancellous bone. Animals fed with low-SFA diets had a significantly greater BMC and cancellous bone strength than those fed with high-SFA diets (Wohl et al., 1998). In addition, the mature cortical bone had less sensitivity to the high-fat diet compared with the immature bone, suggesting a difference in skeletal adaptation between bone modelling in the young and bone remodelling in the adult (Wohl et al., 1998).

Parhami et al. (2001) found that the consumption of an atherogenic high-fat diet (15.8% fat, 1.25% cholesterol and 0.5% cholate) for seven months significantly reduced femoral BMC and BMD compared with a control diet containing 6% fat in atherosclerosis-susceptible C57BL/6 mice.

Cao et al. (2009) had inconsistent results showing that C57BL/6 mice fed with high-fat diets (45% energy as fat) for 14 weeks had increased body weights, reduced bone volume and trabeculae bone in the proximal tibia compared with those fed with a control diet (10% energy as fat) but had no effect on cortical bone mass. However, there was no difference in BMD of either cortical or cancellous bone between the two diet treatments, suggesting that a high-fat diet had no effect on bone mineralization.

Growing chicks fed butter fats, a source of SFAs and CLA, combined with corn oil showed significantly greater bone formation rates, about 60% higher, compared with those given diets enriched in n-6 fatty acids, suggesting a beneficial effect of SFAs on bone modelling (B. A. Watkins et al., 1997).

### **1.9.1.3 Mechanisms of action of saturated fatty acids in bone metabolism**

#### **a. Effects of short-chain saturated fatty acids on osteoblasts**

Whitehead et al. (1986) studied the effects of the short-chain SFAs, butyrate, on a colon carcinoma cell line (LIM 1215) and reported that butyrate significantly increased the activity of alkaline phosphatase (ALP), a biomarker for assessing the differentiation of osteoblasts.

Iwami et al. (1993) observed the direct influence of sodium butyrate on both the murine osteoblast cell line (MC3T3-E1), and murine osteoclast cell line (bone marrow cells). They confirmed the positive effects of butyrate on ALP activity in

MC3T3-E1 cell culture, showing that 0.5 and 1.0 mM of sodium butyrate promote the differentiation of osteoblasts. Moreover, the addition of sodium butyrate into the culture medium before the cells became confluent led to significant ALP activity in the osteoblasts. This suggests that sodium butyrate induced ALP activity by acting on preosteoblasts rather than mature osteoblasts. However, the addition of sodium butyrate at concentrations of 0.5 and 1.0 mM inhibited the protein content, which is a marker for cell growth, suggesting that sodium butyrate suppresses the proliferation of osteoblasts (Iwami & Moriyama, 1993).

Histone deacetylase (HDAC) complexes, including HDAC1, in osteogenic cells (e.g. ROS17/2.8, MC3T3-E1 and primary bone marrow cells) are recruited to the promoter regions of the cell cycle arrest gene (p27) and osteogenic genes (e.g. OSX, osteocalcin and Runx2), which induce histone deacetylation of these promoters to maintain preosteoblasts, and thus block osteoblast differentiation (Lee et al., 2006). Some studies indicate that sodium butyrate is able to increase osteoblast differentiation by suppressing HDACs. During osteoblast differentiation, promoters of osteogenic genes, including OSX and osteocalcin, are hyperacetylated with histone acetyltransferases (HATs), therefore the structures of chromatin become more accessible to osteogenic transcription factors (e.g. OSX and Runx2) for the implementation of osteoblast differentiation (Lee et al., 2006). Additionally, sodium butyrate treatment also significantly increases the mRNA levels of OSX and osteopontin in ROS17/2.8 cells and the mRNA levels of osteocalcin, Runx2, osteopontin and ALP in primary bone marrow cells, suggesting that sodium butyrate enhances osteoblast differentiation not only through suppression of HDAC enzyme activity but also stimulates osteogenic gene expression concomitantly (Lee et al., 2006).

Schroeder et al. (2005) conducted a similar experiment and reported that low concentrations of sodium butyrate (0.1-50  $\mu$ M) stimulated the proliferation of either MC3T3-E1 cells or primary osteoblasts, and a high concentration ( $> 1$  mM) of sodium butyrate resulted in increased cytotoxicity. Sodium butyrate also promoted osteoblast differentiation in calvarial tissues through an increase in Runx2 transcriptional activity (Schroeder & Westendorf, 2005).

Inconsistently, Katono et al. (2008) found that the addition of 0.01 to 100  $\mu$ M sodium butyrate did not affect the proliferation and differentiation of normal human osteoblasts after 12 days culture. However, the formation of mineralized nodules and calcium content of calcified nodules were increased by butyrate in a dose-dependent way, suggesting that butyrate can promote bone formation by stimulating the production of non-collagenous proteins such as bone sialoprotein and osteopontin in osteoblasts. Iida et al. (2011) also observed that the addition of 10  $\mu$ M to 1 mM sodium butyrate had no effect on proliferation in osteoblastic ROS 17/2.8 cells.

3-hydroxybutyrate is not only an intermediate substance of bovine milk lipid generation but also a product from the degradation of long-chain fatty acids in the liver and transported via blood to peripheral tissues as an energy source (Zhao et al., 2007). *In vivo*, it has been shown that the administration of 3-hydroxybutyrate in bilateral ovariectomized rats positively affected bone growth. The administration of 3-hydroxybutyrate *in vitro* to osteoblast MC3T3-E1 cells increased the differentiation and mineralization at the concentration lower than 10  $\mu$ g/ml (Zhao et al., 2007).

#### **b. Effects of short-chain SFAs on osteoclasts**

Sodium butyrate suppressed osteoclast differentiation at the early stage by stimulating the production of OPG from osteoblasts. OPG is an important factor that inhibits the differentiation of mononuclear osteoclasts from osteoclast precursors via inhibiting the RANK-RANKL signalling pathway. However, sodium butyrate did not affect the differentiation and maturity of osteoclasts after RANK-RANKL signalling (Iwami & Moriyama, 1993; Rahman et al., 2003; Katono et al., 2008). In addition, 2.5 mM sodium butyrate has cytotoxic effects on bone marrow cells that also may inhibit the formation of osteoclasts (Iwami & Moriyama, 1993). However, Rahman et al. (2003) indicated that the effect of sodium butyrate was specific for the cells of the osteoclast lineage, rather than cytotoxic.

Not consistent with the data above, Cornish et al. (2008) found that both butyric (C4:0) and caprylic (C8:0) acids at three concentrations of 0.1, 1.0 and 10  $\mu$ g/ml had no apparent effect on osteoclastogenesis in bone marrow cells. Only butyric acid at 10  $\mu$ g/ml showed inhibition in osteoclastogenesis.

### **c. Effects of short-chain saturated fatty acids on bone mineralization and calcium deposition**

Beside the effects of sodium butyrate on the osteoblast differentiation of osteogenic cells, Lee et al. (2006) found that sodium butyrate treatment also enhanced calcium deposition in ROS 17/2.8 cells and primary bone marrow cells. Iida et al. (2011) indicated that sodium butyrate increased the production of PGE<sub>2</sub>, which is able to enhance bone formation, by promoting the expression of cyclooxygenases (e.g. COX-1 and COX-2). In human osteoblasts, sodium butyrate stimulated the formation of mineralized nodules and increased the calcium content of the mineralized nodules in a dose-dependent manner (Katono et al., 2008). This effect of butyrate on the formation of hydroxyapatite crystals or extracellular matrix of bone is induced by the enhanced production of bone sialoprotein and osteopontin. In contrast, sodium butyrate in the concentrations of 10<sup>-8</sup> to 10<sup>-4</sup> were not associated with the expression of type I collagen, which is the major organic component of bone extracellular matrix. The higher level of sodium butyrate (10<sup>-3</sup> M) simulated mineralized nodule formation *in vitro* by accelerating gene and protein expressions of type I collagen and osteopontin (Iida et al., 2011).

### **d. Effects of medium- and long-chain saturated fatty acids on bone cells**

Cornish et al. (2008) studied the relationship between a broad range of fatty acids of bovine milk with cultures of bone marrow cells, osteoblasts and neonatal mouse calvariae. They observed that SFAs with 14-18 carbon atoms of chain length showed maximal inhibition of osteoclastogenesis in both bone marrow cells and osteoclastic RAW 264.7 cells, particularly palmitic acid (C16:0) inhibited 50% of osteoclastogenesis at 10 µg/ml. Moreover, when the RAW 264.7 cells were cultured on ivory slices in the presence of stearic acid, 10 µg/ml and RANKL, there was a 30% decrease in bone resorption area and 19% decrease in cell number. However, bone resorption was not affected by these SFAs in cultures of neonatal mouse calvariae. This evidence implies that SFAs directly affect the preosteoclast cells rather than the activity of mature osteoclasts (Cornish et al., 2008). The antiosteoclastogenic actions of SFAs are shown to be mediated by certain fatty acid receptors, named G protein-coupled receptors (GPRs), which are expressed by bone cells and mediate the biological response to fatty acids (Cornish et al., 2008; Lampe, 2011). For

instance, GPR41 and GPR43 specifically bind short-chain fatty acids, while GPR40 and GPR120 bind medium and long-chain fatty acids. GPR120 is highly expressed in RAW 264.7 cells and a synthetic GPR40/120 agonist inhibits osteoclastogenesis to a degree comparable with the palmitic and stearic acids in cultures of murine bone marrow (Cornish et al., 2008). Additionally, palmitic and stearic acids at concentrations of 0.1 and 1.0  $\mu\text{g/ml}$  had modest effects on the proliferation of osteoblasts, which was assessed via the measurement of thymidine incorporation into osteoblasts, suggesting that the inhibitory effects of these osteoclastogenic fatty acids was specific, rather than a non-specific toxic effect (Cornish et al., 2008).

It was reported that capric acid (C10:0) at 1 mM inhibits lipopolysaccharide (LPS)-induced osteoclastogenesis in RAW 264.7 cells (Park et al., 2011). LPS stimulates osteoclast formation and differentiation, then induces bone resorption and subsequently increases bone loss. LPS strongly increases mRNA expression of inducible nitric oxide synthase (iNOS) and the production of nitric oxide (NO), which acts as a mediator of osteoclastogenesis by enhancing cell fusion and stimulating cell differentiation from mononuclear pre-osteoclasts to multinucleated osteoclasts. Capric acid inhibited LPS-induced iNOS mRNA expression and NO production, therefore suppressed the osteoclastogenesis. This potent inhibitory effect of capric acid on osteoclastogenesis exerts via the pathway of signal transducer and activator of transcription 3 (STAT3), which is a member of the STAT family regulating iNOS gene expression and has a negative effect on the modulation of osteoclastogenesis. LPS markedly stimulates STAT3 phosphorylation, while capric acid inhibits it (Park et al., 2011).

In contrast, Oh et al. (2010) reported completely different results in that SFAs, such as lauric (C12:0) and palmitic (C16:0) acids, increased the number of TRAP (tartrate-resistant acid phosphatase)-positive multinucleated cell, whereas osteoclasts at the early stage were not affected by the exposure to SFAs, suggesting the stimulatory effects of SFAs on osteoclastogenesis by acting on mature osteoclasts, rather than their early precursors. SFAs also promoted osteoclast survival by inhibiting the apoptosis of mature osteoclasts by the signalling pathway of Toll-like receptor 4 (TLR-4), myeloid differentiation factor 88 and NF- $\kappa$ B (MyD88) (Oh et al., 2010).

## **1.9.2 The effect of polyunsaturated fatty acids on bone**

### **1.9.2.1 Biochemistry of essential fatty acids**

Polyunsaturated fatty acids (PUFAs) can be classified into two major families, including the n-6 fatty acids and the n-3 fatty acids. EFAs such as LA and ALA are the PUFAs that cannot be synthesized endogenously due to human body lacking the ability to insert a double bond before carbon-9 in the fatty acid chain; therefore they are only obtained from dietary intake. LA (C18:2, n-6) and ALA (C18:3, n-3) serve as the precursors that can be converted to numerous downstream PUFAs, various eicosanoids (e.g. LTs, lipoxins, PGs and resolvins) and decosanoids through a series of elongation and desaturation steps by the activity of COX and lipoxygenases (LOX). Various vegetable oils (e.g. corn, sunflower, sesame and safflower) contain high concentrations of n-6 fatty acids, which are commonly converted to AA (C20:4 n-6) in body. EPA (C20:5 n-3) and DHA (C22:6 n-3) are synthesized from ALA and are mainly found in cold-water fish (e.g. salmon, tuna herring and mackerel) and fish oils (Weiss et al., 2005; Maggio et al., 2011).

The EFAs are an essential component of the membrane structure in the body, such as the membrane of cells, mitochondria and nuclei as well as of the endoplasmic and sarcoplasmic reticulum (Kruger & Horrobin, 1997). They govern the physicochemical properties of membranes and cell-cell communication by the critical features of the chain length and the number and position of *cis*-double bonds. For example, the fatty acyl unsaturation of EFAs provides a fluid lipid bilayer phase that allows the various membrane proteins, such as enzymes, receptors and ion channels to function optimally. Na<sup>+</sup>, K<sup>+</sup>-ATPase activity is sensitive to the unsaturation of EFAs, while Ca<sup>2+</sup>-ATPase is influenced more by the chain length rather than the degree of unsaturation (Stubbs & Smith, 1990).

### **1.9.2.2 Epidemiological studies**

Bone mineral accrual during childhood and adolescence seems to have a critical effect on preventing osteoporosis in later life. Estimated dietary intakes of PUFAs have been indicated to affect bone growth and modelling in humans (Gunnes & Lehmann, 1996; Högström et al., 2007; Eriksson et al., 2009).

### **a. Bone mineral density**

Both cross-sectional and longitudinal studies conducted in children and adolescences have shown that dietary PUFA intake have positive effects on bone growth (Gunnes & Lehmann, 1996; Högström et al., 2007). It was reported that cortical BMD gain peaked at the age of 16 and 14 years in boys and girls, respectively. In girls, cortical and trabecular BMD values had achieved adult premenopausal values by the age of 16.5 and 15 years, respectively. Dietary PUFA intake was significantly and positively correlated with trabecular BMD accretion in the forearm in 11-year-old girls, but not in boys. 4.8% of trabecular BMD gain was attributed to the intake of PUFAs in those girls. The Northern Osteoporosis and Obesity Study including 78 young men aged 16.7 years showed that the serum concentration of n-3 fatty acids, particularly DHA, were positively associated with BMD of the spine and total body and BMD accumulations at the spine after 8 years of follow-up (Högström et al., 2007). In 85 healthy 8-year old Caucasian children, the total n-6 fatty acids concentration, LA concentration and the ratio of n-6/n-3 fatty acid were negatively associated with BMD. While the AA level and the ratio of AA/LA was positively related with total body BMD and BMC (Eriksson et al., 2009).

In adults, numerous observational studies have reported the relationship between PUFAs and bone density with inconsistent results. In a cross-sectional study, Nawata et al. (2013) found that among healthy Japanese postmenopausal women, daily n-3 PUFAs intake was positively associated with BMD at both the lumbar spine and the femoral neck, while n-6 fatty acid intake was positively correlated with BMD at the femoral neck but not at the lumbar spine. Jarvinen et al. (2012) found a similar positive effect of PUFAs on BMD in the total body and at the lumbar spine, but not at the femoral neck among elderly women without hormone therapy in the Kuopio OSTPRE Fracture Prevention Study. Among the individual fatty acids assessed, increased intakes of ALA (n-3) and LA (n-6) were positively associated with higher BMD for the total body and at the lumbar spine.

Most PUFAs and bone studies have focused on older women, but several recent studies includes both sexes and report sex-specific differences in the effect of PUFAs on bone. The Framingham Osteoporosis Study (FOS) was conducted among a total of 854 old adults (average 75 years of age) to investigate the association between

individual PUFA intake and fish consumption and BMD at the trochanter and femoral neck (Farina et al., 2011b). There was no significant cross-sectional association between BMD and the intakes of PUFAs or fish in either sex. However, a significant interaction was found between AA and EPA+DHA intakes in women. Farina et al. (2012) also assessed the relationship between plasma PUFA concentrations and femoral neck BMD in the FOS among 765 older adults. They found that there were no significant associations between the plasma levels of LA, DHA and AA, and BMD within women in adjusted models. However, men with higher plasma AA levels tended to have a higher BMD at the femoral neck than those with the lowest plasma AA ( $p$ -trend=0.06).

The cross-sectional studies have inherent limitations due to their study design. It is also important to understand that the relationships between PUFA and BMD change over time. The Aberdeen Prospective Osteoporosis Screening Study, a longitudinal study, was conducted in 891 healthy, mainly women aged 45-55 years (Macdonald et al., 2004). After 5-7 years of follow-up, it was observed that dietary intakes of PUFAs and monounsaturated fatty acids (MUFAs) were negatively associated with BMD change at the lumbar spine, suggesting a harmful effect of PUFA consumption on bone health. The longitudinal analysis of the FOS study showed similar results that women with greatest LA intakes tended to lose more BMD at the femoral neck over 4 years ( $p$ -trend=0.06) than those with the lowest intakes. Other EFAs were not associated with BMD among women. Moreover, no significant interactions were observed between EPA+DHA intakes and LA or AA intakes in women. However, men with higher intakes of EPA, DHA or EPA+DHA had less loss of femoral neck BMD. Higher AA intakes were also protective against BMD loss ( $p$ -trend=0.06) in men. Further, among men an interaction between EPA+DHA and AA intakes was found (Farina et al., 2011b). These reports imply a sex specific difference between BMD and PUFA intakes. This negative relationship between PUFAs and BMD observed in women were partially explained by the higher concentrations of lipid peroxidation, which commonly occurs in postmenopausal women with abnormal estrogen levels (Farina et al., 2012).

The increasing ratio of total n-6/n-3 fatty acids in the diet has shown to have a negative effect on BMD in women (Weiss et al., 2005). However, Farina et al.

(2012) reported that no significant association was observed between dietary or plasma n-6/n-3 fatty acids ratio and femoral neck BMD status with both cross-sectional and longitudinal analysis among men and women. Moreover, the optimal ratio that would be of benefit for bone health is unknown and it has increased approximately from 1 to 10 during the past 100 years (Simopoulos, 2006).

### **b. Bone fracture risk**

Several studies have investigated the association between PUFAs and the risk of fracture. However, there is no clear consensus as some of the studies show protective effects of AA and ALA on fracture (Farina et al., 2011a, 2012), while other studies show no effect (Orchard et al., 2010).

The study of Women's Health Initiative reported that the higher dietary intakes of total PUFAs, total n-6 fatty acids or n-6/n-3 ratio were associated with lower total fracture risk ( $p$ -trend=0.019, 0.009 and 0.001, respectively), whereas higher intake of EPA+DHA was correlated with greater total fracture risk ( $p$ -trend=0.010). No significant association was found between individual PUFA intake or n-6/n-3 ratio and hip fracture risk (Orchard et al., 2010).

In the longitudinal Framingham Study, the elderly (aged a mean of 75 years) in the highest quartile of ALA intake had 54% lower fracture risk compared with those in the lowest quartile. AA intake was significantly related with decreased risk of hip fracture within men (80% lower fracture risk in the highest vs. lowest quartile of intake,  $p$ -trend=0.05), but not women. However, other individual PUFA or the ratio of n-6/n-3 fatty acids were not associated with decreased hip fracture risk. The highest level of plasma AA in older adults was associated with a 51% lower risk of hip fracture, but older adults in the highest quintile of plasma LA concentrations had over twice the fracture risk than those in the lowest quintile ( $p$ -trend=0.05). The adjustment for BMI attenuated this association. These results above suggest a protective effect of some individual PUFAs against hip fracture (Farina et al., 2011a).

Inconsistently, two large cohort and long-term studies, the Nurses' Health Study and the Health Professionals Follow-up Study showed that there were no significant associations between any total or individual PUFA intakes and hip fracture risk in

men alone and in the total sample. However, women with low intakes of total PUFAs, total n-6 fatty acids and LA had increased risk of hip fracture ( $p$ -trend=0.05, 0.07 and 0.04, respectively) (Virtanen et al., 2012).

