Effect of calcium and vitamin D fortified milk on bone markers and vitamin D status of active, premenopausal women in Palmerston North, New Zealand

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Effect of calcium and vitamin D fortified milk on bone markers and vitamin D status of active, premenopausal women in Palmerston North, New Zealand

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in

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Rifana Cholidah

2013

Statement of originality

"I hereby declare that this thesis is my own word and that, to the best of my knowledge and belief, it contains no material previously published or written by another person nor material which a substantial extent has been accepted for the qualification of any other degree or diploma of a university or other institution of higher learning, except where due acknowledgement is made in the acknowledgements".

Signed …….

Date …….

Abstract

Objective: To evaluate the effects of 12 weeks supplementation with calcium and vitamin D fortified milk on vitamin D status and bone turnover markers; osteocalcin and C-telopeptide of type 1 collagen, of active, healthy premenopausal women aged 30-45 years old in Palmerston North, New Zealand.

Methods: The study was a repeated measure design. Forty three premenopausal women were recruited. Participants received two daily servings (2 x 30 g) of fortified milk (1200 mg/d calcium and 10 µg/d vitamin D) over 12 weeks. Anthropometric characteristics were measured for screening by body mass index and bone density measurement. Dietary information was collected using an estimated 3-day food record and a food frequency questionnaire. Blood samples were taken for a screening blood test and to measure plasma 25(OH)D_3, interleukin-6, and bone turnover markers; C telopeptide of type I collagen (CTx) and osteocalcin. Usual physical activity levels were estimated using the SPARC short-form New Zealand Physical Activity Questionnaires in face-to-face interviews and were objectively measured using accelerometers in a self-selected group of 25 women.

Results: A significant increase in plasma 25(OH)D_3 was found (from 69.75 ± 15.87 nmol/L at baseline to 87.83 ± 19.06 at week 12, mean ± standard deviation; *p*-value <.0001). There were significant reductions in the levels of CTx (0.31 ± 0.12 to 0.21 ± 0.09 µg/L, *p*-value <.0001) and osteocalcin (22.63 ± 6.64 to 19.64 ± 6.25 µg/L, *p*-value 0.0003). Dietary calcium intake was 1013 ± 367 mg/day and vitamin D intake was 3.9 ± 2.1 µg/day. The duration of physical activity in the questionnaire and accelerometer were 115 ± 74 and 415 ± 319 (light physical activity), 208 ± 225 and 289 ± 143 (moderate physical activity) and 126 ± 130 and 59 ± 61 minutes (vigorous physical activity) respectively.

Conclusion: Calcium and vitamin D fortified milk supplementation improved vitamin D status and decreased bone turnover markers in active premenopausal women aged 30-45 years old over a period of 12 weeks.

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“Then, which of the favours of your Lord will you deny?”

(Holy Quran, Ar-Rahman:13)

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Abbreviations

$1,25(OH)_2D_3$	1,25-dihydroxyvitamin D_3
$25(OH)D_3$	25-hydroxyvitamin D_3
AI	Adequate intake
AMDR	Acceptable macronutrient distribution ranges
ANZFA	Australia New Zealand Food Authority
B-ALP	Bone-specific alkaline phosphatase
BGP	Bone gla protein
BMC	Bone mineral content
BMD	Bone mineral density
BMI	Body mass index
BMUs	Bone Multicellular Units
BTMs	Bone turnover markers
CTx	Cross-linked C telopeptide of type I collagen
CV	Coefficient of variation
CYP	Cytochrome
D_2	Ergocalciferol
D_3	Cholecalciferol
DEXA	Dual energy X-ray absorptiometry
EAR	Estimated average requirement
FFQ	Food frequency questionnaire
GM-CSF	Granulocyte macrophage colony-stimulating factor
HRP	Horseradish peroxidase
ID-LC-MS/MS	Isotope-dilution liquid chromatography–tandem mass spectrometry
IFN-ɣ	Interferon-ɣ
IL	Interleukin
LPA	Light physical activity
MCP-1	Monocyte chemoattractant protein-1
M-CSF	Macrophage colony-stimulating factor
MGP	Matrix ɣ-carboxylic acid (gla) protein
MPA	Moderate physical activity
NCPs	Noncollagenous proteins

NHANES	National Health and Nutrition Examination Survey
NRV	Nutrient reference value
NRVANZ	Nutrient Reference Values for Australia and New Zealand
NTx	N-telopeptide of type I collagen
NZPAQ	New Zealand Physical Activity Questionnaires
NZEO	New Zealand European and Others
OC	Osteocalcin
OPG	Osteoprotegerin
PA	Physical activity
PBM	Peak bone mass
PGE_2	Prostaglandin E_2
PGs	Prostaglandins
PICP	C-propeptide of type I collagen
PTH	Parathyroid hormone
QUS	Quantitative ultrasound
RANK	Receptor activator of nuclear factor kB
RANKL	Receptor activator of nuclear factor kB ligand
RCT	Randomized controlled trial
RDI	Recommended dietary intake
SPARC	Sport and Recreation New Zealand
T_3	Triiodothyroxine
T_4	Tetraiodothyronine
TH	Thyroid hormone
TMB	Tetramethylbenzidin
TNF	Tumor necrosis factor
TSA	Tyramide signal amplification
UL	Upper level of intake
VPA	Vigorous physical activity

Introduction

Age-related bone loss affects all genders, nationalities and ethnicities (Halioua & Anderson, 1989; Li et al., 2012). Females are more affected by age-related bone loss than males due to bone loss after the onset of menopause (Mazess & Barden, 1991), and females also have a smaller skeletal mass than men which contributes to higher architectural damage (Seeman, 2002).

Evidence shows that adequate lifetime calcium intake and physical activity habits of healthy premenopausal women aged 20 to 50 years can increase their peak bone mass (Halioua & Anderson, 1989). A meta-analysis of thirty-three well-designed studies reported an overall positive correlation between calcium intake and bone mass in premenopausal females and the findings were consistent across different study designs (Welten et al., 1995). However, a more recent 12-year prospective study involving 77,761 females aged 34-59 years reported no evidence that higher calcium and milk intakes reduced fracture incidence (Feskanich et al., 1997). In children and adolescents, studies of calcium supplementation indicated a positive correlation between high calcium intake and higher bone mass (Cadogan et al., 1997; Johnston et al., 1992). In adolescent females, a randomized controlled trial evaluating the effect of 18 months milk supplementation showed a significant increase in bone mineral content and bone mineral density. However, no significant effect of milk supplementation was found on markers of bone turnover (Cadogan et al., 1997).

Few studies have been carried out to evaluate the effect of milk supplementation on bone turnover markers, particularly of premenopausal women. In a study it was found that milk supplementation significantly increased vitamin D status over 24 months (Du et al., 2004). A similar result was also reported in a short-term study of postmenopausal women (Kruger et al., 2010). In a short-term study of 82 premenopausal women, it was found that fortified milk supplementation resulted in a significant reduction of bone turnover markers (Kruger et al., 2006).

Studies of physical activity (PA) reported that physical activity benefits bone health, bone mass and bone status in children, adolescents and premenopausal women (Slemenda et al., 1991; Janz et al., 2010; Baxter-Jones et al., 2008; Kanders et al., 1988; Heinonen et al., 2012). The beneficial impacts of physical activity during adolescence remain sustained into young adulthood (Baxter-Jones et al., 2008). However, in premenopausal females, the benefits were not sustained once the exercise discontinued (Heinonen et al., 2012).

Factors such as lifestyle (physical activity), intakes of calcium and vitamin D, and sex hormone status influence development of peak bone mass as well as on going bone health (Cooper & Eastell, 1993; Heaney & Weaver, 2003; Hind & Burrows, 2007; Sakuma et al, 2007). As nutrition is essential for bone health, several other vitamins and minerals have also been identified to have significant impact on bone health and metabolism (Bonjour et al., 2010; Cashman, 2002). However, to date, limited milk supplementation studies have been performed to evaluate vitamin D status and bone turnover markers in physically active premenopausal women.

Chapter 1

Literature review

Section 1. Bone

1.1. Function

The function of every bone depends on its site. There are six main functions of bones, which are to provide structural support, protect vital organs, movement, maintain electrolyte and acid-base balance, and to produce blood cells by hematopoietic tissue in the red bone marrow (Dempster, 2006; Pocock & Richards, 2006).