### **1.9.2.3 Intervention studies**

#### **a. Human studies**

In a human randomised controlled trial (RCT), the femoral neck BMD was maintained in the intervention group with PUFA intake compared with a significant decrease in the placebo group (Lappe et al., 2013). The intervention group also had significantly increased BMD at Ward's triangle (+2.3%) compared with those in the control group who had bone loss (-1.1%). Moreover, the interventional diet had significantly positive effects on bone turnover showed by biomarkers (e.g. bone alkaline phosphatase and type I collagen cross-linked N-telopeptide). However, some studies showed no effect of PUFAs supplementation on BMD or bone markers (Bassey et al., 2000; Poulsen et al., 2007).

Diets rich in very long-chain PUFAs, such as EPA (C20:5, n-3) and DHA (C22:6, n-3) showed positive effects on bone mass (Lappe et al., 2013), but not ALA (C18:3, n-3). Although ALA can be converted into EPA and further DHA, the conversion is inefficient. Just 6% of the dietary intake of ALA can be converted to EPA and 3.8% can be converted to DHA in human with high dietary SFA intake (Poulsen et al., 2007). Additionally, high-ALA diets seem to provide more substance for conversion to EPA, while inhibit further conversion from EPA to DHA by suppressing the last steps of C24:5 n-3 desaturation in DHA synthesis (Gibson et al., 2011).

#### **b. Animal studies**

In spite of the lack of evidence showing the effects of dietary EFAs on bone metabolism in humans, a large number of studies have been conducted in animals.

A diet supplemented with 10% flaxseed oil (main source of ALA) in growing mice showed no effect on bone strength or mass at femurs and lumbar vertebrae compared with a 10% corn oil diet (Cohen & Ward, 2005). However, n-3 PUFAs with a longer carbon chain length such as DHA are positively associated with BMC in the femurs of growing rats (Li et al., 2003). In the lipid composition analysis of femoral marrow and periosteal tissue, a diet rich in DHA resulted in a higher DHA

content in the long bone tissue compartments. This increased level of DHA in femoral tissues was significantly associated with greater femur BMC. A higher content of total n-3 PUFA also had a positive effect on femur BMC. Rats fed with docosapentaenoic acid (DPA, C22:5, n-6) had higher DPA content in femoral marrow and periosteal tissue, whereas they had the lowest femur BMD and BMC concentrations compared with other treatments. Moreover, the ratio of n-6/n-3 PUFAs in different femoral compartments, including marrow neutral lipids and periosteal polar lipids, were inversely correlated with femur BMC. Green et al. (2004) reported that male weanling rats fed with a diet containing n-6/n-3 ratio of 1.4:1.0 had greater femur BMD than rats fed with the ratio of 7.1:1.0. However, no effect of the diet rich in n-3 fatty acids on femur weight, length, bone area (BA) and BMC was observed.

Numerous studies have indicated that a high dietary intake of the very long-chain n-3 PUFAs such as EPA and DHA is beneficial for maintaining BMC and BMD in OVX mature rodents (Sun et al., 2003; Watkins et al., 2005; Watkins et al., 2006). Poulsen et al. (2006) studied the effect of EFA alone on BMD in OVX rats. No difference in BMD was observed between rats fed with low-EFA diet (0.1 g/body weight EFA+0.5% calcium) and OVX controls (0.5% calcium alone). Rats fed with high-EFA diet (1.0 g/body weight EFA+0.5% calcium) had a significantly lower BMD than OVX controls and sham controls, suggesting a potential detrimental effect of high doses of pure EFA on OVX-induced bone loss. A dietary ratio of 5:1 n-6/n-3 long-chain PUFAs significantly increased the concentration of DHA in femoral marrow, periosteum, and preserved bone mass in OVX rats compared with the ratio of 10:1 (Watkins et al., 2006).

#### **1.9.2.4 Mechanisms of action of polyunsaturated fatty acids in bone metabolism**

##### **a. Effects of USFAs on calcium balance**

The dietary intake of EFAs affects calcium metabolism in the body (Claassen et al., 1995a; Claassen et al., 1995b; Kruger et al., 1995; van Papendorp et al., 1995). Animal studies found that fish oil rich in EPA and DHA may increase intestinal calcium absorption and decrease faecal and urinary calcium excretion, thus enhancing overall calcium balance (Kruger et al., 1995; van Papendorp et al., 1995).

The ratio of n-6/n-3 PUFAs seems to be an important factor for bone status. A GLA:EPA ratio of 3:1 resulted in 41.5% increase in intestinal Ca absorption, 27% decrease in faecal Ca excretion compared with the LA:ALA ratio of 3:1 (Claassen et al., 1995a; Claassen et al., 1995b). There was a negative relationship between dietary n-3 PUFA level and urinary calcium. The calcium balance and calcium content in bone ash significantly increased in the 3:1 GLA:EPA group by 41.5% and 24.7%, respectively, compared with the control (Claassen et al., 1995a).

Calcium absorption in the duodena mainly consists of three steps: uptake via calcium channels in the brush border membrane, transport by the carrier protein (e.g. Ca binding protein) through the cell, and extrusion of Ca through the basolateral membrane by Ca-ATPase and the Na-Ca exchanger (Kruger & Horrobin, 1997; Haag et al., 2003). The *in vitro* study has suggested that DHA increased intestinal Ca absorption by promoting Ca-ATPase activity in basolateral membrane compared with EPA-treated or non-treated membranes (Haag et al., 2003).

Several factors such as cyclic AMP and PTH have effects on the regulation of Ca reabsorption in the kidneys. PGs can regulate the intra-renal cyclic AMP concentration. Particularly, high urinary PGE<sub>2</sub> level positively correlates with renal Ca excretion (Kruger & Horrobin, 1997). The dietary intake of EPAs can affect PG synthesis. EPA is the precursor of PGE<sub>3</sub>, PGI<sub>3</sub> and LTC<sub>5</sub>, which elicit different biological activity to their structurally analogous counterparts such as PGE<sub>2</sub> and PGI<sub>2</sub> derived from AA. The increase in dietary EPA intake can change the eicosanoid metabolites from the n-6 to n-3, thus the production of PGE<sub>2</sub> is decreased, leading to the reduction of urinary calcium excretion. Moreover, EPA can incorporate into the renal cell membranes, changing the membrane composition by replacing AA in the phospholipid fractions. In the presence of high intracellular Ca, changes in membrane composition may result in the release of EPA via a Ca-dependent way, affecting PG synthesis (Kruger & Horrobin, 1997).

#### **b. Effects of PUFAs on bone metabolism**

Bone density is defined by the balance between bone formation and bone resorption, which is regulated by numerous specific systemic hormones (e.g. PTH, calcitonin, insulin, growth hormone, sex steroids, etc.) and local factors (e.g. IGF-1, TGF-β

super family, cytokines, PGs, etc.) produced in bone. Studies in human and animal models have shown that EFAs have beneficial effects on bone metabolism measured by changes in the levels of bone biomarkers.

Subjects that consumed low-SFA milk containing fish oil, oleic acid and vitamins for 1 year had a significant increase in plasma OPG (an inhibitor for bone breakdown) by 17.7%, RANKL (a stimulator for bone resorption) by 7% and the ratio of OPG/RANKL by 10%. Osteocalcin (a marker for bone formation and bone turnover) significantly increased by 22% in the intervention group. However, subjects consuming skimmed milk had no significant change in all bone turnover markers. In this study, milk fortified with n-3 fatty acids improved bone formation markers. However, it was difficult to attribute this beneficial effect on bone to the fortification of EPA and DHA or to the decreased amount of SFA and the increased level of oleic acid in the fortified milk compared with control milk (Martin-Bautista et al., 2010). A smaller human trial showed that an interventional group supplemented with 900 mg n-3 PUFAs had no change in bone formation markers, including serum osteocalcin or bone-specific ALP, while the level of urinary pyridinoline (Pyd, a bone resorption marker) significantly reduced. These results indicate that supplementation of n-3 PUFAs may inhibit bone resorption, but has no effect on bone formation (Salari Sharif et al., 2010). Similarly, Griel et al. (2007) found that middle-age adults that consumed high levels of plant-sourced dietary n-3 PUFAs (e.g. ALA) and consequently reduced the ratio of n-6/n-3 for 6 weeks had significantly decreased serum N-telopeptides (NTx, a bone resorption marker for type I collagen breakdown) and maintained levels of serum ALP, suggesting that n-3 PUFAs have protective effect on bone metabolism by reduction of bone resorption and maintenance in bone formation.

Watkins et al. (2000a) confirmed the results of their human studies in animal trials. They reported that the high dietary intake of n-3 PUFAs or the low dietary ratio of n-6/n-3 (1.19 vs. 23.76) was positively associated with activity of serum bone-specific ALP and negatively associated with bone PGE<sub>2</sub> production in growing male rats, suggesting the beneficial effects of n-3 PUFAs on bone formation. Inconsistently, an animal trial investigated the effects of different ratio of GLA:EPA on bone status in growing rats measured by urinary excretion of bone resorption

markers such as Pyl, deoxypyridinoline (Dpyd) and hydroxyproline (Hyp) (Claassen et al., 1995b). Male rats fed with high GLA:EPA ratios (1:1, 3:1 and 1:3) had increases in BMC and a significant decrease in bone matrix collagen degradation compared with controls fed with a LA:ALA ratio of 3:1. In OVX rats, loss of estrogen can increase collagen degradation and bone turnover rate. Dietary supplementation of GLA and EPA combined with estrogen-implanted corrected bone loss due to OVX through significantly reducing the urinary excretion of both Hyp and Dpyd and lowering serum osteocalcin levels compared with both sham and OVX control. Therefore, EFAs potentiated the effect of estrogen on bone status and affected both bone formation and bone resorption (Schlemmer et al., 1999).

### **c. Effects of PUFAs on osteoblasts**

The mesenchymal stem cell (MSC) is the precursor that can differentiate into either osteoblasts or adipocytes. During the process of aging, MSCs tend to differentiate into adipocytes instead of into osteoblasts, thus the amount of adipose in bone marrow increases and osteoblasts decrease, which contributes to osteoporosis in the elderly (Maurin et al., 2002; Shen et al., 2006). Peroxisome proliferator-activated receptors (PPARs), including three iso-types of PPAR- $\alpha$ , PPAR- $\beta/\delta$  and PPAR- $\gamma$  in the mammals, are expressed by osteoblasts (Jackson & Demer, 2000; Maurin et al., 2005). The activation of PPARs (e.g. PPAR- $\alpha$ , PPAR- $\delta$  or PPAR- $\gamma$ 1) by the binding to specific ligands can promote differentiation of pre-osteoblasts into mature osteoblasts with enhanced ALP activity and matrix calcification, while the activation of PPAR- $\gamma$ 2 can stimulate differentiation of mesenchymal progenitors into adipocytes (Jackson & Demer, 2000; Poulsen et al., 2007; Casado-Díaz et al., 2012).

*In vitro* studies have described PUFAs and their metabolites of PG as endogenous ligands for the PPARs (Yu et al., 1995; Maurin et al., 2002; Poulsen et al., 2007). DHA and AA decreased the proliferation of human primary osteoblastic cells in a dose-dependent way, but did not lead to cell apoptosis (Maurin et al., 2002). Therefore, the age-related bone loss is potentially due to the release of PUFAs by mature adipocytes, which inhibited osteoblast proliferation (Maurin et al., 2002). The short-term anti-proliferative effects of these PUFAs were attributed to the inhibition of the G1-to-S-shape transition in the cell cycle. Moreover, both DHA and AA have been shown to activate PPAR-  $\gamma$  (Yu et al., 1995; Maurin et al., 2005). For the

long-term effects of PUFAs on osteoblastogenesis, the cell cycle withdrawal and cessation of cell proliferation are associated with induction of osteoblastic differentiation (Maurin et al., 2005).

Additionally, the n-3 PUFAs promote osteoblastogenesis by preventing the formation of products that inhibit osteoblastogenesis. n-3 PUFAs indirectly inhibit production of the LT family formed by 5-LOX activity, which can inhibit the bone-forming activity of osteoblasts (Poulsen et al., 2007). DHA and EPA also favour the osteoblastogenesis via the increase in the expression of osteogenic markers and the OPG/RANKL ratio in osteoblasts, whereas AA promotes osteoclastic activity by decreasing OPG/RANKL ratio and stimulates the differentiation of human mesenchymal stem cell into adipocytes (Casado-Díaz et al., 2012).

#### **d. Effects of PUFAs on osteoclasts**

*In vitro* studies have shown that n-3 PUFAs such as DHA and EPA inhibited osteoclastogenesis and osteoclast activation induced by RANKL in the culture of murine bone marrow cells and the monocytic cell line RAW 264.7 compared with the n-6 PUFAs LA and AA (Sun et al., 2003; Rahman et al., 2008). N-3 PUFAs have been reported to have an anti-inflammatory effects due to the inhibition in pro-inflammatory cytokines gene expression (e.g. TNF- $\alpha$ , IL-1 and IL-6), which increase the COX-mediated production of PUFA metabolites, including PGE<sub>1</sub>, PGE<sub>2</sub> and PGE<sub>3</sub> (Sun et al., 2003; Yuan et al., 2010). PGE<sub>2</sub> has been shown to have a biphasic effect that it promotes osteoblastogenesis in low concentrations and promotes bone resorption in high concentrations (Poulsen et al., 2007). PGE<sub>2</sub>, together with TNF- $\alpha$ , increase the gene expression of RANKL in both stromal cells and pre-B-cells, which promotes osteoclast progenitors by the RANK-RANKL interaction and stimulates the maturation of osteoclasts. It also decreases the secretion of OPG, thus affecting the balance between OPG-RANKL signalling (Sun et al., 2003). Compared with EPA, DHA strongly inhibits osteoclastogenesis at the late stage of cell-cell fusion to multinuclear osteoclasts, which was identified by gene expression analysis (Akiyama et al., 2013). Furthermore, resolvin D1 and neuroprotectin D1, which are metabolites of DHA via the LOX pathway and act as

anti-inflammatory mediators, have been indicated to inhibit RANKL-induced osteoclastogenesis (Yuan et al., 2010)

In addition, the NF- $\kappa$ B signalling pathway also has an essential role in the generation and activation of osteoclasts. Sun et al. (2003) reported that DHA and EPA suppressed RANKL-induced NF- $\kappa$ B activation in the culture of murine bone marrow-derived macrophages, while LA and AA had no effect. Consistently, the TRAP enzyme activity and osteoclastogenesis measured by TRAP staining in primary bone marrow cells, which are considered as markers for osteoclast maturation, were inhibited by DHA and EPA, whereas LA and AA had no effect (Sun et al., 2003).

AA and GLA may increase osteoclastogenesis via stimulating the expression of membrane-bound RANKL and inhibiting secretion of OPG by osteoblasts indirectly, therefore decreasing the ratio of OPG/RANKL. However, both DHA and EPA have no effect on membrane-bound RANKL secretion, although DHA may inhibit OPG secretion, suggesting a protective effect on bone (Coetzee et al., 2007; Poulsen et al., 2008).

## **1.10 The relationship between dairy proteins and bone**

### **1.10.1 Epidemiological studies**

Approximately 22-30% of bone mass and about 50% of the volume of bone is protein (Dawson-Hughes, 2003; Heaney & Layman, 2008). During continuous bone matrix turnover and remodelling, cross-linking of collagen molecules undergoes posttranslational amino acid modifications, involving hydroxylation of proline and lysine, whereas collagen fragments released by proteolysis cannot be reutilized to form new bone matrix. Therefore, daily dietary protein intake is needed to maintain bone status (Heaney & Layman, 2008). Insufficient or low intake of protein due to total dietary nutrition insufficiency is associated with frailty and osteoporotic fracture in elderly people (Hannan et al., 2000)

Observational studies have shown that long-term high consumption of dairy products increased protein intake in pre- and peri-pubertal children, which was associated with increased lumbar spine BMD, total body BMC and bone geometry (Chan et al., 1995; Alexy et al., 2005; Bounds et al., 2005), suggesting that dietary protein intake

could be important for stimulating bone modelling and increasing bone strength in childhood and adolescence.

Among studies of adults, evidence for the association between protein consumption and bone mass and fracture risk is inconsistent, including protection, harm and no effect. Hannan et al. (2000) reported that higher intakes of both animal and total protein were associated with increased BMD in the femoral neck and spine in the elderly in the Framingham Osteoporosis Study. Similarly, the Rancho Bernardo Study has indicated that for every 15 g/day more in animal protein intake increased BMD at the hip, the femoral neck, the spine and total body, whereas vegetable protein had no effect on BMD in the elderly (Promislow et al., 2002). Rapuri et al. (2003) found that postmenopausal women with a higher protein intake had a significant increase in BMD at the spine (7%), mid-radius (6%) and total body (5%) compared with those had lower protein intake in the cross-sectional study, whereas no effect of protein intake on the rate of bone loss was observed in the longitudinal study. Sellmeyer et al. (2001) reported that there was no significant association between the ratio of animal to vegetable protein intake and BMD among the elderly women in the Study of Osteoporosis Fractures. Recently, Darling et al. (2009) conducted a systematic review of all relevant studies and indicated that the protein intake was significantly and positively associated with BMD or BMC at the main clinical relevant sites in the cross-sectional surveys. The dietary intake of protein explained 1-2% of BMD. The cohort studies also showed no deleterious influence of protein intake in bone (Darling et al., 2009).

Increased dietary protein was related with an increased risk of forearm fracture in women in the Nurses' Health Study, particularly the intake of animal protein (Feskanich et al., 1996). However, no relationship was observed between protein intake and hip fracture incidence. Sellmeyer et al. (2001) observed a higher bone loss rate at the femoral neck and an increased risk of hip fracture in women with a high dietary ratio of animal to vegetable protein compared with those with the low ratio. In contrast, the Framingham Osteoporosis Study reported that elderly with a higher dietary protein intake had decreased hip fracture risk than those with a lower intake of protein (Misra et al., 2011). There was no relationship between dietary intake of

protein and fracture risk in either the systematic review or the meta-analysis (Darling et al., 2009).

### **1.10.2 Intervention studies**

A meta-analysis showed a significant positive effect of protein supplementation on BMD at the lumbar spine, while no effect was observed on hip fracture risk. No effect of milk basic protein (MBP), mainly milk whey protein, or soy supplementation alone was found on lumbar spine BMD (Darling et al., 2009).

Aoe and colleagues (Aoe et al., 2001; Yamamura et al., 2002; Aoe et al., 2005; Uenishi et al., 2007) conducted a series of RCTs in healthy young and menopausal women to investigate the effects of MBP supplementation on BMD and bone metabolism. MBP is the basic protein fraction in milk and mainly consist of whey protein, which is a by-product of cheese or casein manufacture (Aoe et al., 2001). Both young and menopausal women with 40 mg/day MBP supplements for 6 months had a higher rate of BMD gain in the lumbar vertebrae, left calcaneus or radius than those in placebo group (Aoe et al., 2001; Yamamura et al., 2002; Aoe et al., 2005; Uenishi et al., 2007). There was no difference between the two groups in bone formation markers such as serum osteocalcin and bone-specific ALP in menopausal women (Aoe et al., 2005), while in young women aged  $21.3 \pm 1.2$  years in the MBP group had significantly increased levels of serum osteocalcin at 6 months (Uenishi et al., 2007). The bone resorption marker NTx or Dpyd in urine significantly decreased in the MBP group of both young and menopausal women at 6 months compared with the placebo group (Aoe et al., 2001; Yamamura et al., 2002; Aoe et al., 2005; Uenishi et al., 2007). These results suggest that daily MBP supplementation in adult women is effective in preventing bone loss and that the increase in BMD seems to be modulated through potent inhibition of bone resorption and moderate promotion of bone formation by MBP supplementation.

In contrast, Zou et al. (2009) reported that there was no effect of daily 40 mg MBP supplementation on BMD and bone metabolism in young health women (mean age  $19.6 \pm 10.6$  years), although it was effective in inhibiting bone resorption.