1.2. Structure

Bones are generally classified into two main types: long bones such as the femur, tibia, fibula and radius, and flat bones such as the cranium, scapula and ribs. The long bones can be categorised into three regions: the epiphysis (edges), diaphysis (hollow shaft), and the metaphysis, the section under the growth plate which resides between epiphyses and diaphysis. The diaphysis is comprised mainly of compact bone, while the metaphysis and the epiphysis contain trabecular bone that is surrounded by a shell of cortical bone (Figure 1.1) (Dempster, 2006; Marieb & Hoehn, 2006).

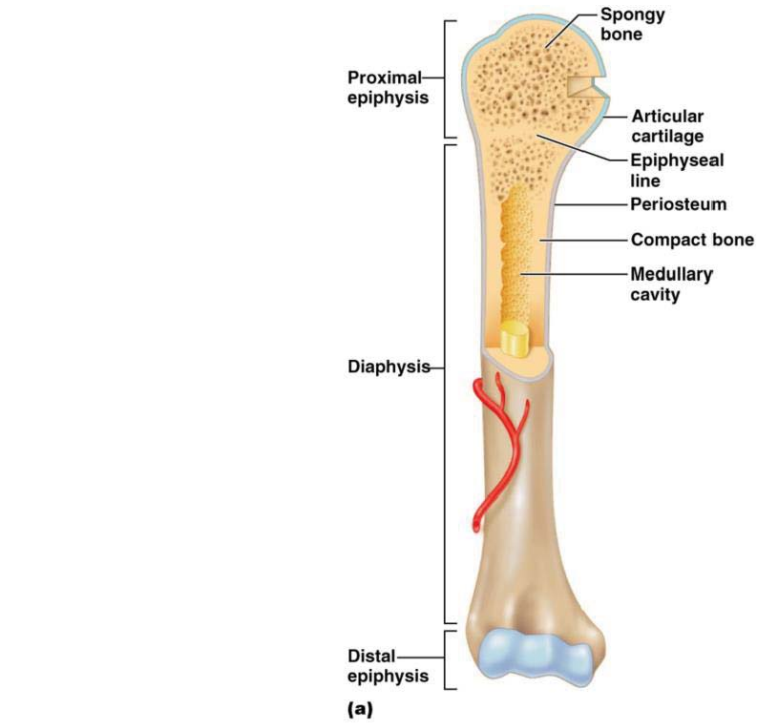

Figure 1.1. The structure of long bone, showing epiphysis, epiphyseal line (growth plate) and diaphysis (Marieb & Hoehn, 2006), reproduced with permission from Pearson Education.

The adult skeleton is comprised generally of two forms of bone: cortical (compact) bone and trabecular (spongy) bone (Figure 1.2). Around 80% of bone in adult is composed of cortical bone and 20% is trabecular, however the relative ratio of these two forms of bone depends on the skeletal site. For instance the proportion of trabecular : cortical bone in human vertebrae is around 75:25, in the diaphysis of radius is approximately 95:5, and in the femoral head is 50:50 (Dempster, 2006).