### **1.10.3 Mechanisms of proteins in bone metabolism**

#### **1.10.3.1 Dietary protein optimises IGF-1 levels**

Serum IGF-1 plays a key role in regulating bone metabolism and is an important determinant for bone growth and density (Yakar et al., 2002). Dietary protein can affect bone metabolism through regulating the hepatic production and action of IGF-1. In the rat model, protein restriction seems to decrease bone mass and strength by an early reduction in plasma IGF-1, impairs cortical bone formation by inducing osteoblast resistance to IGF-1 and result in a later increase in bone resorption, suggesting an uncoupling between bone formation and resorption (Ammann et al., 2000; Bourrin et al., 2000). Protein restriction has been indicated to decrease plasma levels of IGF-1 by inducing a hepatic resistance to the action of growth hormone, and by an increased metabolic clearance rate of IGF-1 (Ammann et al., 2000; Rizzoli & Bonjour, 2004). On the other hand, increasing protein consumption is positively related to circulating IGF-1 levels (Hoppe et al., 2004a; Hoppe et al., 2004b; Budek et al., 2007). It has been shown that a high consumption of milk or total protein, but not meat protein increased serum IGF-1 concentrations in children, suggesting a differential effect of milk protein on bone metabolism compared with the intake of meat protein (Hoppe et al., 2004a; Hoppe et al., 2004b; Budek et al., 2007).

#### **1.10.3.2 Protein and intestinal calcium absorption**

It have been reported in human studies that high protein (daily 2.1 g/kg diet) promoted intestinal calcium absorption compared with the low (daily 0.7 g/kg diet) or moderate protein diet (daily 1.0 g/kg diet) assessed by dual isotope methods (Kerstetter et al., 1998; Kerstetter et al., 2005). Low protein intake (daily 0.7 g/kg diet) reduced calcium absorption, but increased the circulating levels of PTH and  $1,25(\text{OH})_2\text{D}_3$ , which could increase calcium absorption in turn. However, Heaney (2000) and Dawson-Hughes et al. (2002) found no effect of protein intake on calcium absorption. Heaney (2000) conducted a longitudinal study in 191 middle-aged Roman Catholic nuns and reported that there was no association between intestinal calcium absorption and dietary intake of protein. The average daily protein intake was about 1 g/kg diet, and the mean calcium intake was 17.6 mmol/day. Dawson-Hughes et al. (2002) had similar findings in the elderly with the age over 65 years. The discrepancies between studies were explained by the large

inter-individual variability in calcium absorption and various factors that may influence calcium absorption (Kerstetter et al., 2005; Thorpe & Evans, 2011).

The evidence of animal studies support the positive effect of protein intake on intestinal calcium absorption. In rats, increasing dietary protein improved calcium absorption in the intestine through increasing transcellular calcium uptake (Bennett et al., 2000; Gaffney-Stomberg et al., 2010).

#### **1.10.3.3 Protein and urinary calcium excretion**

*In vivo* studies in both human and animal models have shown that increasing dietary protein intake increased urinary calcium excretion (Kerstetter et al., 1998; Bennett et al., 2000; Kerstetter et al., 2005; Gaffney-Stomberg et al., 2010; Cao et al., 2011). However, faecal calcium significantly decreased during the high protein diet compared with the low protein diet (Kerstetter et al., 1998; Gaffney-Stomberg et al., 2010), suggesting that high-protein diets induce a shift of the endogenous excretion of calcium from the faeces to the urine (Gaffney-Stomberg et al., 2010).

#### **1.10.3.4 Regulation of bone cells by dietary protein**

Several *in vitro* studies have shown the effect of MBP on bone cells (Takada et al., 1996; Kawakami, 2005; Xu, 2009). The protein induced a dose-dependent increase in proliferation and differentiation of osteoblastic MC3T3-E1 cells. It also stimulated bone formation by promoting the production of osteocalcin and IGF-1 and increasing the ratio of OPG/RANKL (Xu, 2009). Moreover, MBP increased the content of collagen-specific Hyp and total protein in the osteoblasts (Takada et al., 1996).

The effects of MBP on the activity of osteoclasts and bone resorption were studied in both *in vivo* and *in vitro* studies (Takada et al., 1997; Toba et al., 2001). It has been reported that MBP was effective in inhibiting the formation of osteoclasts and suppressing osteoclast-mediated bone resorption, suggesting that MBP maintained the balance of bone remodelling by the suppression of bone resorption and the stimulation of bone formation (Takada et al., 1997; Toba et al., 2001).

## **Section 4. Motivation and objectives**

### **1.11 Objectives of the study**

The main objective of this study was to investigate the effects of a milk lipid fraction (MLF) on bone status in growing rats. The experiments were conducted both *in vivo* and *in vitro*, including:

1. Animal study to evaluate the effects of MLF on bone growth and bone parameters such as BMC, BMD and biomechanical properties in growing rats.
2. *In vitro* cell culture experiments to evaluate the effects of MLF on the development and activity of bone cells, specifically osteoblasts and osteoclasts.

### **1.12 Hypotheses**

MLF consumption increases bone mass at the femur, lumbar spine and whole body in growing female rats.

The biomechanical strength of the femur would be greater among rats fed with the MLF diet.

The MLF stimulates the proliferation, differentiation and mineralization of MC3T3-E1 osteoblast.

The MLF inhibits osteoclastogenesis in RAW264.7 cells.

## Chapter 2. Materials and Methods

### 2.1 Animal study

#### 2.1.1 Animals

There were one hundred and eight 3-month old female Sprague-Dawley rats weighing at least 200 g were housed individually in shoe box cages, with *ad libitum* access to chow pellets and reverse-osmosis water. The rats were housed in a light-controlled room (12 hour day/night cycle) and at a temperature of  $22 \pm 2^{\circ}\text{C}$ . All rats were ranked by baseline femoral BMD, and then randomly allocated into three groups, including a control group (n=48), a MLF-250 group (n=30) and a MLF-500 group (n=30), so that there were minimal differences in BMD between each group. The trial ran for 13 weeks. The study length was chosen based on the results of previous studies conducted in our laboratory. 45 rats (n=15 for each group) were selected to euthanize for biomechanical testing at month 3, while 63 continued into a second arm of the trial after ovariectomy. The trial was approved by the Massey University Animal Committee, protocol 13/07.

#### 2.1.2 Diets

The rats were fed the control or test diets for 13 weeks.

##### Stage 1: One week acclimatization (Week 0)

All rats were allowed one week to acclimatize to a powdered control diet, having previously being fed a commercial rat chow manufactured by the Massey University Feed Mill to AIN-93G and AIN-93M specification and was formulated to contain 0.5% calcium (w/w) (standard for the housing facility).

##### Stage 2: Test diets feeding period (Week 1-12)

Rats were fed 20 g/day of the control diet or one of MLF diets, which were balanced for calcium, fat and protein.

During the course of the study rats in the control group were fed a standard powdered rat maintenance diet (0.5% calcium w/w). The MLF-250 group was fed 250 mg/rat/day of the test MLF diet, and the MLF-500 group was fed 500 mg/rat/day of the MLF diet. These doses were chosen based on dosages which had shown an

effect in a similar dietary intervention study in mice. Daily feed intake and body weight for each rat was recorded during the study period.

Dietary composition of the control and MLF diets is shown in Table 2.1, and typical composition of MLF is shown in Table 2.2.

**Table 2.1** Dietary composition of the control and MLF diets.

Ingredient	Control diet g/kg of diet	MLF diet g/kg of diet
Sodium caseinate	15.00	13.32
MLF		3.13 <sup>a</sup>
Cystine	0.27	0.27
Glycine	0.33	0.33
Methionine	0.15	0.15
Glutamine	0.70	0.70
Calcium carbonate	1.25	1.25
Cellulose	5.00	5.00
Vitamin mix	1.00	1.00
Mineral mix	5.00	5.00
Corn oil	5.00	3.87
Sucrose	4.00	4.00
Starch	62.30	61.99
Total	100.00	100.00

a: this MLF is 53.73% protein and 36.2% total fat (w/v) from a particular batch.

**Table 2.2** Typical composition of the MLF.

Composition	%
Protein	50.1
Total lipids	35.6
Saturated fatty acids	22.0
Monounsaturated fatty acids	9.4
Polyunsaturated fatty acids	2.1
Trans fatty acids	1.6
Cholesterol	0.12
Lactose	7.8
Ash	5.2
Moisture	2.7

## **2.1.3 Dual Energy X-Ray Absorptiometry (DEXA) Scans**

### **2.1.3.1 Procedures**

Animals (n=108) were scanned for baseline measurements at week 0. Repeat scans were conducted *in vivo* at week 11. Rats were weighed and anaesthetised with an appropriated dose of anaesthetic ie. 0.05 ml/100 g body weight. The anaesthetic was a mixture of 0.2 ml Acepromazine (ACP) + 0.5 ml Ketamine + 0.1 ml Xylazine + 0.2 ml sterile water, and was administrated via an intra-peritoneal injection using a 1 ml syringe with 25 G x 5/8 needle. In rats, the suitable level of anaesthesia occurred in approximately 5-10 minutes after injection and remained for up to 2 hours.

Bone mineral status was measured with a Hologic Discovery A bone densitometer (Hologic Inc, Bedford, USA). A daily Quality Control (QC) scan was conducted to ensure precision met with the required coefficient of variation. Rats were positioned supine with appropriate angles between the femur and tibia, and spine and femur. Each rat underwent a whole body scan and 3 regional high-resolution scans of the spine and left and right femurs. The coefficients of variations (CVs) for scans of lumbar spine and femoral BMD measurements were 0.42-0.46% at the baseline (Week 0) and 0.28-0.49% at Week 12.

### **2.1.3.2 Introduction of DEXA**

The fundamental principle of dual energy X-ray absorptiometry (DEXA) is to measure the transmission of X-rays with high- and low-photon energies (usually 100keV and 44keV) through the body in order to assess the amount of different body composition – bone mineral and soft tissue (Crabtree et al., 2007). Bone mineral is a physically dense composition primarily consists of calcium and phosphorus molecules, which have relatively higher atomic numbers. Soft tissue is a mixture of skin, fat, muscle and water, which have lower physical density and a lower effective atomic number compare with bone mineral, because their chemical constituents mainly are carbon, hydrogen and oxygen. Therefore, bone mineral and soft tissues have different attenuation of the X-ray beam at the same photon energy. The exponential equations are used to calculate body composition with various mass attenuation coefficients (Crabtree et al., 2007).

DEXA is considered as the “gold standard” for BMD measurements in humans (Adams, 2013). Several studies have shown that it provides a reasonably accurate

and precise technique (< 2% of error) to assess BMD at both peripheral and central sites in small animals (Bertin et al., 1998; Nagy & Clair, 2000).

The strengths of DEXA are short scan times (< 5 minutes) and low radiation exposure (1-6  $\mu\text{Sv}$ ). One limitation is that the measurements of bone are size-dependent as the DEXA image is a 2-dimensional image of a 3-dimensional object. It provides an “areal” bone mineral density (BMD;  $\text{g}/\text{cm}^2$ ) of integral (trabecular and cortical) bone rather than a true volumetric density of bone (Crabtree et al., 2007; Adams, 2013). There is no consideration about bone depth. This means that a small bone will have a lower areal BMD than a large bone (Crabtree et al., 2007). It has been suggested that adjusting BMC measurements of whole body for several factors such as bone area, bone area for height, height for age and body weight (Crabtree et al., 2007; Adams, 2013). Alternatively, the corrections can be made by calculating bone mineral apparent density (BMAD), or by the relation between BMC and lean muscle mass, because the loading of skeleton produced by muscular activity strongly predicts BMC (Adams, 2013).

## **2.1.4 Biomechanical Testing**

### **2.1.4.1 Procedures**

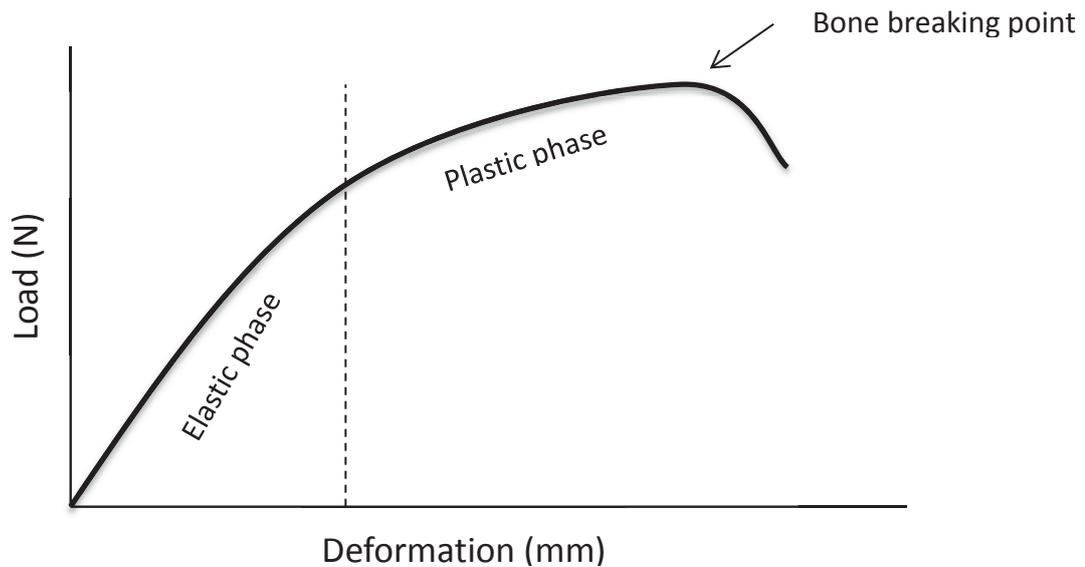
The left femurs of rats were scraped clean of flesh and stored in phosphate-buffered saline (PBS) at room temperature until testing. The wet weight, length, shaft width and thickness of femurs were measured using a balance with a readability of 0.0001 g and a digital calliper with a readability of 0.01 mm, respectively. The middle-point of femur diaphyseal was marked. A three-point bending test was conducted using a biomaterial testing machine (Shimadzu Ezi-test, Kyoto, Japan) to measure maximum load, maximum stroke, break load, break stress, break stroke, break strain, elasticity and energy absorption capacity of the femoral diaphysis. A preload (10 N) was first applied to the mid-shaft of femur with a support span of 15 mm and a test speed of 50 mm/min and then the bending load (60 N) was applied until failure.

### **2.1.4.2 Introduction of biomechanical testing**

The purpose of biomechanical tests is to assess the mechanical properties of bones. Various test techniques are available, such as tension, 3- or 4-point bending, compression, torsion and fatigue testing. In 3-point bending test, the bone is

positioned onto two supports, and then a single-pronged loading device is applied to the opposite surface at the precisely middle point between the two supports (Sharir et al., 2008).

The bending test includes three phases: elastic phase, plastic phase and ultimate bone breaking point (Rubin & Rubin, 2006; Sharir et al., 2008). During biomechanical testing, these three phases can be measured and plotted on a Load-Deformation Curve (Figure 2.1). Elastic phase occurs during the initial stage of a load, which bends the bone. There is a linear increase in deformation when the load increases, reflecting the stiffness of the bone. The stiffer the bone, the higher value of elasticity, and the steeper the linear portion of the load-deformation curve. However, if the load is removed during this phase, the bone will return to its original shape and size immediately, without permanent damage. The stiffness of a bone is determined by the percentages of the collagen fibres and the hydroxyapatite crystals in the composition, and their maturity and organization. When the load increases further, the bone moves to the plastic phase. The increase in deformation of the bone is no longer linear with the applied load. The bone is unable to revert to its original shape when the force is removed, and permanent damage begins to accrue within the bone. The plastic phase first occurs through ultrastructural microcracks in the hydroxyapatite and then the damage of the collagen fibres. As the load continues in the plastic phase, the bone will finally break, namely the ultimate breaking point. This bone breaking point is also considered as exceeding the ultimate strain or the ultimate stress, reflecting the mineral content of the bone.



**Figure 2.1** Load-Deformation Curve of three-point bending test, showing the 3 phases of bone breaking, including elastic phase, plastic phase and ultimate bone breaking point.

#### 2.1.4.3 Definition of Parameters Measured

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

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

Break Load (N): the force applied to bone that causes a break.

Break Stress ( $\text{N}/\text{mm}^2$ ): break load per unit area of bone.

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

Break Strain (%): the fractional change in dimension of the bone, i.e. degree of bending.

Elasticity ( $\text{N}/\text{mm}^2$ ): the intrinsic stiffness or Young's modulus of the bone, i.e. the maximum slope of the load-strain curve divided by area.

Plasticity ( $\text{N}/\text{mm}$ ): the extrinsic stiffness of the bone, i.e. the maximum slope of the load-deformation curve.

Energy (J) absorption: the total amount of energy absorbed by bone tissue before it breaks.

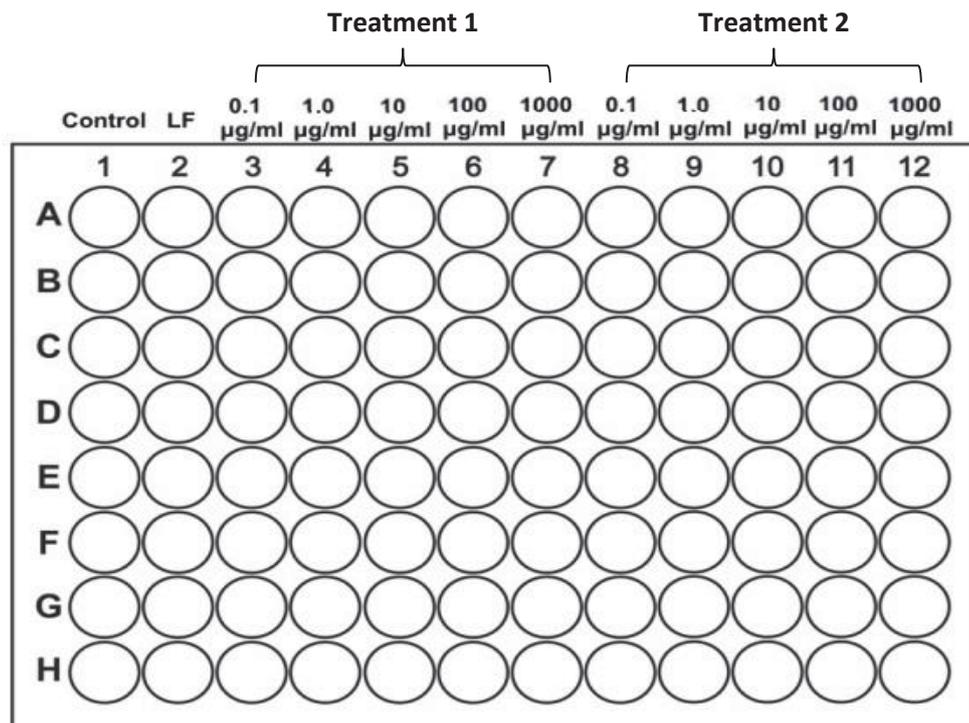
## **2.2 Cell culture**

### **2.2.1 MC3T3-E1 subclone 4 pre-osteoblast like cells**

#### **2.2.1.1 Colorimetric osteoblast proliferation assay**

##### **a. Cell culture**

The MC3T3-E1 sub-clone 4 murine pre-osteoblast like cells (ATCC® CRL-2593™) were grown in MEM $\alpha$  medium (Invitrogen 12571-063, Gibco) containing 10% heat-inactivated fetal bovine serum (FBS) (Invitrogen Cat. No 10093-144) supplemented with 0.25% gentamicin reagent solution (10 mg/ml; Invitrogen 15710-064). 100  $\mu$ l/well of cell suspension was seeded into 96-well flat bottom tissue culture plates at a density of  $0.4 \times 10^5$  cells/ml and incubated at 37°C under 5% CO<sub>2</sub> for 24 hours (day 1). The cell number was counted using hemocytometer. The experiment was conducted in triplicate (repeated in three culture plates) on the same day. On day 2, growth arrest was induced by removing the old MEM $\alpha$  plus 10% FBS media and replacing it with fresh MEM $\alpha$  plus 0.1% bovine serum albumin (BSA) media (0.20  $\mu$ m syringe filter [Raylab Cat. No. BE30CS20AS] sterilised) and incubated at 37°C under 5% CO<sub>2</sub> for 24 hours. On day 3, MLF powder was dissolved in warm MEM $\alpha$  with 0.1% BSA on a protein basis (53.73% of protein content in MLF) and vigorously agitated, a 1 mg/ml MLF stock solution was made (i.e. 1.8612 mg of MLF powder was required to dissolve in 1 ml media), sterilised using a 0.20  $\mu$ m syringe filter and then used to create test dilutions (0.1, 1.0, 10, 100, 1000  $\mu$ g/ml) in MEM $\alpha$  with 0.1% BSA. A 1,000  $\mu$ g/ml lactoferrin stock solution in MEM $\alpha$  with 0.1% BSA was prepared as a positive control and filter sterilised by 0.20  $\mu$ m syringe filter. The old media in the plates was replaced with 1,000  $\mu$ g/ml lactoferrin (repeat in 8 wells), 0 control (MEM $\alpha$  with 0.1% BSA, repeat in 8 wells) and MLF dilutions from low to high concentrations (each treatment was repeated in 16 wells) (see Figure 2.2). The plates were incubated for 48 hours at 37°C under 5% CO<sub>2</sub>.



**Figure 2.2** Arrangement of control and treatments in a 96-well cell culture plate in the osteoblast proliferation assay. Control: MEM $\alpha$  with 0.1% BSA; LF: lactoferrin 1 mg/ml. Test dilutions were repeated in twice on one culture palte (treatment 1 and 2).

### **b. Methyl-thiazolyl tetrazolium (MTT) assay**

Following the 48 hours of incubation with MLF, 10  $\mu$ l/well of MTT solution (5 mg/ml in PBS, 0.20  $\mu$ m filter sterilised) was added to the 96-well plates, and then incubated at 37°C under 5% CO<sub>2</sub> for 3-4 hours until the purple-blue crystals formed. In this assay, MTT is reduced by butanedioic acid reductase, which is produced in the mitochondria of living cells, to form amethyst formazan crystal. The plates were centrifuged for 10 minutes at 2000 rpm and the media carefully removed from each well. The crystals in the plates were dissolved by adding 100  $\mu$ l/well of dimethyl sulfoxide (DMSO) and the plate allowed to sit for 5-10 minutes at room temperature. The optical density was measured at 550 nm using a Elx 808 micro-plate reader (BioTek Instrument Inc, Vermont, USA). The value of optical density at 550 nm is directly proportional to the number of living osteoblast and indirectly reflects the reproductive activity of cells (Xu, 2009). Results were presented as the mean readings of optical density derived from triplicate 96-well cell culture plates.

### 2.2.1.2 Osteoblast differentiation assay

#### a. Reagents and recipes

##### 1% (v/v) formaldehyde

2.70 ml formaldehyde (37%) dissolved in 100 ml Milli-Q water

##### Tris Buffered Saline (TBS), pH 9.5

2.42 g Tris, 8.77 g NaCl, 100 ml Milli-Q water, 1 ml Triton-X 100, 0.1 g  $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$ , Adjust pH to 9.5 using 1M HCl

##### ALP test reagent (0.05M p-nitrophenyl phosphate [p-NPP] in TBS, pH 9.5)

139.18 mg of p-NPP dissolved in 7.5 ml TBS (pH 9.5)

##### 1% (w/v) crystal violet in PBS

1 g of crystal violet powder dissolved in 100 ml pre-warmed PBS (~37°C)

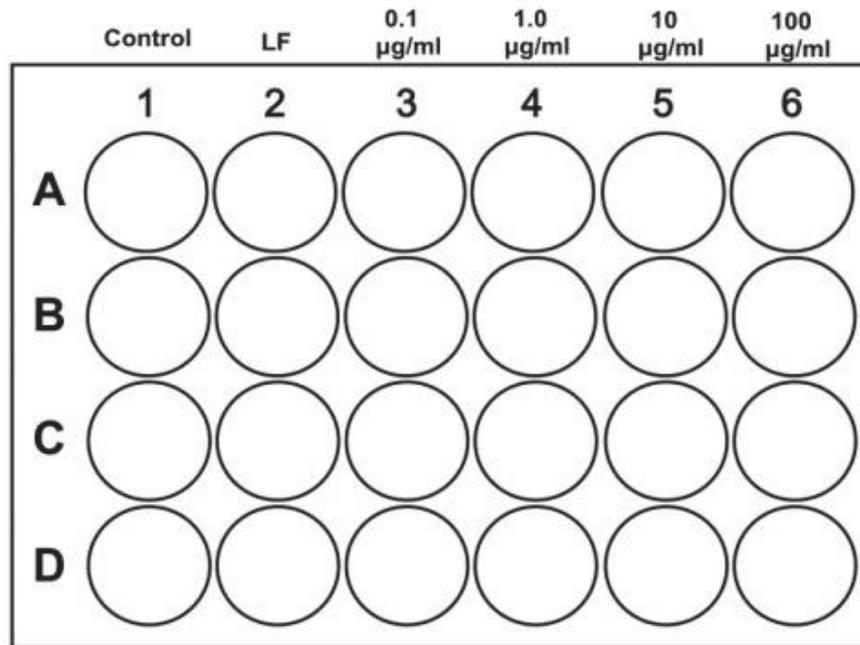
##### 0.2% (v/v) Triton-X 100

30  $\mu\text{l}$  of Triton-X 100 (100%) dissolved in 15 ml PBS

#### b. Cell culture

The murine osteoblast-like cells, MC3T3-E1 Clone 4 (ATCC® CRL-2593™) were cultured as previously described in the osteoblast cell proliferation assay. 1 ml/well of cell suspension was seeded into 24-well flat bottom culture dishes (surface area: 2  $\text{cm}^2$ ) at a density of  $0.2 \times 10^5$  cells/ml and incubated at 37°C under 5%  $\text{CO}_2$  for 72 hours (day -3). The cell number was counted using hemocytometer. The experiment was conducted in triplicate (repeated in three culture plates) on the same day. After 3 days (day 0), the cells became 80% confluent in each well. MLF powder was dissolved in the osteogenic media (MEM $\alpha$  with 10% FBS, 10 mM  $\beta$ -glycerophosphate, and 50  $\mu\text{g}/\text{ml}$  ascorbic acid on a protein basis (53.73% of protein content in MLF) at the concentrations of interest (0.1, 1.0, 10, 100  $\mu\text{g}/\text{ml}$ ), i.e. 1.8612 mg of MLF powder was required to dissolve in 1 ml osteogenic media to make a 1 mg/ml MLF stock. 100  $\mu\text{g}/\text{ml}$  lactoferrin stock solution in osteogenic media was prepared as a control and filter sterilised by 0.20  $\mu\text{m}$  syringe filter. The old media in the 24-well plates was replaced with i) lactoferrin positive control (100  $\mu\text{g}/\text{ml}$ ), ii) a 0 control (osteogenic media), and iii) MLF dilutions from low to

high concentration (0.1, 1.0, 10, 100  $\mu\text{g/ml}$ ). The control or each treatment was conducted in four replicates per plate (see Figure 2.3). The plates were then incubated for 72 hours at 37°C under 5% CO<sub>2</sub>. On day 3 and 6, the old osteogenic media was replaced with fresh osteogenic media prepared as in day 0 containing the dissolved lactoferrin (100  $\mu\text{g/ml}$ ), blank and MLF (0.1, 1.0, 10, 100  $\mu\text{g/ml}$ ) every 72 hours.



**Figure 2.3** Arrangement of control and treatments in a 24-well cell culture plate in the osteoblast differentiation and mineralization assay. Control: osteogenic media; LF: lactoferrin 100  $\mu\text{g/ml}$ .

### c. ALP activity assay

On day 9, the old osteogenic media in the 24-well plates were completely removed and the cells washed once with PBS (0.5 ml/well). The cells were fixed with 300  $\mu\text{l/well}$  with 1% formaldehyde for 15 minutes at room temperature.

Alkaline phosphatase measurement: the cells were washed again with PBS prior to incubation with 300  $\mu\text{l/well}$  of ALP test reagent for 1 hour at 37°C. Two samples (100  $\mu\text{l}$  aliquots) of the ALP test reagent from each well were transferred into a 96-well plate and the absorbance was read at 405 nm using a micro-plate reader. The remaining ALP test reagents were washed from the 24-well plates with PBS.

Crystal violet measurement: 300  $\mu$ l of 1% crystal violet was added into each well of the 24-well plates, which were incubated for a further hour at 37°C. Plates were then gently washed by immersion in running tap water for 15 minutes and air dried for 24 hours. On day 10, 500  $\mu$ l/ml of 0.2% Triton-X 100 was added to each well and the plate was allowed to sit for 90 minutes at room temperature. Two samples (150  $\mu$ l aliquots) from each well were transferred to a 96-well plate and the absorbance was read at 405 nm using 0.2% Triton-X100 as a blank.

ALP plays a critical role in the calcification of bone and is a good marker for assessment of osteoblast differentiation. It can hydrolyse the ester bond of organic phosphate compounds under alkaline conditions. ALP is not only important for increasing phosphate level required for hydroxyapatite crystallization but also hydrolyses the substances (e.g. ATP and pyrophosphate), which inhibit calcification (Katono et al., 2008). The effect of MLF on osteoblast differentiation was quantified by expressing ALP levels per cell number, achieved by measuring absolute ALP levels in each well, followed by quantification of cell number using crystal violet absorbance. ALP activity was expressed as an index by dividing p-NPP absorbance levels by the intensity of the crystal violet absorbance.

### **2.2.1.3 Osteoblast mineralization assay**

#### **a. Reagents and recipes**

0.1% (w/v; 1mg/ml) Alizarin Red S (Sigma, A5533-25G):

1 mg of Alizarin Red S dissolved in 1 ml of Milli-Q water, adjusted to pH 5.5 with 0.1M ammonium hydroxide (freshly made).

10% Cetylpyridinium Chloride (Sigma, C0732-100G):

10% solution (w/w) was made in Milli-Q water.

#### **b. Cell culture**

The murine osteoblast-like cells, MC3T3-E1 subclone 4 (ATCC® CRL-2593™) were cultured as previously described for the osteoblast cell proliferation assay. 1 ml/well of cell suspension was seeded into 24-well flat bottom culture dishes (surface area: 2 cm<sup>2</sup>) at a density of  $0.2 \times 10^5$  cells/ml and incubated at 37°C under 5% CO<sub>2</sub> for 72 hours (day -3). The cell number was counted using hemocytometer. The

experiment was conducted in triplicate. After 3 days (day 0), the cells became 80% confluent in each well. MLF powder was dissolved in osteogenic media (MEM $\alpha$  with 10% FBS, 10 mM  $\beta$ -glycerophosphate, and 50  $\mu$ g/ml ascorbic acid on a percentage protein basis (53.73% of protein content in MLF) at the concentrations of interest (0.1, 1.0, 10, 100  $\mu$ g/ml), i.e. 1.8612 mg of MLF powder was required to dissolve in 1 ml osteogenic media to make a 1 mg/ml MLF stock. A 100  $\mu$ g/ml lactoferrin stock solution in osteogenic media was prepared as a control and filter sterilised by 0.20  $\mu$ m syringe filter. The old media in the 24-well plates was replaced with i) a lactoferrin (100  $\mu$ g/ml), ii) blank (osteogenic media), and iii) MLF dilutions from low to high concentrations (0.1, 1.0, 10, 100  $\mu$ g/ml). The 24-well plates were covered with sterile AeraSeal film sterile for the duration of the MLF treatment (A9224, Sigma-Aldrich). Each treatment or control was conducted in 4 replicates per plate (see Figure 2.3). Plates were then incubated for 72 hours at 37°C under 5% CO<sub>2</sub>. On day 3, 6, 9, 12, 15, 18 and 21, the old osteogenic media were replaced with fresh osteogenic media prepared as in day 0 containing the dissolved lactoferrin (100  $\mu$ g/ml), blank and MLF (0.1, 1.0, 10, 100  $\mu$ g/ml) every 72 hours. The AeraSeal films was changed each time media and treatments were refreshed.

### **c. Alizarin red histochemistry assay**

On day 24, the old osteogenic media in the 24-well plates was removed and the cells were washed once with PBS (0.5 ml/well). Then, the cells were fixed with ice-cold 100% ethanol (0.5 ml/well) at room temperature for 1 hour in the dark. The ethanol was carefully aspirated and plates were allowed to briefly air dry. 0.5 ml of 0.1% (w/v; 1 mg/ml) Alizarin Red reagent (pH 5.5) was added to cover each well and the plates left to stain overnight at room temperature. On day 25, the plates were gently washed by immersing in running tap water for 15 minutes to remove excess dye and air dried in a fume hood at room temperature. The calcification nodules produced by differentiated osteoblasts were stained by alizarin red solution, which reacts with calcium and stains calcification parts with red colour (Isama & Tsuchiya, 2003). Visual identification and photography of the Alizarin Red-positive nodules in each well were performed. 0.5 ml of 10% cetylpyridinium chloride was added to each well to de-stain the alizarin-red and these plates were left overnight. On day 26, two aliquots (150  $\mu$ l) of the alizarin-red solubilised in 10% cetylpyridinium chloride were

transferred from each well to 96-well plates and the absorbance was read at 550 nm using micro-plate reader. The absorbance reflecting the degree of Alizarin red staining of mineralised nodule in the plates.

## **2.2.2 Raw 264.7 osteoclastogenesis assay**

### **2.2.2.1 Cell culture**

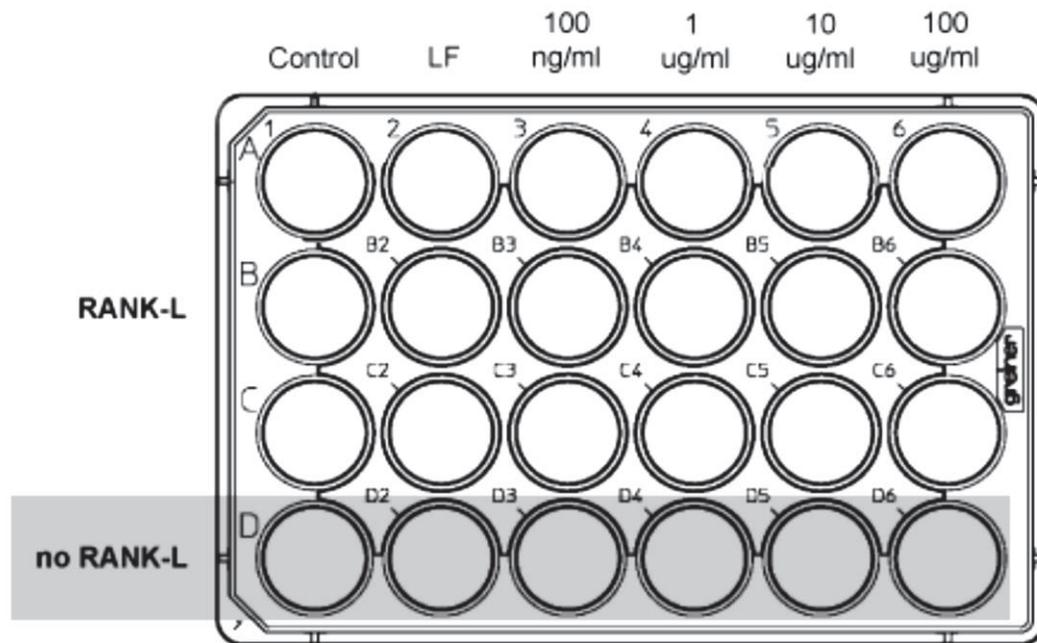
Mouse RAW 264.7 cells are a macrophage cell line and were obtained from ATCC (Rockville, MD, USA). Cells were cultured in 24-well plates and maintained in Dulbecco's Modified Eagle Media (DMEM) liquid (Invitrogen Cat. No 11995-073) supplemented with 1% antibiotic-antimycotic (100X) liquid (Invitrogen Cat. No 15240-062) and 10% heat-inactivated FBS (Invitrogen Cat. No 10093-144) in a 37°C humidified incubator containing 5% CO<sub>2</sub>.

Mouse TRANCE (rmRANKL) (R&D Cat. No 462-TEC-010) was reconstituted in sterile PBS with 0.1% BSA.

### **2.2.2.2 Tartrate-resistant acid phosphatase (TRAP) staining assay**

RAW 264.7 Cells were seeded (day 0) in the stock solution of each control and MLF sample at a density of  $1.5 \times 10^4$  cells/ml. The cell number was counted using hemocytometer. The control and MLF treatments were 10% FBS/DMEM containing 100 µg/ml lactoferrin or a MLF (0-100 µg/ml) sample. 1 ml of RANKL-free control and MLF samples was added into the bottom row of a 24 well plate (see Figure 2.4). 1.5 ul of RANKL was added into the control and MLF treatment wells (3 per treatment) followed by 1 ml of sample and cells. The experiment was conducted in triplicate (repeated in three culture plates) on the same day. The plates were incubated at 37°C under 5% CO<sub>2</sub> for 72 hours. On day 3, the media were completely replaced and fresh RANKL, control or MLF sample re-added. On day 5, 30 µl of the cell culture media from each well was added to a 96-well plate in duplicate and stained for tartrate-resistant acid phosphatase (TRAP) using Sigma 387-A kit (1% fast garnet base, 1% sodium nitrite, 1% Naphtol AS-BI, 4% acetate, 2% tartrate and 91% milli-Q water). The optical density of the 96-well plates was read at 550 nm using a micro-plate reader. The cells in the 24-well plates were fixed using fixative (25 ml citrate solution + 65 ml acetone + 8 ml 37% formaldehyde – stable for 2 months under 2-8°C) and stained for TRAP using TRAP solution and hemotoxylin.

The 24-well plates were air-dried and photographed of 200X using an Olympus IX71 inverted microscope with Olympus XC50 digital camera (Olympus Corporation of the Americas, USA). Three random fields from each well were recorded. The



**Figure 2.4** Arrangement of control and treatments in a 24-well cell culture plate in the osteoclastogenesis assay.

number of multinucleated cells per well ( $\geq 3$  nuclei) was counted. .

### 2.3 Statistical analyses

All results were analysed using IBM SPSS Statistics version 21 (IBM Corporation, USA). Power calculation of animal study was based on previously conducted studies. Groups of animals and test concentrations in cell culture experiments were compared using one-way ANOVA, followed by post-hoc testing of least-significant difference (LSD) and Duncan's Multiple Range Test, with a p-value of less than 0.05 considered statistically significance and a p-value of less than 0.01 considered highly significant. All cell culture experiments were performed in triplicate. Values and graphs are shown as mean  $\pm$  standard error of the mean (SEM).

## Chapter 3. Results

### 3.1 Diet analysis

The concentrations of protein, fat and minerals in experimental diets were analysed. The compositions are listed in Table 3.1.

**Table 3.1** Diet analysis of experimental diets.

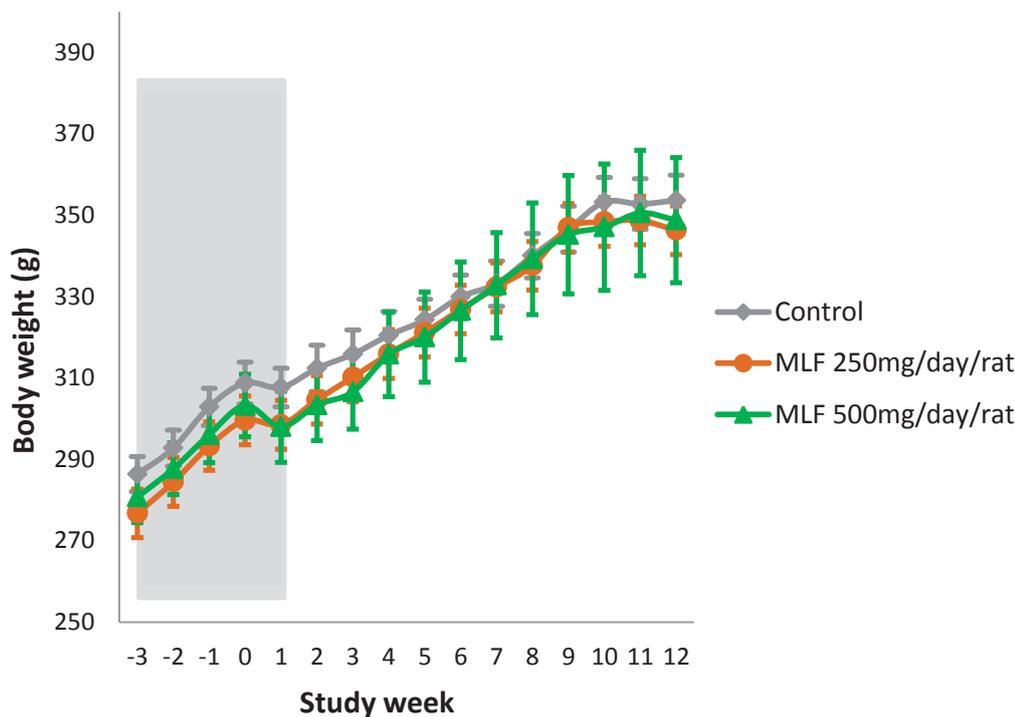
	Control diet	MLF diet
Dry matter (%)	92.0	92.2
Ash (%)	3.4	3.6
Protein (%)	15.7	16.2
Fat (%)	5.3	4.8
Ca (mg/kg)	5,114	5,250
Mg (mg/kg)	714	738
K (mg/kg)	5,371	5,180
P (mg/kg)	3,671	3,820
Zn (mg/kg)	44	44

### 3.2 Body weight and feed intake

The body weight of each rat was recorded weekly. The change in body weight between Week -3 and Week 12 is shown in Figure 3.1. Feed intake for each rat was recorded daily. The weekly and daily changes in feed intake between Week 2 and Week 12 are shown in Figure 3.2 and 3.3.