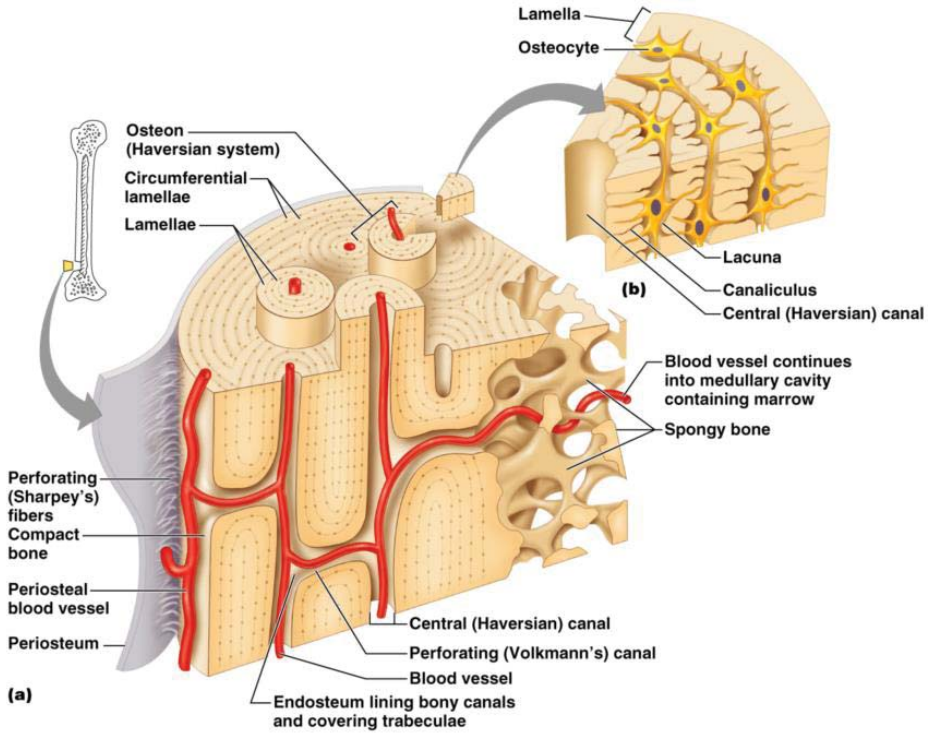

Figure 1.2. Microscopic structure of bone showing cortical (compact) and trabecular (spongy) bone (Marieb & Hoehn, 2006), reproduced with permission from Pearson Education.

All bone surfaces, except at the joints, are sheathed by fibrous connective tissue named the periosteum. This outer fibrous layer of bone contains blood vessels to nourish the bone, as well as the osteoblasts, osteoclasts, and nerve endings. The inner layer of bone which connects directly with the marrow, is known as the endosteum. This membranous sheath also contains blood vessels, osteoblasts and osteoclasts (Dempster, 2006).

1.3. Components of Bone

Bone mainly consists of matrix and bone cells. The matrix of skeleton is made up of around one-third organic and two-thirds inorganic components (Saladin, 2007). Bone matrix is physiologically mineralized and continually renewed throughout life as a result of bone remodelling (Robey & Boskey, 2006).

The organic matrix contains type I collagen, a triple helix molecule consisting of two identical α1 chains and one α2 chain (Brodsky & Persikov, 2005), and noncollagenous proteins (NCPs) such as osteonectin, matrix ɣ-carboxylic acid (gla) protein (MGP), osteocalcin (bone gla protein [BGP]), and protein S. The organic matrix of bone provides flexibility and elasticity to bone (Robey & Boskey, 2006).

The inorganic (mineral) matrix of bone is primarily made up of 85% hydroxyapatite, a crystalized calcium phosphate salt [$Ca_{10}(PO_4)_6(OH)_2$], 10% calcium carbonate ($CaCO_3$), and small amounts of magnesium, sulphate, sodium, carbonate, potassium, fluoride, and hydroxide ions (Saladin, 2007). The mineral components of bone provide load bearing strength and mechanical rigidity to the bone (Robey & Boskey, 2006).

There are four main bone cell types: osteogenic cells, osteoblasts, osteoclasts, and osteocytes.

1.3.1. Osteogenic cells

Osteogenic cells are pluripotent stem cells which have self-regeneration into the majority of other bone cells, adipocytes, chondrocytes, tendon cells, and myocytes. They are also known as mesenchymal stem cells (Aubin et al., 2006). These cells proliferate regularly and some of them transform into osteoblasts (Figure 1.3). Osteogenic cells are found in the endosteum and the central canals (Saladin, 2007). They function to provide multiple cell lineages (Aubin et al., 2006).

1.3.2. Osteoblasts

Osteoblasts are derived from pluripotent mesenchymal stem cells (Figure 1.3) (Raggatt & Partridge, 2010). Osteoblasts are mostly angular or cuboidal shaped, and are found on the bone surface under the endosteum and periosteum, and the internal marrow cavity (Pocock & Richards, 2006). Osteoblasts are very similar in morphology to that of fibroblasts in cell culture (Ducy et al., 2000).

The main functions of the osteoblasts are bone formation and controlling osteoclast differentiation (Karsenty, 2008). The formation of new bone has mainly three consecutive stages: the production and maturation of osteoid matrix, followed by mineral deposition of the matrix. The process of matrix production and mineralization occurs at the same rate in normal adult bone, thus the balance between these two processes is maintained (Hadjidakis & Androulakis, 2006). The osteoid is produced by osteoblasts by rapid deposition of collagen. Consequently, there is an increased mineralization rate to meet the increased production of collagen. In the final phase,

the production of collagen decreases while mineralization persists until the osteoid is completely mineralized (Hadjidakis & Androulakis, 2006).