#### 3.2.1 Body weight

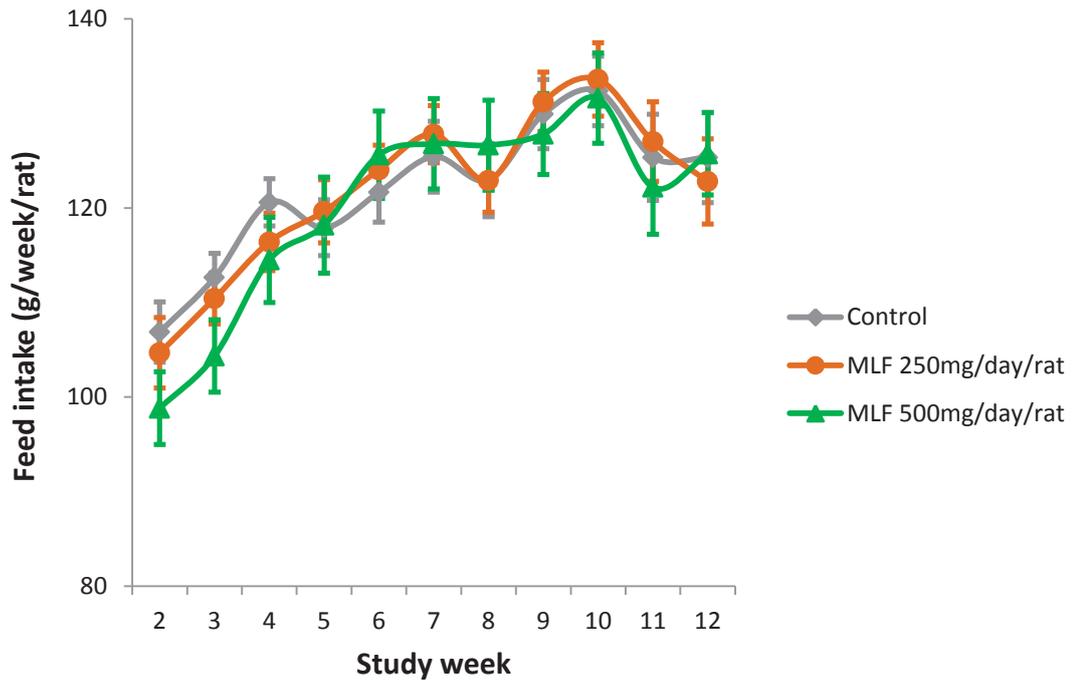
Body weight increased from about 280 to 350 g/rat during this period (Figure 3.1). In Week 1, body weight slightly decreased when the rats were switched from the chow to test diets. From Week 10, the body weight plateaued and remained relatively constant. There was no significant difference between groups for changes in body weight.



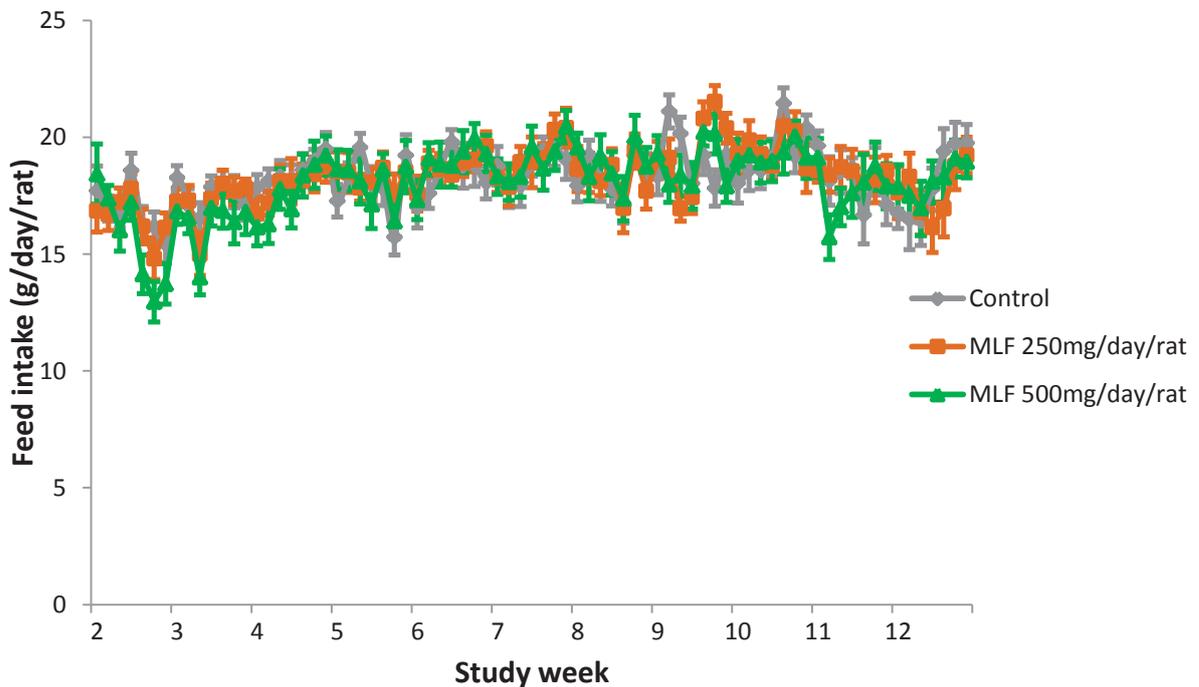
**Figure 3.1** Weekly body weight measurements in Sprague-Dawley rats fed base (Control; n=15) or one of two experimental diets: MLF at 250 mg/day/rat (n=15) or at 500 mg/rat/day (n=15). The shaded area indicates an acclimatisation period where all rats were fed a 0.5% calcium (w/w) rat chow pellet diet between Week -3 to -1, and a powdered diet at Week 0. All data are means±SEM.

### 3.2.2 Feed intake

Weekly feed intakes increased in all three groups from approximately 100 g/week/rat at Week 2 to 130 g/week/rat at Week 10, and then decreased to 125 g/week/rat at Week 12 (Figure 3.2). Daily feed intake varied between 13-22 g/day/rat and was 18.2 g/day/rat on average during the study period (Figure 3.3). There was no significant difference between groups in either weekly or daily feed intake (data not shown).



**Figure 3.2** Weekly feed intake for Sprague-Dawley rats fed base (Control; n=15) or one of two experimental diets: MLF at 250 mg/day/rat (n=15) or at 500 mg/day/rat (n=15). All data are means  $\pm$  SEM.



**Figure 3.3** Daily feed intake for Sprague-Dawley rats fed base (Control; n=15) or one of two experimental diets: MLF at 250 mg/day/rat (n=15) or at 500 mg/day/rat (n=15). All data are means  $\pm$  SEM.

### 3.3 Body composition of rats

The body composition of rats, including fat mass, lean mass, body weight and fat percentage was measured at Week 0 and Week 11 using DEXA. At Week 0, control rats had the greatest fat mass, lean mass, body weight and fat percentage compared with MLF-250 (MLF 250 mg/day) and MLF-500 (MLF 500 mg/day) groups. At Week 11, the fat mass, lean mass, body weight and fat percentage of all groups increased. The MLF-250 group had the greatest fat mass and fat percentage compared with control and MLF-500 groups. The MLF-500 group had the greatest lean mass and body weight when compared with the control and MLF-250 groups. However, there was no significant difference between any of the groups in terms of rat weight, fat and lean mass and fat percentage at both the Week 0 and Week 11 (Table 3.2).

**Table 3.2** Body composition of rats fed with control or MLF diets at the Week 0 (Baseline) and Week 11. Results are represented as means  $\pm$  SE.

Measurement		Dietary treatment			<i>p</i> -Value
		Control (n=48)	MLF 250 mg/day (n=30)	MLF 500 mg/day (n=30)	
Fat mass (g)	Week 0	42.989 $\pm$ 1.881	41.651 $\pm$ 1.977	40.946 $\pm$ 2.606	0.776
	Week 11	74.630 $\pm$ 3.808	79.309 $\pm$ 4.562	73.560 $\pm$ 7.306	0.731
Lean mass (g)	Week 0	257.751 $\pm$ 3.046	256.446 $\pm$ 4.211	256.722 $\pm$ 3.183	0.957
	Week 11	267.967 $\pm$ 3.592	267.982 $\pm$ 4.586	273.838 $\pm$ 3.901	0.527
Body Weight (g)	Week 0	300.740 $\pm$ 3.963	298.097 $\pm$ 5.274	297.668 $\pm$ 4.828	0.865
	Week 11	342.597 $\pm$ 5.175	347.291 $\pm$ 6.360	347.398 $\pm$ 8.859	0.825
Fat percentage (%)	Week 0	14.141 $\pm$ 0.523	13.872 $\pm$ 0.530	13.545 $\pm$ 0.678	0.761
	Week 11	21.421 $\pm$ 0.906	22.571 $\pm$ 1.092	20.318 $\pm$ 1.375	0.417

Body weight, fat mass, lean mass and fat percentage increased in all groups following 12 weeks of feeding (Table 3.3). The MLF-250 group had the largest increase in fat mass and fat percentage, while the MLF-500 group showed the greatest increase in lean mass and body weight. There were no significant differences between groups for the changes of body weight, fat mass and fat percentage during the study period. However, the rats fed with 500 mg/day MLF had

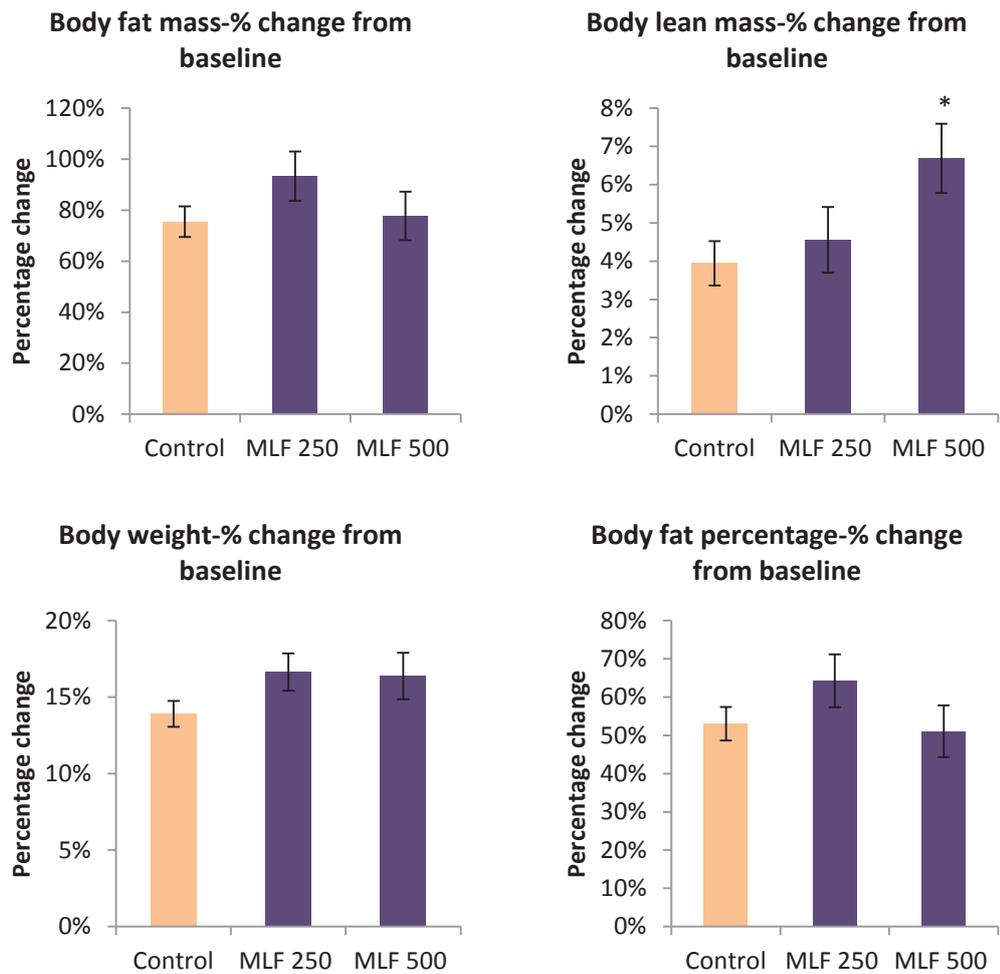
a significantly higher increase in lean body mass ( $p=0.03$ ) than those fed with control or 250 mg/day MLF diet.

**Table 3.3** Changes of DEXA measures of body composition following 12 weeks of feeding control or MLF diets.

Measurements		Dietary treatments		
		Control (n=48)	MLF 250 mg/day (n=30)	MLF 500 mg/day (n=30)
Fat mass (g)	Mean	31.64 <sup>a</sup>	37.66 <sup>a</sup>	32.61 <sup>a</sup>
	SE	2.73	3.80	5.36
Lean mass (g)	Mean	10.22 <sup>a</sup>	11.54 <sup>a</sup>	17.12 <sup>b</sup>
	SE	1.51	2.08	2.25
Body weight (g)	Mean	41.86 <sup>a</sup>	49.19 <sup>a</sup>	49.73 <sup>a</sup>
	SE	2.61	3.50	5.16
Fat percentage (%)	Mean	7.28 <sup>a</sup>	8.70 <sup>a</sup>	6.77 <sup>a</sup>
	SE	0.62	0.90	0.95

Different superscripts (a, b) represent a significant difference between groups in the same row at  $p < 0.05$ .

The MLF-500 group exhibited a greater percentage change in body lean mass (6.7%) from Week 0 compared with the control (3.9%) and MLF-250 (4.6%) groups. This percentage change in the MLF-500 group was statistically significant compared with the control group ( $p = 0.01$ ). The MLF-250 group had the highest percentage increase in body fat mass (93.3%), body weight (16.6%), and fat percentage (64.3%) than the control and MLF-500 groups. However, there was no significant difference between groups (Figure 3.4).



**Figure 3.4** Percentage changes of body composition from baseline after 12 weeks of feeding control or MLF diets. Rats were fed with a base (Control; n=48) or one of two experimental diets: MLF at 250 mg/day/rat (n=30) or at 500 mg/rat/day (n=30). Bars represent Means  $\pm$  SEM. Values were calculated by  $(\text{Value}_{\text{Week 11}} - \text{Value}_{\text{Week 0}}) / \text{Value}_{\text{Week 0}}$ . \* indicates statistical significance from the control at  $p < 0.05$ .

### 3.4 Bone density

#### 3.4.1 Femurs

Both left and right femurs were measured for each animal and the results for both were pooled and averaged. At Week 0, the MLF-500 group had the highest *in vivo* femur bone area and BMC when compared with the control and MLF-250 group. The control and MLF-250 groups had greater *in vivo* femur BMD than the MLF-500 group. At Week 11, MLF-500 groups had the greatest *in vivo* femur bone area, BMC

and BMD compared with control and MLF-250 groups. However, these differences between groups were not significant (Table 3.4).

**Table 3.4** *In vivo* femur bone area, bone mineral content (BMC) and bone mineral density (BMD) of rats at Week 0 (Baseline) and Week 11. Results are represented as means  $\pm$  SE.

Measurement		Dietary treatment			<i>p</i> -Value
		Control (n=48)	MLF 250 mg/day (n=30)	MLF 500 mg/day (n=30)	
Area (cm <sup>2</sup> )	Week 0	1.433 $\pm$ 0.012	1.435 $\pm$ 0.012	1.446 $\pm$ 0.011	0.727
	Week 11	1.503 $\pm$ 0.013	1.504 $\pm$ 0.014	1.521 $\pm$ 0.015	0.640
BMC (g)	Week 0	0.422 $\pm$ 0.006	0.422 $\pm$ 0.006	0.423 $\pm$ 0.005	0.986
	Week 11	0.461 $\pm$ 0.006	0.465 $\pm$ 0.007	0.473 $\pm$ 0.007	0.508
BMD (g/cm <sup>2</sup> )	Week 0	0.294 $\pm$ 0.002	0.294 $\pm$ 0.003	0.292 $\pm$ 0.003	0.865
	Week 11	0.306 $\pm$ 0.002	0.309 $\pm$ 0.003	0.310 $\pm$ 0.003	0.496

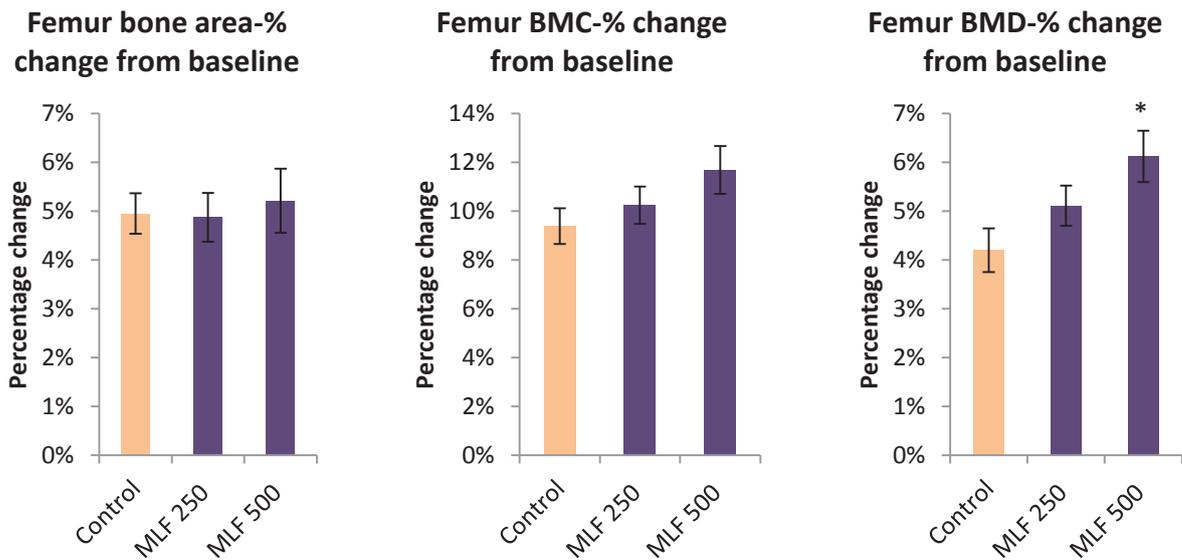
Femur bone area, BMC and BMD increased in all three groups during 12 weeks of feeding. The MLF-500 group showed the greatest increase in femur BMC and BMD compared with control and MLF-250 groups (Table 3). The MLF-500 group also had the greatest increase in femur bone area. However, there was no significant difference between groups (Table 3.5).

**Table 3.5** Changes of *in vivo* femur bone area, bone mineral content (BMC) and bone mineral density (BMD) changes after 12 weeks of feeding control or MLF diets.

Measurement		Dietary treatments		
		Control (n=48)	MLF 250 mg/day (n=30)	MLF 500 mg/day (n=30)
Area (cm <sup>2</sup> )	Mean	0.071 <sup>a</sup>	0.070 <sup>a</sup>	0.075 <sup>a</sup>
	SE	0.006	0.007	0.009
BMC (g)	Mean	0.039 <sup>a</sup>	0.043 <sup>ab</sup>	0.050 <sup>b</sup>
	SE	0.003	0.003	0.004
BMD (g/cm <sup>2</sup> )	Mean	0.012 <sup>a</sup>	0.015 <sup>ab</sup>	0.018 <sup>b</sup>
	SE	0.001	0.001	0.002

Different superscripts (a, b) represent significant difference between groups in the same row at *p* < 0.05.

The MLF-500 group had greater percentage change of femur BMD (6.1%) from Week 0 compared with control (4.2%) and MLF-250 group (5.1%). This percentage change in the MLF-500 group was statistically significant compared with the control group ( $p = 0.004$ ). The MLF-500 group also had the highest percentage change in femur bone area (5.2%) and femur BMC (11.7%) compared to control (5.0% and 9.4%, respectively) and MLF-250 groups (4.9% and 10.2%, respectively). There was no significant difference between the groups (Figure 3.5).



**Figure 3.5** Percentage change for femur bone area, BMC and BMD from baseline after 12 weeks of feeding control or MLF diets. Rats were fed with a base (Control;  $n=48$ ) or one of two experimental diets: MLF at 250 mg/day/rat ( $n=30$ ) or at 500 mg/rat/day ( $n=30$ ). Bars represent means  $\pm$  SEM. Values were calculated by  $(\text{Value}_{\text{Week 11}} - \text{Value}_{\text{Week 0}}) / \text{Value}_{\text{Week 0}}$ . \* indicates statistical significance from the control group at  $p < 0.05$ .

### 3.4.2 Lumbar spine

At Week 0, the MLF-500 group had the largest *in vivo* lumbar spine bone area, BMC and BMD, compared with the control and MLF-250 group. At Week 11, the MLF-500 group also had the greatest *in vivo* lumbar spine bone area, BMC and BMD compared with the control and MLF-250 groups. However, these differences between groups were not significant at either Week 0 or Week 11 (Table 3.6).

**Table 3.6** *In vivo* lumbar spine bone area, bone mineral content (BMC) and bone mineral density (BMD) of rats at Week 0 (Baseline) and Week 11. Results are represented as means  $\pm$  SE.

Measurement		Dietary treatment			<i>p</i> -Value
		Control (n=48)	MLF 250 mg/day (n=30)	MLF 500 mg/day (n=30)	
Area (cm <sup>2</sup> )	Week 0	1.948 $\pm$ 0.020	1.964 $\pm$ 0.022	1.973 $\pm$ 0.023	0.698
	Week 11	2.081 $\pm$ 0.018	2.081 $\pm$ 0.022	2.110 $\pm$ 0.023	0.565
BMC (g)	Week 0	0.453 $\pm$ 0.008	0.459 $\pm$ 0.008	0.462 $\pm$ 0.009	0.712
	Week 11	0.500 $\pm$ 0.008	0.510 $\pm$ 0.009	0.521 $\pm$ 0.010	0.246
BMD (g/cm <sup>2</sup> )	Week 0	0.232 $\pm$ 0.002	0.233 $\pm$ 0.002	0.234 $\pm$ 0.002	0.791
	Week 11	0.240 $\pm$ 0.002	0.245 $\pm$ 0.002	0.247 $\pm$ 0.003	0.130

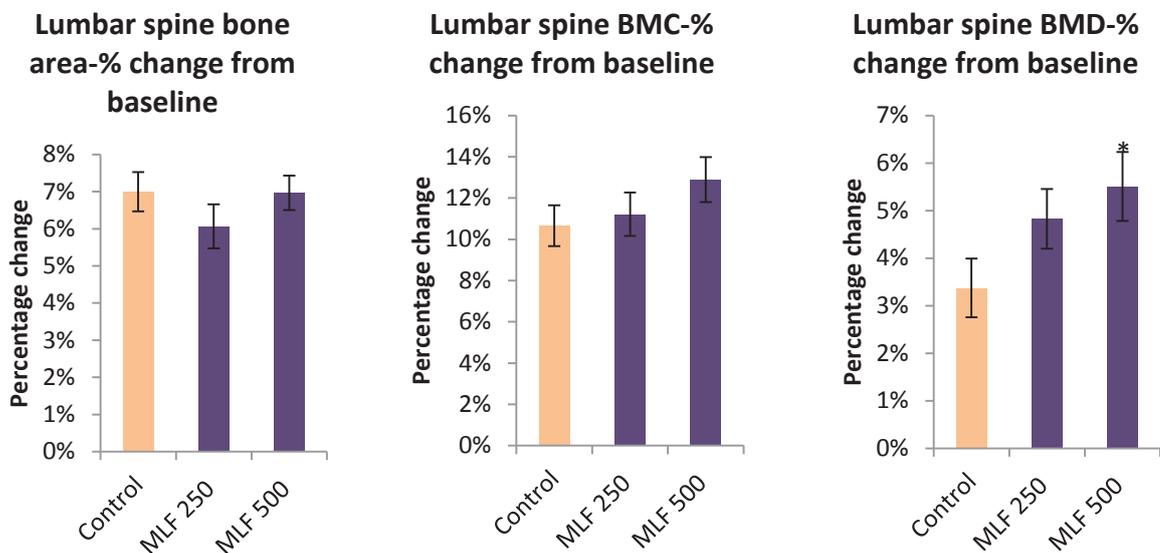
Lumbar spine bone area, BMC and BMD increased in all groups following 12 weeks of feeding. The MLF-500 group had the largest lumbar spine BMD compared with the control group and MLF-250 group. The MLF-500 group also had the highest increases in lumbar spine bone area and BMC. However, these differences were not significant between groups (Table 3.7).

**Table 3.7** Changes of *in vivo* lumbar spine area, bone mineral content (BMC) and bone mineral density (BMD) after 12 weeks of feeding control or MLF diets.

Measurement		Dietary treatment		
		Control (n=48)	MLF 250 mg/day (n=30)	MLF 500 mg/day (n=30)
Area (cm <sup>2</sup> )	Mean	0.133 <sup>a</sup>	0.118 <sup>a</sup>	0.136 <sup>a</sup>
	SE	0.009	0.011	0.009
BMC (g)	Mean	0.047 <sup>a</sup>	0.051 <sup>a</sup>	0.059 <sup>a</sup>
	SE	0.004	0.005	0.005
BMD (g/cm <sup>2</sup> )	Mean	0.008 <sup>a</sup>	0.011 <sup>ab</sup>	0.013 <sup>b</sup>
	SE	0.001	0.001	0.002

Different superscripts (a, b) represent significant difference between groups in the same row at  $p < 0.05$ .

The MLF-500 group had a greater percentage change in lumbar spine BMD (5.5%) from Week 0 compared with the control (3.4%) and MLF-250 group (4.9%). This was a statistically significant difference between the MLF-500 group and the control group ( $p=0.023$ ). The MLF-500 group also had the largest percentage increase in lumbar spine BMC (12.9%) compared with the control (10.7%) and MLF-250 group (11.2%). The control group had the highest percentage increase in lumbar spine bone area (7.0%) compared with the MLF-250 group (6.1%) and MLF-500 group (6.9%). However, these differences were not significant between groups (Figure 3.6).



**Figure 3.6.** Percentage change of lumbar spine bone area, BMC and BMD from baseline after 12 weeks of feeding control or MLF diets. Rats were fed with a base (Control;  $n=48$ ) or one of two experimental diets: MLF at 250 mg/day/rat ( $n=30$ ) or at 500 mg/rat/day ( $n=30$ ). Bars represent Means  $\pm$  SEM. Values were calculated by  $(\text{Value}_{\text{Week 11}} - \text{Value}_{\text{Week 0}}) / \text{Value}_{\text{Week 0}}$ . \* indicates statistical significance from the control group at  $p < 0.05$ .

### 3.4.3 Whole body

At both Week 0 and Week 11, the MLF-500 group had the greatest whole body bone area, BMC and BMD compared with the control and MLF-250 group. However, these differences between groups were not significant at either Week 0 or Week 11 (Table 3.8).

**Table 3.8** *In vivo* whole body bone area, bone mineral content (BMC) and bone mineral density (BMD) of rats at the Week 0 (Baseline) and Week 11. Results are represented as means  $\pm$  SE.

Measurement		Dietary treatment			<i>p</i> -Value
		Control (n=48)	MLF 250 mg/day (n=30)	MLF 500 mg/day (n=30)	
Area (cm <sup>2</sup> )	Week 0	61.371 $\pm$ 0.591	61.395 $\pm$ 0.721	61.649 $\pm$ 0.579	0.948
	Week 11	67.052 $\pm$ 0.651	67.599 $\pm$ 0.877	67.994 $\pm$ 1.040	0.705
BMC (g)	Week 0	9.817 $\pm$ 0.133	9.796 $\pm$ 0.168	9.916 $\pm$ 0.145	0.850
	Week 11	11.252 $\pm$ 0.140	11.429 $\pm$ 0.188	11.589 $\pm$ 0.222	0.388
BMD (g/cm <sup>2</sup> )	Week 0	0.160 $\pm$ 0.001	0.159 $\pm$ 0.001	0.161 $\pm$ 0.001	0.709
	Week 11	0.168 $\pm$ 0.001	0.169 $\pm$ 0.001	0.170 $\pm$ 0.001	0.245

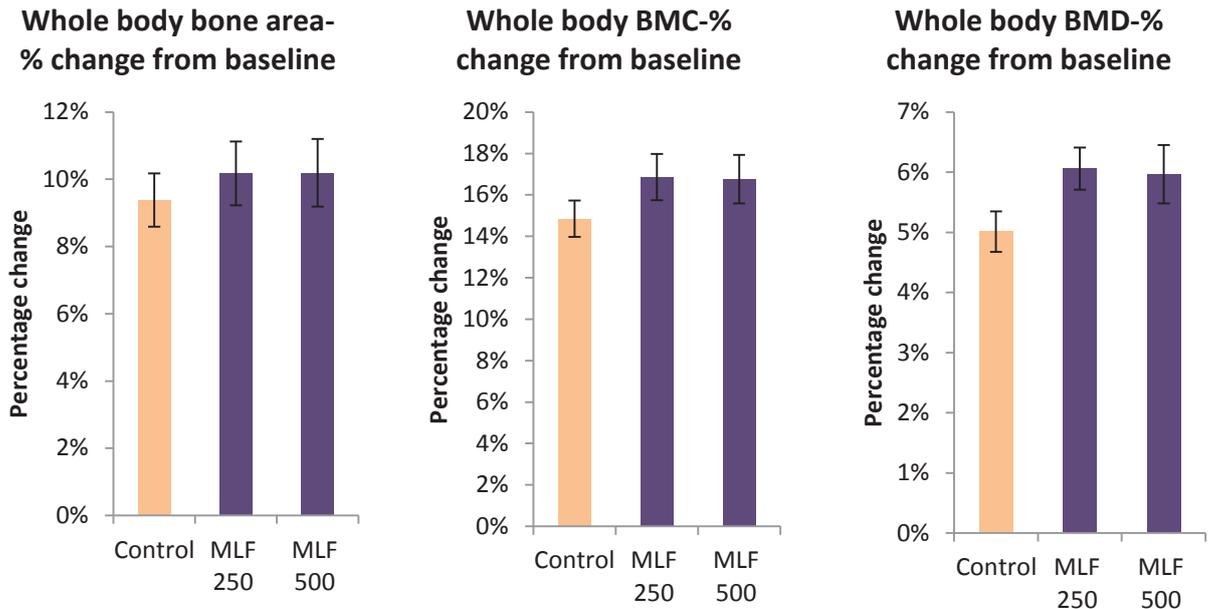
Whole body bone area, BMC and BMD increased in all groups following 12 weeks of feeding. The MLF-500 group had highest increase in whole body bone area and BMC compared with the control group and MLF-250 group. MLF-250 and MLF-500 groups had higher increases in whole body BMD than control group. These differences were not significant between groups (Table 3.9).

**Table 3.9** Changes of *in vivo* whole body bone area, bone mineral content (BMC) and bone mineral density (BMD) after 12 weeks of feeding control or MLF diets.

Measurement		Dietary treatment		
		Control (n=48)	MLF 250 mg/day (n=30)	MLF 500 mg/day (n=30)
Area (cm <sup>2</sup> )	Mean	5.680 <sup>a</sup>	6.204 <sup>a</sup>	6.345 <sup>a</sup>
	SE	0.463	0.581	0.647
BMC (g)	Mean	1.435 <sup>a</sup>	1.634 <sup>a</sup>	1.673 <sup>a</sup>
	SE	0.078	0.104	0.121
BMD (g/cm <sup>2</sup> )	Mean	0.008 <sup>a</sup>	0.010 <sup>a</sup>	0.010 <sup>a</sup>
	SE	0.001	0.001	0.001

a: represent that there was no significant difference between groups in the same row at  $p < 0.05$ .

The MLF-500 group had the largest percentage change in whole body bone area (10.2%) from Week 0 compared with the control (9.4%) and MLF-250 group (10.2%). The MLF-250 group had the highest percentage increase in whole body BMC (16.9%) and BMD (6.1%) compared with the control (14.9% and 5.0%, respectively) and MLF-500 group (16.8% and 6.0%, respectively). These differences were not significant between groups (Figure 3.7).



**Figure 3.7** Percentage changes of whole body bone area, BMC and BMD from baseline after 12 weeks of feeding control or MLF diets. Rats were fed with base (Control; n=48) or one of two experimental diets: MLF at 250 mg/day/rat (n=30) or at 500 mg/rat/day (n=30). Bars represent Means  $\pm$  SEM. Values were calculated by  $(\text{Value}_{\text{Week 11}} - \text{Value}_{\text{Week 0}}) / \text{Value}_{\text{Week 0}}$ .

### 3.5 Biomechanics

Rats in the MLF-500 group had the largest left femur shaft length and largest wet weight compared with the control and MLF-250 groups. The control group had the greatest femur shaft width, shaft thickness, max load, max stroke, break load, break stress, break stroke, break strain and energy compared with MLF-250 and MLF-500 groups. MLF-250 group had the highest value of elasticity compared to the control and MLF-500 groups. However, there was no significant difference between groups for these biomechanical measurements (Table 3.10).

**Table 3.10** Dimensional and biomechanical results from three-point bending tests on the left femurs following 12 weeks of feeding a control or MLF diets. Results are represented as means  $\pm$  SE.

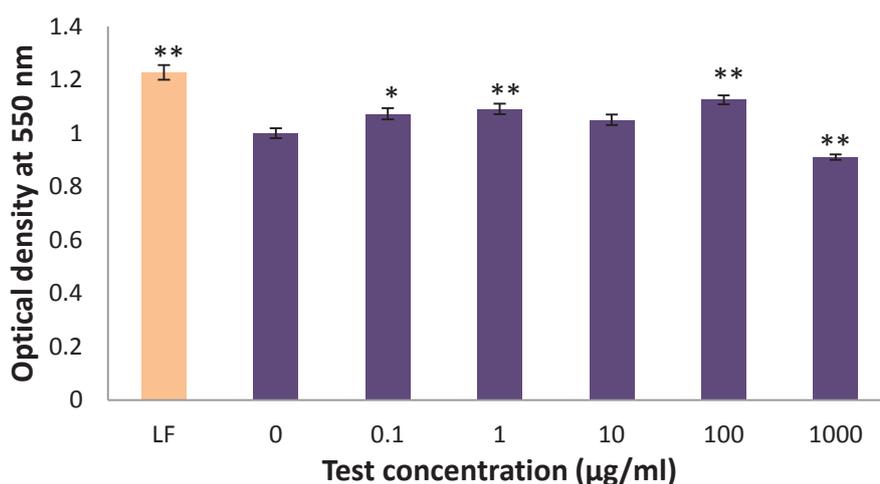
Measurement	Dietary treatment			<i>p</i> -Value
	Control (n=15)	MLF 250 mg/day (n=15)	MLF 500 mg/day (n=15)	
Shaft Width (mm)	4.142 $\pm$ 0.098	4.077 $\pm$ 0.055	4.097 $\pm$ 0.067	0.826
Shaft Thickness (mm)	3.253 $\pm$ 0.054	3.173 $\pm$ 0.038	3.207 $\pm$ 0.022	0.376
Length (mm)	37.581 $\pm$ 0.233	37.541 $\pm$ 0.335	37.633 $\pm$ 0.260	0.973
Wet weight (g)	1.061 $\pm$ 0.023	1.048 $\pm$ 0.025	1.081 $\pm$ 0.016	0.555
Max load (N)	177.130 $\pm$ 5.717	166.548 $\pm$ 3.665	166.781 $\pm$ 4.122	0.189
Max stroke (mm)	1.517 $\pm$ 0.064	1.433 $\pm$ 0.047	1.414 $\pm$ 0.046	0.352
Break load (N)	176.651 $\pm$ 5.553	164.073 $\pm$ 3.716	164.617 $\pm$ 4.054	0.096
Break stress (N/mm <sup>2</sup> )	91.921 $\pm$ 3.980	90.628 $\pm$ 3.186	88.600 $\pm$ 3.36	0.799
Break stroke (mm)	1.531 $\pm$ 0.064	1.459 $\pm$ 0.049	1.456 $\pm$ 0.055	0.570
Break strain (%)	13.354 $\pm$ 0.722	12.373 $\pm$ 0.489	12.445 $\pm$ 0.473	0.410
Elasticity (N/mm <sup>2</sup> )	1059.442 $\pm$ 68.983	1090.421 $\pm$ 51.190	1068.577 $\pm$ 35.436	0.916
Energy (J)	0.170 $\pm$ 0.013	0.153 $\pm$ 0.099	0.155 $\pm$ 0.010	0.489

## 3.6 Cell culture

### 3.6.1 Osteoblasts

#### 3.6.1.1 Proliferation

Lactoferrin 1,000  $\mu\text{g/ml}$  and MLF at 0.1, 1.0 and 100  $\mu\text{g/ml}$  significantly increased the proliferation of MC3T3-E1 sub-clone 4 pre-osteoblasts ( $p < 0.001$ ,  $p = 0.018$ ,  $p = 0.003$  and  $p < 0.001$ , respectively). Cells cultured in 100  $\mu\text{g/ml}$  MLF had the highest proliferation compared with those cultured in other concentrations of MLF. However, MLF at 1,000  $\mu\text{g/ml}$  significantly decreased cell proliferation ( $p = 0.004$ ). MLF at 10  $\mu\text{g/ml}$  had a slight but non-significant effect on the proliferation of pre-osteoblasts (Figure 3.8).

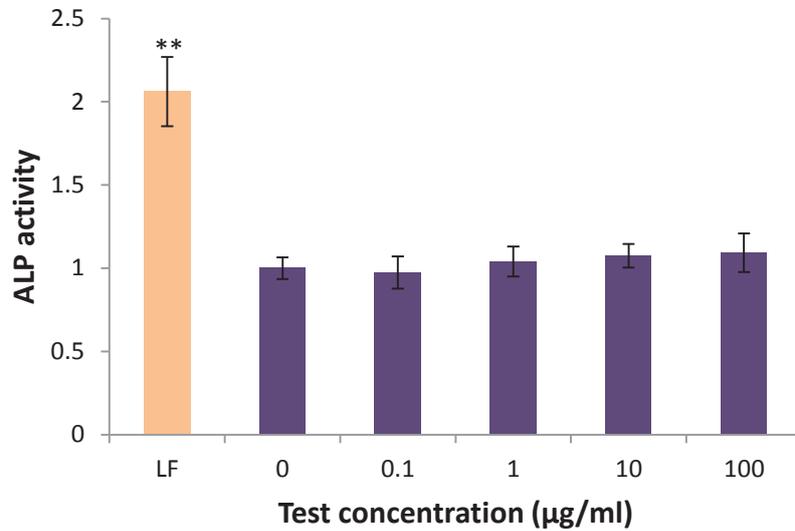


**Figure 3.8** Effect of MLF (0-1,000  $\mu\text{g/ml}$ ) on cell proliferation in MC3T3-E1 sub-clone 4 pre-osteoblasts. Bars represent cell proliferation for each treatment relative to the 0  $\mu\text{g/ml}$  control. Lactoferrin (LF) was included as a control at 1,000  $\mu\text{g/ml}$ . Data are means  $\pm$  SEM. Asterisks indicate statistical significance from the 0 control, with \* for  $p < 0.05$  and \*\* indicating  $p < 0.01$ .