In bone remodelling, osteoblasts play numerous vital roles such as expression of osteoclastogenic factors, synthesis of the organic matrix of the bone (collagen, glycoproteins and proteoglycans), and performing mineral deposition (calcification) (Karsenty, 2008; Pocock & Richards, 2006).

The osteoblastic lineage cells consist of various types of cells including immature and mature osteoblasts (Gori et al., 2000). The cells of the osteoblastic lineage influence the differentiation of the osteoclast. If there is a temporary change in the number of mature osteoblasts, bone formation is affected but osteoclastic bone resorption is sustained (Corral et al., 1998). This suggests that osteoclastogenesis is affected by immature osteoblasts while mature osteoblasts are responsible for bone matrix synthesis and bone mineralisation (Raggatt & Partridge, 2010). Some factors such as stress and fractures promote osteogenic cells to proliferate faster generating osteoblasts to restore and rebuild the bone (Saladin, 2007).

Osteoblasts and cells from the intestines, kidney, liver, spleen, lung, mammary tissue, and skin produce osteoprotegerin (OPG), a soluble decoy receptor and glycoprotein, a member of the tumor necrosis factor (TNF) receptor superfamily (Neville-Webbe, et al., 2004; Theoleyre et al., 2004). OPG inhibits osteoclast differentiation, osteoclastogenesis (Simonet et al., 1997), and binding of receptor activator of nuclear

factor kB (RANK) to receptor activator of nuclear factor kB ligand (RANKL) on the cell surface of osteoblast lineage cells (Khosla, 2001).

Osteoblasts and stromal cells express RANKL on the surface membrane. RANKL is important in the osteoclast maturation and function and joint destruction in rheumatoid arthritis (Khosla, 2001; Kong et al., 1999; Eghbali-Fatourechi et al., 2003). Additionally, osteoblasts and stromal cells produce macrophage colony-stimulating factor (M-CSF), a cytokine that induces differentiation and proliferation of mononuclear cells into macrophages, regulated by osteoclastogenic molecules such as parathyroid hormone (PTH) (Udagawa et al., 1990; Owens & Chambers, 1993; Yang et al., 1996; Teitelbaum, 2000).

1.3.3. Osteoclasts

Osteoclasts are of hematopoietic origin (Figure 1.3) (Boyle et al., 2003) and the principal cells in bone resorption. These giant multinucleated cells are derived from the fusion of several stem cells of the monocytes/macrophage family (Teitelbaum, 2000). Osteoclast differentiation is influenced by two important osteoclastogenic cytokines, RANKL and M-CSF (Teitelbaum, 2007; Vaananen & Laitala-Leinonen, 2008). The M-CSF receptor, a subfamily of the tyrosine kinase growth factor receptor, is responsible for the M-CSF cellular responses (Yang et al., 1996). Together with RANKL, a potent stimulator of osteoclast differentiation and activation, M-CSF promotes osteoclastogenesis (Yasuda et al., 1998; Teitelbaum, 2000). Furthermore, RANK, which is expressed on the surface of mature osteoclasts and osteoclast lineage cells, and chondrocytes, has a central role in osteoclastogenesis (Hsu et al., 1999; Kong et al., 1999; Khosla, 2001; Cao, 2011; Eghbali-Fatourechi et al., 2003; Bell, 2003). The highest

RANK expression is seen in the osteoclasts within area of active bone resorption (Hsu et al., 1999).

In contrast, OPG acts as a negative regulator of osteoclastogenesis and loss of functional OPG causes excessive osteoclastogenesis resulting osteoporosis (Simonet et al., 1997). The evidence indicates that the ratio of RANKL/OPG determines the level of the differentiation and function of osteoclasts and the regulation of bone remodelling and bone loss (Kong et al., 1999; Hofbauer et al., 2000). The mechanism by which preosteoblasts or stromal cells regulates osteoclast development is known as OPG/RANKL/RANK pathway. Proinflamatory cytokines such as TNFα, interleukin (IL)-1 and IL-6, which increase with increased bone resorption, are capable of promoting osteoclast activity via the regulation of the OPG/RANKL/RANK pathway (Figure 1.3) (Khosla, 2001; Cao, 2011).