#### 3.6.1.2 Differentiation

Lactoferrin significantly increased ALP activity in MC3T3-E1 sub-clone 4 pre-osteoblasts. ALP activity appeared to increase in response to MLF concentration although the increase was not statistically significant. Cells cultured in 100  $\mu\text{g/ml}$  of MLF had the highest ALP activity compared with cells cultured in other MLF

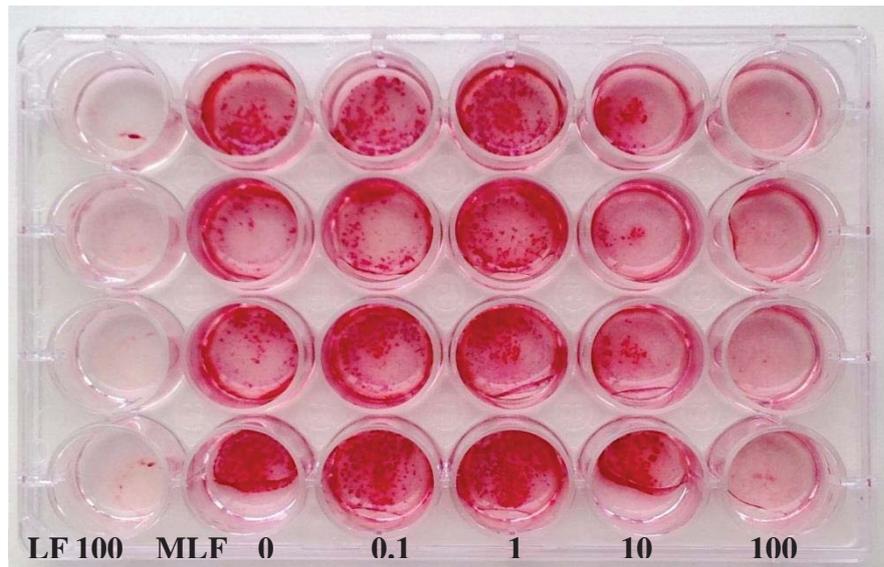
concentrations. However, there was no significant difference between any of the groups (Figure 3.9).



**Figure 3.9** Effect of MLF on cell differentiation in MC3T3-E1 sub-clone 4 pre-osteoblasts. Bars represent alkaline phosphatase (ALP) activity of each treatment relative to the 0 µg/ml control. Lactoferrin (LF) was included as a control at 100 µg/ml. Data are Means  $\pm$  SEM. Asterisks indicate statistical significance from the 0 control, with \* for  $p < 0.05$  and \*\* indicating  $p < 0.01$ .

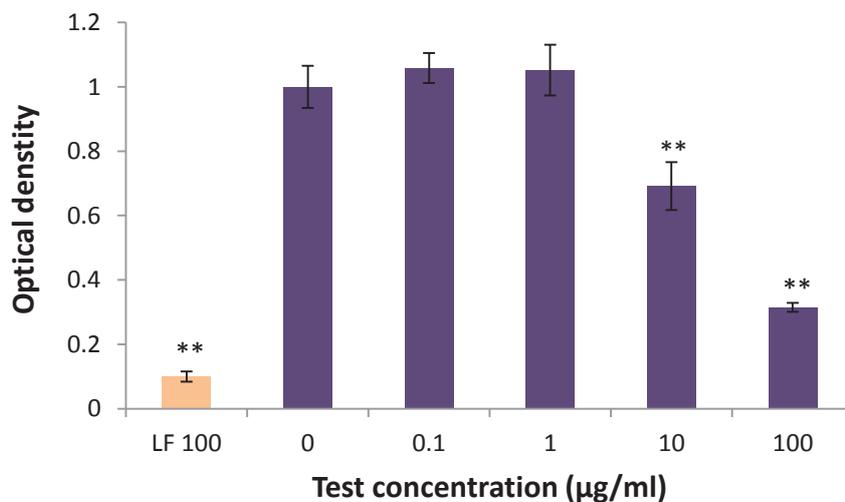
### 3.6.1.3 Mineralization

After staining with alizarin red, the presence of red mineralized nodules was evident in wells treated with 0-10  $\mu\text{g/ml}$  of MLF. The wells of MLF 100  $\mu\text{g/ml}$  had few nodules, while the wells of lactoferrin 100  $\mu\text{g/ml}$  had no visible red nodules (Figure 3.10).



**Figure 3.10** Representative photo of 24-well plate for cell mineralization experiment in MC3T3-E1 sub-clone 4 pre-osteoblasts stained with alizarin red. LF: lactoferrin ( $\mu\text{g/ml}$ ); MLF: milk lipid fraction ( $\mu\text{g/ml}$ ).

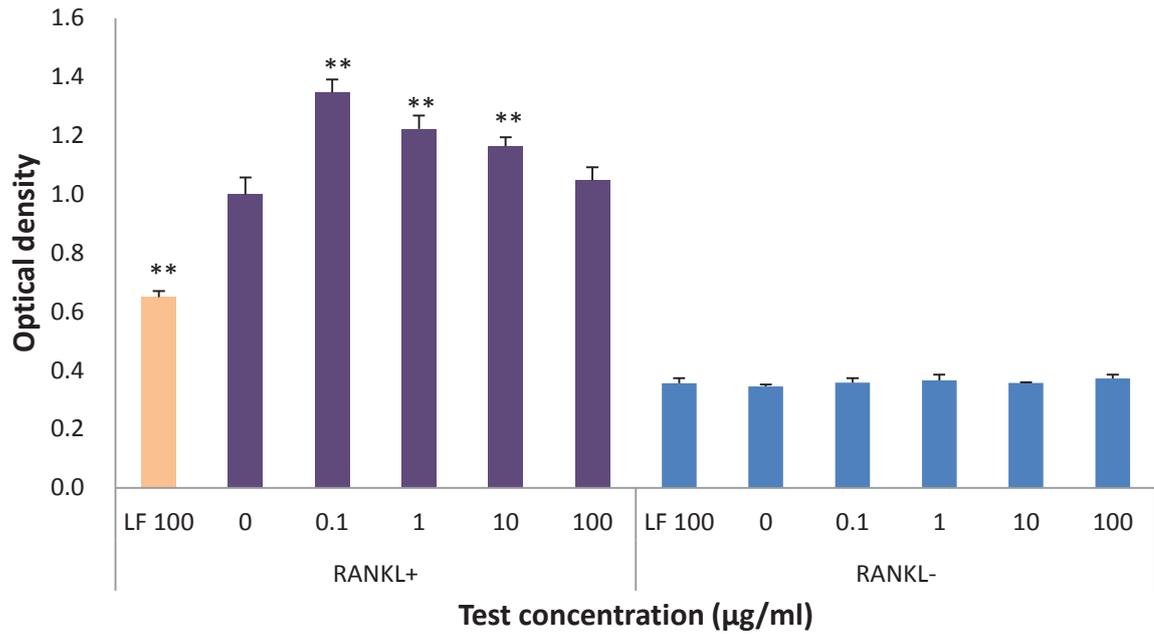
The 0.1 and 1.0  $\mu\text{g/ml}$  MLF treatments slightly increased the mineralization of MC3T3-E1 sub-clone 4 pre-osteoblasts. 0.1  $\mu\text{g/ml}$  of MLF had the greatest effect on the mineralization of cells compared with 0 and 1.0  $\mu\text{g/ml}$ . MLF at 10 and 100  $\mu\text{g/ml}$  and lactoferrin at 100  $\mu\text{g/ml}$  significantly inhibited the mineralization in MC3T3-E1 sub-clone 4 pre-osteoblasts compared with the 0  $\mu\text{g/ml}$  control ( $p < 0.001$ ,  $p < 0.001$  and  $p < 0.001$ , respectively) (Figure 3.11).



**Figure 3.11** Effect of MLF on cell mineralization in MC3T3-E1 sub-clone 4 pre-osteoblasts. Bars represent alizarin red optical densities for each treatment relative to the 0 µg/ml. Lactoferrin (LF) was included as a control at 100 µg/ml. Data are Means ± SEM. Asterisks indicate statistical significance from the 0 control, with \* for  $p < 0.01$ .

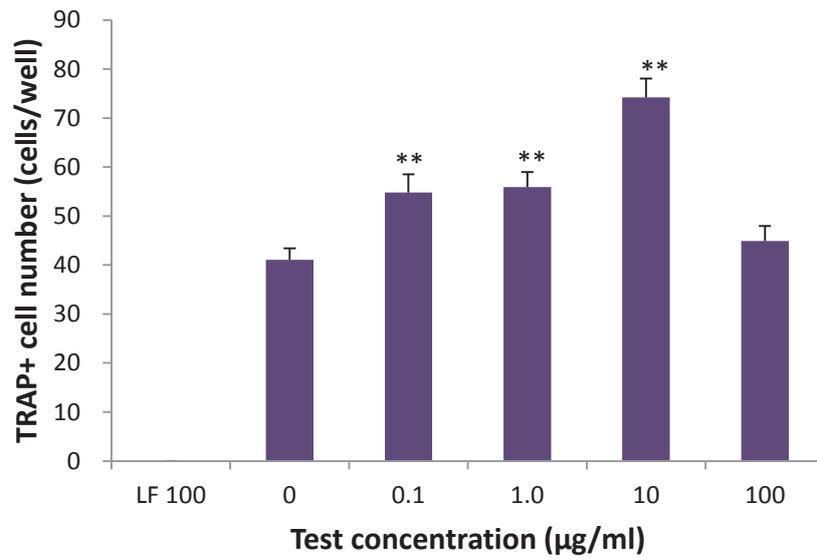
### 3.6.2 Osteoclasts

The MLF at concentrations of 0.1, 1.0 and 10 µg/ml significantly increased osteoclastogenesis in RAW 264.7 cells cultured with RANKL ( $p < 0.001$ ,  $p < 0.001$  and  $p = 0.004$ , respectively). 0.1 µg/ml of MLF had the highest effect on osteoclastogenesis compared with cells treated with concentrations of 1.0 and 10 µg/ml. MLF at 100 µg/ml slightly increased osteoclastogenesis, but this was not significant. 100 µg/ml of lactoferrin significantly decreased osteoclastogenesis in RAW 264.7 cells cultured with RANKL ( $p < 0.001$ ). There was no effect of MLF or lactoferrin on osteoclastogenesis in RAW 264.7 cells cultured without RANKL (Figure 3.12).



**Figure 3.12** Effect of MLF (0.1-100 µg/ml) on osteoclastogenesis in RAW 264.7 cells. Bars represent TRAP staining optical densities for each treatment relative to the 0 control with (+) or without (-) RANKL. Lactoferrin (LF) was included as a control at 100 µg/ml. Data are Means  $\pm$  SEM. \*\*: significantly different vs. 0 at  $p < 0.01$ .

The MLF significantly increased the number of TRAP-positive cells in RAW 264.7 cells cultured with RANKL at 0.1, 1.0 and 10 µg/ml ( $p < 0.001$ ,  $p = 0.001$  and  $p = 0.009$ , respectively). MLF at 10 µg/ml had the greatest effect on increasing the cell number compared with the lower concentrations of 0.1 and 1.0 µg/ml. 100 µg/ml of MLF slightly increased the number of TRAP-positive cells, but this was not significant. Lactoferrin inhibited the number of TRAP-positive cells (Figure 3.13).



**Figure 3.13** Effect of MLF (0.1-100 µg/ml) on the number of TRAP-positive cells. Bars represent the means of cell number per well after treatments. Lactoferrin (LF) was included as a control at 100 µg/ml. Data are Means  $\pm$  SEM. \*: significantly different vs. 0 at  $p < 0.01$ .

## **Chapter 4. Discussion**

### **Introduction**

The current study evaluated the effects of dietary MLF intake on bone parameters in growing female Sprague-Dawley rats over the period of 12 weeks. Bone mass properties, including BA, BMC and BMD, at the femur, lumbar spine and whole body were measured *in vivo* by DEXA. Biomechanical properties of the left femur were assessed using 3-point bending test.

Bone undergoes a continuous turnover process, involving the coupled processes of bone formation by osteoblasts and bone resorption by osteoclasts. At the cellular level, bone turnover includes the coordination of the number and activity of osteoblasts and osteoclasts. Therefore, the direct effects of MLF on the preosteoblast-like MC3T3-E1 clone 4 cells and RAW 264.7 osteoclastic cells were evaluated.

### **4.1 Animal study**

#### **4.1.1 Effect of MLF on bone density**

In female Sprague-Dawley rats, peak cancellous bone mass accumulates steadily during the first three months of life. During this time, approximately 20% of the endocortical bone surface and 43% of the periosteal bone surface are active in forming new bone. The bone formation activity will rapidly decrease in the following five months after peak bone mass. Peak bone mass is maintained until eight months of age. Age-related cancellous bone loss begins at nine months of age and continues until rats are approximately 12 months old. The endocortical bone formation almost completely ends after nine months of age, whereas a low rate of periosteal bone formation continues until the rats are 16 months old (Li et al., 1991). Given the average life span of Sprague-Dawley rats is 26 months, eight months of age is equivalent to 24-25 years in human life, supposing the average life span is about 75 years in humans. Peak bone mass is achieved at the same time (24-25 years) in humans (Li et al., 1991). Because rats have similar skeletal characteristics to humans, it is an appropriate model for the study of nutritional, endocrine and

environmental factors that can affect peak bone mass (Li et al., 1991; Lelovas et al., 2008).

The increases in BA, BMC and BMD in the control group were within the expected natural growth rate of bone during the study period. At the end of the study, the results of the DEXA scan showed that rats in the high-dose MLF group had the greater *in vivo* BA, BMC and BMD compared with the control and low-dose groups at the femur, lumbar spine and whole body although these results were not statistically significant (refer to Table 3.4, 3.6 and 3.8). Moreover, the high-dose MLF diet resulted in the greatest increase in BMC at the femur and BMD at the femur and lumbar spine compared with the control and low-dose diets. It also resulted in a significant percentage increase in BMD at the femur and lumbar spine (refer to Table 3.5 and 3.7 and Figure 3.5 and 3.6). These results suggest that MLF has a beneficial effect over and above the natural growth rate on bone development. This evidence supports the hypothesis that the addition of MLF in diet could increase bone mass.

Peak bone mass is primarily affected by genetic factors and is also dependent on dietary nutrient intake and physical activity (Mundy, 2000). Genetic factors are estimated to contribute approximately 70% of the variance to bone mass (Högström et al., 2007). Environmental factors such as dietary nutrient intake, physical activity and hormones are estimated to account for approximately 30% of the acquisition of bone mass, and these factors can be influenced.

The MLF contained approximately 36% fat, including a large amount of SFA, moderate MUFA content and a small amount of PUFAs (refer to Table 2.2). The exact fatty acid composition of MLF is not known but milk fat is usually high in palmitic, stearic, myristic, lauric, oleic and conjugated linoleic acids (Li & Watkins, 1998; Jensen, 2002). Previous studies have shown that the dietary intake of these fatty acids affects bone density in both humans and animal models (Gunnes & Lehmann, 1996; Trichopoulou et al., 1997; Bruce A Watkins et al., 1997; Gronowitz et al., 2006; Eriksson et al., 2009).

Epidemiological studies have reported that saturated fat intake is significantly positively correlated with BMD gain, particularly in cortical bone in children

(Gunnes & Lehmann, 1996). MUFA intake, which is mostly derived from olive oil in the Greek diet, was positively associated with forearm BMD in adults (Trichopoulou et al., 1997).

Dietary fat intake has been indicated to influence serum fatty acid profiles, which may further play a role in skeletal health. Male chicks fed a diet containing 36.4% SFA (palmitic and stearic acids) and 54% MUFA in total fat had a higher level of SFA and lower level of PUFA in blood compared with those fed with the diets rich in MUFA or PUFA (Burlikowska et al., 2010). The fatty acid pattern in serum, similarly to the fat in the diet, were high levels in palmitic, stearic and oleic acids (Burlikowska et al., 2010). Deshimaru et al. (2005) analysed the composition of fatty acid in human bone marrow aspirates and reported that the concentrations of palmitic and oleic acid were higher in bone marrow than in serum phospholipids. CLA, the positional and geometric isomer of LA, naturally occur in several foods but are rich in dairy products and ruminant meat (Watkins & Seifert, 2000). Milk fat is the main dietary source of CLA (Li & Watkins, 1998). In an animal study, dietary CLA intake resulted in the highest content of CLA isomers in bone periosteum and marrow, while brain contained the lowest concentration of isomers (Li & Watkins, 1998).

These alterations in serum or bone marrow lipid pattern, which reflects habitual dietary fat intake, may modulate bone formation. Studies in humans have shown that serum fatty acid profiles are associated with bone mineralisation (Gronowitz et al., 2006; Eriksson et al., 2009). In children, serum palmitic acid levels are positively correlated with BMD at the femur (Eriksson et al., 2009). Similarly, Gronowitz et al. (2006) found a positive correlation between serum palmitic acid level and BMC at the lumbar spine and femoral neck. Moreover, fatty acid status only influenced BMD in children, but not in adults. However, there was an inverse but weak association between serum stearic acid level and whole body BMD (Eriksson et al., 2009).

Studies in animal models observed similar positive effects of fatty acid intake on bone properties. Chicks fed with butter fat, which contained greater levels of SFA and natural CLA isomers, had nearly 60% higher bone formation rate compared with those fed with higher n-6 PUFA diet (Bruce A Watkins et al., 1997). Several studies have brought to light the beneficial influence of dietary CLA supplementation in animal models (Kelly & Cashman, 2004; Banu et al., 2006; Park & Pariza, 2008).

Banu et al. (2006) reported that the dietary intake of CLA increased bone mass in both cancellous and cortical bone in young male mice. Co-supplementation of 0.5% CLA and 1.0% calcium improved total bone content and bone density in sham-operated mice. In the OVX mice, CLA increased bone strength without adverse effect on BMC during a bone loss period (Kelly & Cashman, 2004). Similarly, Park and Pariza (2008) analysed 21 feeding studies used mice (both female and male) and reported that mice fed with 0.5% CLA containing diet and enhanced calcium content (0.66%) had improved bone mass compared with those fed CLA with regular calcium content (0.5%). However, these studies did not specify the effect of different CLA isomers on bone parameters. The C9t11-CLA isomer represents approximately 80% of the CLA isomers in dairy fat (Rahman et al., 2011). However, it was found that the t10c12-CLA isomer had a more potent effect on decreasing bone marrow adiposity along with increasing BMD in femoral, lumbar and tibial regions in aging female mice compared with the c9t11-CLA isomer (Rahman et al., 2011).

Dietary fats affect bone formation mainly through the regulation of local and hormonal factors and calcium metabolism. The consumption of oleic acid fortified milk in adults increased plasma levels of osteocalcin and the ratio of OPG/RANKL, markers of bone formation (Martin-Bautista et al., 2010). Moreover, SFA intake was associated with increased production of bone growth factors such as IGF-1 and decreased the *ex vivo* PGE<sub>2</sub> production, which may promote bone formation (Bruce A Watkins et al., 1997). CLA directly inhibited *ex vivo* PGE<sub>2</sub> biosynthesis in rat bone organ culture by competition with other PUFAs in the formation of AA (Li & Watkins, 1998). AA as the precursor of PEG<sub>2</sub> is important, particularly in the young, Watkins et al. (1997) showed that a low level of PEG<sub>2</sub> stimulated bone formation in chicks, while a high level inhibited bone formation. CLA also suppressed bone resorption measured by decreased TRAP activity and lowered urinary Pyr and Dpyr in OVX rats (Kelly & Cashman, 2004). Moreover, bone metabolism in rats fed with CLA may be altered by influencing IGF-1, IGF binding proteins, the production of cytokines such as IL-1 and IL-6, and TNF (Watkins & Seifert, 2000). CLA may also affect bone mass indirectly by modulating calcium metabolism. CLA supplementation over eight weeks in young growing rats increased calcium absorption, making more calcium available for calcification, but had no effect on

bone mass or bone metabolism (Kelly et al., 2003). However, dietary CLA supplementation in OVX rats seemed to have no effect on calcium absorption, bone mass and mineral content (Kelly & Cashman, 2004). CLA increased PTH levels without effect on  $1,25(\text{OH})_2\text{D}_3$  by modulating  $\text{PGE}_2$  production, which may contribute to the increased calcium absorption and the improvement in overall bone mass (Park et al., 2013).