Osteoclasts have a main role in bone remodelling by removing and resorbing brittle and damaged bone. They are responsible for bone resorption starting from proliferation of osteoclast, followed by with the degradation of the organic and inorganic bone matrix by the mature osteoclast (Teitelbaum, 2007). Osteoclast lineage cells produce a number of cytokines such as TNF, IL-1, IL-6, IL-11, and transforming growth factor β (TGF-β) with an essential role in the osteoclastogenesis, and differentiation of osteoclast stem cells into mature osteoclasts (Suda et al., 1997; Hofbauer et al., 2000).

Osteoclasts usually reside at or near the bone surfaces which undergo erosion (Pocock & Richards, 2006). They have a lot of mitochondria and acidic vacuoles containing lysosomal enzymes and acid phosphates. The acidification of bone surface in contact with osteoclasts mediates the resorption of inorganic components of the bone matrix, and lysosomal enzymes absorb the organic fraction (Stenbeck, 2002). One side of the osteoclasts, that bordering the bone surface, has a ruffled border of microvilli resulting in increased cell surface area, and therefore increased bone resoption (Figure 1.4) (Saladin, 2007). The Ruffled border, which has features similar to lysosomal membranes, acts as an exit site for lysosomal proteases and protons. The size of ruffled border may vary depending on the cell activity, it can be very large, in order to provide larger surface area for material exchange and acid circulation (Stenbeck, 2002).

The bone resorption cycle has five subsequent phases: osteoclast's migration to the resorption site, binding to the bone surface, formation and polarization of new membrane regions, degradation and elimination of the bone matrix elements, and lastly either osteoclast apoptosis or osteoclast reoccurrence in the non-resorbing phase (Vaananen et al., 2000). A strongly sealed compartment, the so-called sealing zone, is established between osteoclasts and the bone surface, covers the resorption site from its surroundings and allows the extracellular acidification (Figure 1.4) (Blair et al., 2002; Stenbeck, 2002).

In the bone resorption process, osteoclasts experience prompt and significant changes in polarity, thus the resting cell generates two new membrane domains: the sealing

zone and ruffled border, a specific membrane organelle formed by the fusion of intracellular acidic vesicles which face the resorption lacuna, the fluid filled area (Vaananen & Horton, 1995). Various matrix degrading proteinases and a low pH promote the degradation of bone matrix components in the resorption lacuna (Hill et al., 1994).

During the bone resorption, the expression of vacuole type proton ATPase (V-ATPase) releases a large number of acid equivalents to dissolve the bone mineral. Protons required for the V-ATPase are provided by carbonic anhydrase II, a cytoplasmic enzyme which converts CO_2 and water into bicarbonate and protons (Stenbeck, 2002). After dissolution of the mineral fraction, several proteolytic enzymes degrade the organic component. Tartrate-resistant acid phosphate (TRAP), a commonly used osteoclast marker, is located in transcytotic vesicles of osteoclasts. TRAP can produce very destructive reactive oxygen species (ROS) which can degrade collagen (Vaananen et al., 2000). Cathepsine K, a member of lysosomal cysteine protease, is expressed by active osteoclasts to degrade type I collagen, the primary component of organic bone matrix (Costa et al., 2011).