Additionally, there is increasing evidence that consuming PUFAs, especially n-3 PUFA, results in greater bone formation rate, BMC and BMD in various bone sites of growing rats (Watkins et al., 2000b; Watkins et al., 2003; Green et al., 2004; Poulsen et al., 2007; Li et al., 2010; Lukas et al., 2011).

However, some studies showed that fat intake is potentially harmful for bone health in both human and animal models. In human adults, SFA intake was negatively associated with BMD at the hip site, particularly in men less than 50 years old (NHANES III study) (Corwin et al., 2006). High SFA intake was associated with increased hip fracture risk in a dose-dependent manner, while MUFA and PUFA intakes may reduce total fracture risk in aging women (Orchard et al., 2010). Long-term intakes of PUFA and MUFA were negatively associated with BMD change at femoral neck in aging women (Macdonald et al., 2004). Zernicke et al. (1995) observed that growing rats fed long-term (two years) with high-SFA-sucrose diets rich in palmitic and stearic acids (39%) had significantly lower bone volume and cross-sectional area at lumbar vertebra and smaller cortical shell in the femoral neck compared with the rats fed with low-fat and complex carbohydrates diet. The thinner cortical bone of the femoral neck consequently resulted in lower mechanical properties for rats fed high-SFA diets. Similarly, Cao et al. (2009) and Macri et al. (2012) have shown that growing mice (six weeks old) and rats (21 days old) fed with high-SFA diet for 14 and eight weeks, respectively had decreased cancellous bone mass in the tibia or lumbar spine, but not cortical bone.

Wolf et al. (2000) found dietary fat intake was positively associated with fractional calcium absorption. However, specific fatty acids in the diet, particularly SFA, adversely affect the absorption of dietary calcium by the formation of insoluble calcium soaps in the intestine (Zernicke et al., 1995; Moussavi et al., 2008). Moreover, dietary fat may decrease intestinal membrane fluidity that can reduce

calcium uptake by brush border membrane vesicles (Brasitus et al., 1985; Thomson et al., 1986). The adverse effects of a high-fat diet on intestinal calcium metabolism may be more effective in cancellous bone, which has a faster turnover rate than cortical bone (Wohl et al., 1998).

In agreement with previous observations, the findings in the current study indicate that the MLF promoted skeletal health in growing young female rats. However, it is not known which specific bioactive components of MLF have the beneficial role in bone health as the MLF powder is a complex mixture.

#### **4.1.2 Biomechanics**

The three-point bending test was used to measure mechanical properties of the left femur in this study. Rats in the high-dose MLF group had the greatest length and wet weight of the left femur compared to the control and low-dose groups (refer to Table 3.10). Rats in the low dose group had a slight but non-significant positive effect on the elasticity. Overall, the MLF had no significant effect on most bone mechanical parameters during the study period. The hypothesis that intake of MLF would improve biomechanical strength of bone is not supported by these findings. These results suggest that although both BMC and BMD were higher in the MLF-500 group compared to the control or MLF-250 group, it was not sufficient to lead to a significant physiological change in the mechanical properties of the bone during the study period.

The midpoint of femur is predominantly composed of cortical bone, which determines the biomechanical properties of the femur. Cortical bone has a lower turnover rate than trabecular bone (Wohl et al., 1998; Cao et al., 2009). Diet-induced changes in bone strength can occur sooner in bones containing mostly trabecular bone (e.g. the vertebra) than in bones containing mostly cortical bone (e.g. femur) (Wohl et al., 1998; Cao et al., 2009). Studies have indicated that a diet rich in SFA (8% palmitic acid) can produce adverse effects on bone strength of cancellous bone in roosters, whereas there was no significant effect of dietary SFA intake on cortical bone mechanical properties. A previous study has shown that changes in bone metabolism markers such as specific growth factors or bone turnover markers may not cause functional alterations in biomechanical strength of bone. Turner et al.

(1997) found that in rabbits fluoride treatment increased BMC by 10% and serum IGF-1 by 40%, but the bone strength of femurs and vertebrae decreased by 19% and 25%, respectively. On the other hand, the alterations in bone strength induced by dietary intake may not be completely explained by changes in BMC or BMD. Young female rats fed with purified lignan from flaxseed had improved bone strength at the adolescent age without significant changes in femur bone area or BMC (Ward et al., 2001).

Therefore, although the MLF increased femoral BMC and BMD, it did not result in a change in biomechanical properties. This may be due to the short duration of the study that was not sufficient for affecting the cortical bone mechanical properties.

## **4.2 Bone cells**

### **4.2.1 Osteoblasts**

The effect of MLF on the proliferation, differentiation and mineralization was assessed in osteoblastic MC3T3-E1 clone 4 cells. MC3T3-E1 cells are isolated from mouse calvaria. They have the ability to produce ALP and collagenous protein and to mineralize extracellular matrix (Iwami & Moriyama, 1993). They are useful for studying the effects of bioactive compound on osteoblast function.

#### **4.2.1.1 Proliferation**

The MLF, particularly at the concentration of 100 µg/ml significantly increased cell proliferation of MC3T3-E1 osteoblasts (refer to Figure 3.8), indicating that the MLF is a promoter of osteoblast proliferation *in vitro*. However, the MLF at 1,000 µg/ml significantly reduced cell proliferation compared with the blank control, suggesting that the MLF or a component of the sample could inhibit osteoblast proliferation at high concentrations.

Previous studies provide supportive evidence for the findings in this study. Cornish et al. (2008) demonstrated that fatty acids such as palmitic, stearic and oleic acids at 0.1 and 1.0 µg/ml could stimulate thymidine incorporation by mature osteoblasts, suggesting a weak positive effect (1.2- to 1.3-fold increase) of SFA on the proliferation of primary rat osteoblasts.

PUFAs have been reported to either inhibit or stimulate cell proliferation *in vitro*. The n-3 PUFA, DHA and EPA, have always been shown to inhibit cell proliferation from primary human osteoblast (Maurin et al., 2002), smooth muscle (Lötters et al., 2013) and various human pancreatic cancer cell lines (Rizzoli, 2014), whereas n-6 PUFAs such as AA have various effects on cell proliferation according to the experimental conditions and the cell types (Maurin et al., 2002; Sofi et al., 2010; Aune et al., 2012).

#### **4.2.1.2 Differentiation and mineralization**

ALP is a representative enzyme for evaluating osteoblast differentiation (Isama & Tsuchiya, 2003; Zhao et al., 2007; Katono et al., 2008). In the presence of 0.1 to 100 µg/ml, of MLF, MC3T3-E1 cells produced increasing ALP activity in a dose-dependent manner after 9-days of culture (refer to Figure 3.9). However, these results were not statistically significant, suggesting that 9-day of cell culture in MLF may not be sufficient to stimulate the differentiation of MC3T3-E1 cells.

In the Alizarin red S staining assay, the MC3T3-E1 cells were cultured with the MLF for a longer period (24 days). Calcium deposition in cells was evaluated. After 24-day cell culture, MLF led to increased but non-significant calcium deposition in MC3T3-E1 cells, particularly at the concentrations up to 1 µg/ml, compared with the blank control. MLF at the concentrations of 10 and 100 µg/ml significantly inhibited the mineralization (refer to Figure 3.11). These results suggested that the low levels of MLF have no statistically significant effect on mineralization in osteoblasts, whereas the high concentrations of MLF could suppress the formation of calcified nodules.

Calcium deposition occurs in the mature osteoblasts whereas ALP occurs in less-differentiated osteoblasts (Xu, 2009). The ALP activity assay showed that high levels of MLF led to increased but non-significant osteoblast differentiation, while the mineralization assay indicated that the MLF in high concentrations resulted in inhibition in calcium nodule formation, suggesting that high concentration of MLF could inhibit the function of mature osteoblasts rather than suppress the differentiation of pre-osteoblasts.

Previous studies reported a negative effect of fatty acids on osteoblast differentiation in various cell models. Rabbit serum containing approximately 39% palmitic acid, 21% oleic acid and 21% linoleic acid of total fatty acid inhibited the expression of ALP in cells and led to adipocyte differentiation in rat osteoblastic ROS17/2.8 cells, mouse calvaria-derived MB1.8 osteoblasts and human bone tumour SaOS-2/B10 and MG-63 cell lines (Diascro et al., 1998). Moreover, the addition of a fatty acid mixture containing palmitic, oleic and linoleic acids to cell culture medium also induced adipocyte-like differentiation of both ROS17/2.8 and SaOS-2/B10 cells (Diascro et al., 1998). Free fatty acids act as ligands for PPAR $\gamma$  that are not only activate the differentiation of preadipocytes into adipocytes, but also suppress the formation of osteoblasts from MSCs (Diascro et al., 1998; Kim et al., 2013).

Deshimaru et al. (2005) found that oleic acid at the concentration of 10-100  $\mu$ M significantly promoted the osteoblast differentiation of ST2 cells synergistically in the presence of BMP-2, whereas there was no either promoting or suppressing effect of oleic acid on adipocyte differentiation of ST2 cells with oil-red O staining, suggesting that oleic acid may only promote the differentiation of pre-osteoblasts with BMP-2 as it can induce osteoblast differentiation in both MSCs and pre-osteoblasts. Moreover, it has been reported that CLA decreased bone marrow adiposity along with increased BMD (Rahman, 2011). CLA may reduce adipogenesis and improve osteoblastogenesis by inhibiting PPAR $\gamma$ , which suppresses osteoblastogenesis during the differentiation of MSCs (Park et al., 2013).

In the current study, it was found that the MLF, which is rich in SFA, had no deleterious effect on osteoblast differentiation. Other fatty acids of the MLF such as MUFA and PUFA could also exert their effects on the differentiation of osteoblasts.

The function and survival of osteoblasts are also affected by SFA. Bone marrow fats exerted a lipotoxic effect on the function and survival of osteoblasts at the concentrations of 250-1000  $\mu$ M (Elbaz et al., 2010). They predominantly consisted of palmitate and stearate that were biosynthesised by adipocytes. *In vitro* studies have shown that co-culture of normal human osteoblasts with human adipocytes resulted in significantly decreased differentiation, mineralization and the expression of ALP, osteocalcin, osterix and Runx2. Additionally, the presence of adipocytes led to increased osteoblast apoptosis. This lipotoxicity effect of adipocytes can be reverted

by suppression of fatty acid biosynthesis (Elbaz et al., 2010). Similarly, palmitate treatment, at a high concentration of 100-250  $\mu\text{M}$ , decreased cell survival and resulted in apoptosis in a time- and dose-dependent way in the human fetal osteoblast 1.19 cell line (Kim et al., 2008). Lu et al. (2012) reported that the prolonged exposure (72 h) to palmitate induced apoptosis in both human bone marrow-derived and umbilical cord-derived MSCs. The lipotoxicity of SFA may explain the inhibition of mineralization in osteoblasts cultured with high MLF concentrations. Palmitate results in osteoblast apoptosis through reducing the activity of extracellular regulated kinase (ERK), which is a member of mitogen-activated protein kinases (MAPKs) and plays an important role in cell growth, differentiation and apoptosis of osteoblasts and cell survival of osteoclast (Kim et al., 2008; Lu et al., 2012).

Some studies have reported that n-3 PUFA such as AA inhibit the differentiation of human MSC into osteoblasts and promote adipocyte differentiation. However, DHA and EPA stimulate osteoblast differentiation but do not affect the differentiation of adipocytes (Casado-Díaz et al., 2012).

The lactoferrin control did not promote osteoblast mineralization as expected. Lactoferrin at concentrations of 100  $\mu\text{g/ml}$  and higher is capable of stimulating the formation of calcified mineral nodules and increasing the mineralized area in osteoblasts isolated from fetal rat calvaria (Cornish et al., 2004). Similarly, bovine lactoferrin at 1  $\mu\text{M}$  enhanced the extracellular matrix calcification induced by human osteoblast-like MG63 cells, which were cultured on collagen-coated plates (Takayama & Mizumachi, 2008). This discrepancy from previous studies could be explained by the different experimental cells used in this study or the degradation of lactoferrin sourced from milk.

The findings in this study support the hypothesis that the MLF would stimulate the proliferation of osteoblasts but does not provide evidence for an increase in differentiation and mineralization of the MC3T3-E1 osteoblast.

#### **4.2.2 Osteoclasts**

The effects of MLF on osteoclast development were evaluated in a mouse macrophage line RAW 264.7. These cells can be induced to differentiate into

osteoclasts in the presence of RANKL. This experimental system is used to assess the ability of MLF to act directly on osteoclast formation. Osteoclasts are characterized by high secretion of TRAP, which is a metalloenzyme and generates destructive oxygen species that can destroy bone structure (Park et al., 2013). Multinucleated osteoclasts are positive for TRAP staining whereas mononuclear osteoclast precursors have low TRAP activity, thus TRAP activity is used as a marker for osteoclasts (Park et al., 2011). At first, the differential effect of MLF on total TRAP activity was measured in RANKL-induced RAW 264.7 cells. Multinucleated osteoclasts are the mature osteoclasts that have the ability to resorb bone. Next, the effect of MLF on the formation of TRAP-positive multinucleated cells (MNCs) was examined.

In the current study, RANKL caused the formation of osteoclasts in RAW 264.7 cells. The total TRAP activity was significantly increased by the MLF at 0.1  $\mu\text{g/ml}$  and higher concentrations compared with the control group, suggesting MLF stimulates the differentiation of osteoclasts, particularly at 0.1  $\mu\text{g/ml}$  (refer to Figure 3.12). The number of newly developed osteoclasts, which are evaluated as MNCs staining positively for TRAP, was significantly increased in a dose-dependent way from 0.1 to 10  $\mu\text{g/ml}$ . This result suggested that MLF at the concentrations of 10  $\mu\text{g/ml}$  and lower can stimulate the formation of active osteoclasts (refer to Figure 3.13). MLF at 100  $\mu\text{g/ml}$  had no significant effect on the total TRAP activity or the formation of mature osteoclasts, suggesting that high concentrations of MLF did not affect osteoclastogenesis. Lactoferrin at 100  $\mu\text{g/ml}$  showed inhibitory effects on osteoclastogenesis. MLF significantly suppressed the total TRAP activity and completely inhibited the formation of multinucleated osteoclasts. It was consistent with the finding that lactoferrin at 100  $\mu\text{g/ml}$  completely inhibited osteoclastogenesis in mouse bone marrow cells (Cornish & Naot, 2010).

The MLF rich in SFA showed a promoting effect on osteoclastogenesis up to 10  $\mu\text{g/ml}$  but not significant at 100  $\mu\text{g/ml}$  in this study. Consistently, Oh et al. (2010) reported that lauric and palmitic acids at the concentrations of 20-100  $\mu\text{M}$  significantly increased M-CSF and RANKL-induced formation of TRAP-positive MNCs in mouse bone marrow cells in the later stage of osteoclastogenesis. When removed, the cytokines such as RANKL or M-CSF that can promote osteoclast

survival from cultures of differentiated osteoclasts, lauric or palmitic acid alone, prevented the apoptosis of mature osteoclasts through the TLR-4 and MyD88 signalling pathways (Oh et al., 2010).

Contrary to the findings above, the inhibition of osteoclastogenesis by several milk fractions and fatty acids in various cell types had been reported. SFAs ranging in chain length from 14-18 carbon atoms, inhibited RANKL-induced osteoclastogenesis in bone marrow cultures, particularly myristic, palmitic and stearic acids at 10 µg/ml (Cornish et al., 2008). In the culture of RAW 264.7 cells, palmitic and stearic acids had similar anti-osteoclastogenesis actions. Park et al. (2011) found that 1 mM of capric acid (C10:0) significantly reduced the number of LPS-induced TRAP-positive MNCs and decreased TRAP gene expression in RAW264.7 cells by inhibiting NO production in the STAT3 pathway. Fatty acids with more than 20 carbon atoms have poor solubility that affects the assessment, but behenic acid (C22:0) at up to 1 µg/ml had no effect on osteoclastogenesis in RAW 264.7 cells (Cornish et al., 2008). CLA, particularly the t10c12-CLA isomer, led to a reduction in osteoclastogenesis and bone resorption pit formation in the culture of RAW 264.7 cells stimulated with RANKL (Rahman et al., 2011). The modulation of RANKL signalling pathway contributes to this inhibitory effect of CLA on osteoclastogenesis (Rahman et al., 2006).

The findings describing the effects of PUFAs on osteoclastogenesis are inconsistent. Some studies reported that PUFAs, particularly DHA, inhibit osteoclastogenesis (Cornish et al., 2008; Rahman et al., 2008; Yuan et al., 2010). However, others observed increased osteoclastogenesis by EPA and AA (Yuan et al., 2010).

Bone cells express several fatty acid receptors such as GPRs 40, 41 and 43 in osteoclasts, GPR 84 in bone marrow and GPR 120 in both osteoblasts and osteoclasts (Wang et al., 2006; Cornish et al., 2008). These receptors can be activated by various ligand types, including fatty acids, amino acids, ions and nucleotides and modulate cell biological response to fatty acids (Wang et al., 2006; Lampe, 2011). It has been identified that short- and long-chain SFAs act as ligands for GPRs 40, 41, 43 and 120, and medium-chain SFAs as ligands for GPR 84 (Cornish et al., 2008). The effect of medium- and long-chain fatty acids on osteoclastogenesis is likely modulated, at least in part, through GPRs.

The discrepancies between the findings in this study and previous studies (Cornish et al., 2008; Rahman et al., 2011) may be possibly explained by the different experiment systems and cell types used in this study and the complexity of the lipid composition in the MLF sample. The findings in this study do not support the hypothesis that MLF would inhibit osteoclastogenesis in the RAW264.7 model.

### **4.3 Limitations of the study and future improvements**

First, the duration of animal trial was short. The current study used 3-month old female Sprague-Dawley rats to evaluate the effect of MLF consumption on bone growth. The test diets were fed to rats for 12 weeks. In female Sprague-Dawley rats, there is a marked increase in body weight and bone turnover rate in the first 3 months of age. In the following five months, the activity of bone formation rapidly decreases. The peak bone mass is maintained until eight months of age (Li et al., 1991). Therefore, a future long-term study is needed to assess the effect of MLF on bone growth in the growing rats. Moreover, the long-term effects of MLF consumption on bone status at specific bone sites such as cortical bone and cancellous bone are required to be evaluated.

Second, the current study has shown a positive effect of the MLF consumption on bone health in growing animals, whereas the effects of MLF on bone status in later life, particularly its effect on osteoporosis, is interesting and need to be investigated in the future.

Third, the influence of dietary MLF intake in serum or bone marrow lipid pattern as well as the association between the alterations of serum or bone marrow lipid and bone status and bone turnover markers such as OPG, RANKL, Pgd and NTx, which reflect bone formation and resorption, are required to be investigated in future studies.

Finally, this study tested the effects of the MLF on the MC3T3-E1 osteoblasts and RAW264.7 osteoclasts. These findings could be further confirmed in primary cell culture are used in the future studies.

#### **4.4 Conclusion**

In conclusion, the oral administration of MLF to the growing rats improved acquisition of bone tissue and had a favourable effect on peak bone mass over a period of 12 weeks.

The results of *in vitro* experiments indicated that the MLF could directly act on bone cells. The MLF increased the proliferation of MC3T3-E1 pre-osteoblast cell line, but there were no effect on osteoblast differentiation and the higher concentration of MLF may inhibit the function of mature osteoblasts. Additionally, the MLF stimulated the osteoclastogenesis of RAW264.7 cells. These results are contrary to those reported previously. The composition of MLF may need to be further refined and subfractionated as using a complex mixture of components such as in MLF can present many challenges in an *in vitro* system.

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