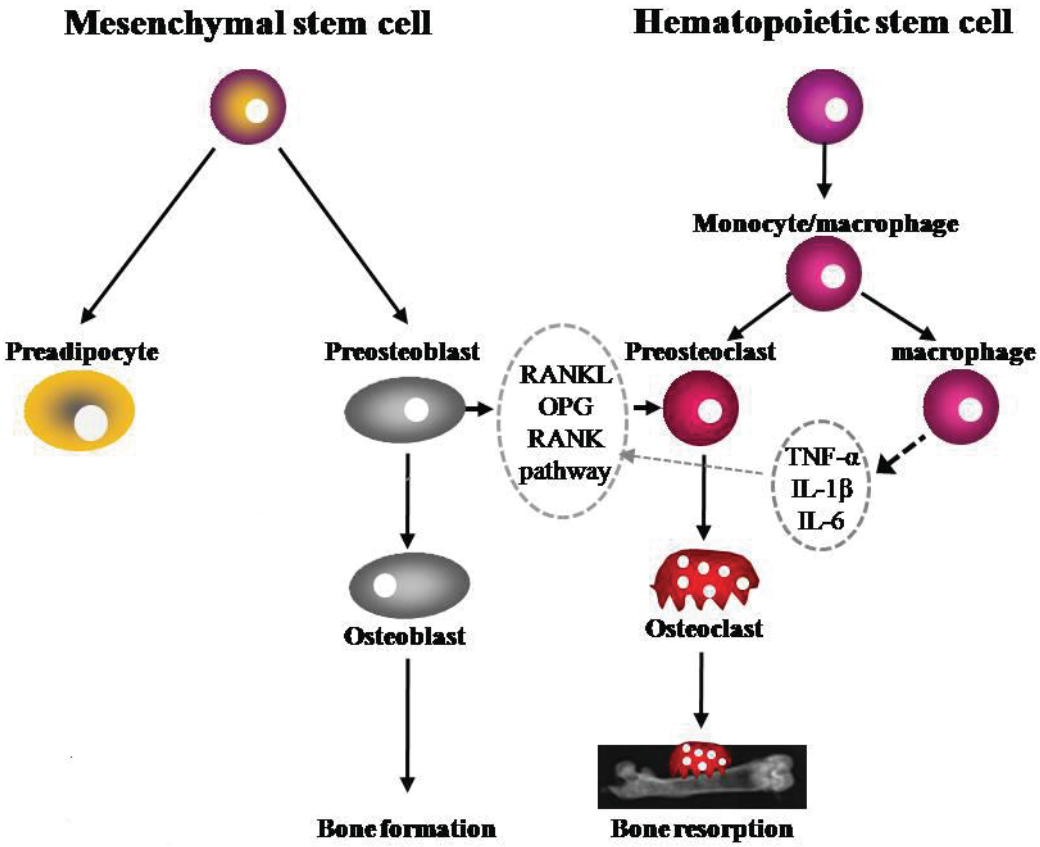

Figure 1.3. Osteoblast and osteoclast origin, adapted from Cao, (2011). Osteoblasts are derived from mesenchymal stem cells that proliferate into preosteoblast, and subsequently into osteoblasts, while osteoclasts are developed from monocyte/macrophage precursors of hematopoietic progenitors that proliferate into preosteoclast, and then into osteoclasts. Cytokines such as TNFα, IL-1 and IL-6 are capable to modulate osteoclastofenesis via OPG/RANKL/RANK pathway.

1.3.4. Osteocytes

Osteocytes are mature osteoblasts that have become trapped in lacunae within the matrix (Bonewald, 2007). After bone matrix mineralisation, the adjacent osteocytes build a complex network via the dendrite-like processes from canaliculi (tunnels) throughout the mineralized matrix (Figure 1.4). By their dendrite-like processes, osteocytes communicate with other nearby osteocytes as well as with osteoblasts on the bone surface (Kamioka, et al., 2001).

This connection between osteocytes allows the transfer of nutrients and chemical signals, and removal of metabolic waste to the nearest blood vessels (Saladin, 2007). Also, the connection enables osteocytes to regulate dissolution and deposition of calcium in the perilacuno-pericanaliculi space and afterward to the extracellular fluid. This transport is known as osteocytic osteolysis, and is beneficial to transfer calcium when plasma calcium concentrations reduce (Pocock & Richards, 2006; Kaiser et al., 2012).

Numerous functions have been suggested for osteocytes in bone metabolism including acting as a calcium sensor (Kamioka, et al., 2001; Ypey, 1992), acting as a mechanosensor (strain sensors) (Kleinnulend et al., 1995; Kaiser et al., 2012), and a regulator of osteoid matrix maturation and mineral deposition (Mikuni-Takagaki et al., 2009).

Osteocytes promote homeostatic maintenance of both bone density and plasma concentrations of calcium and phosphate. As the strain sensors, when bones have a burden, osteocytes promote a flow in the canaliculi and lacunae, then produce biochemical signals that control the bone remodelling by adjusting bone shape and density as the adaptation to stress (Saladin, 2007; Kaiser et al., 2012).

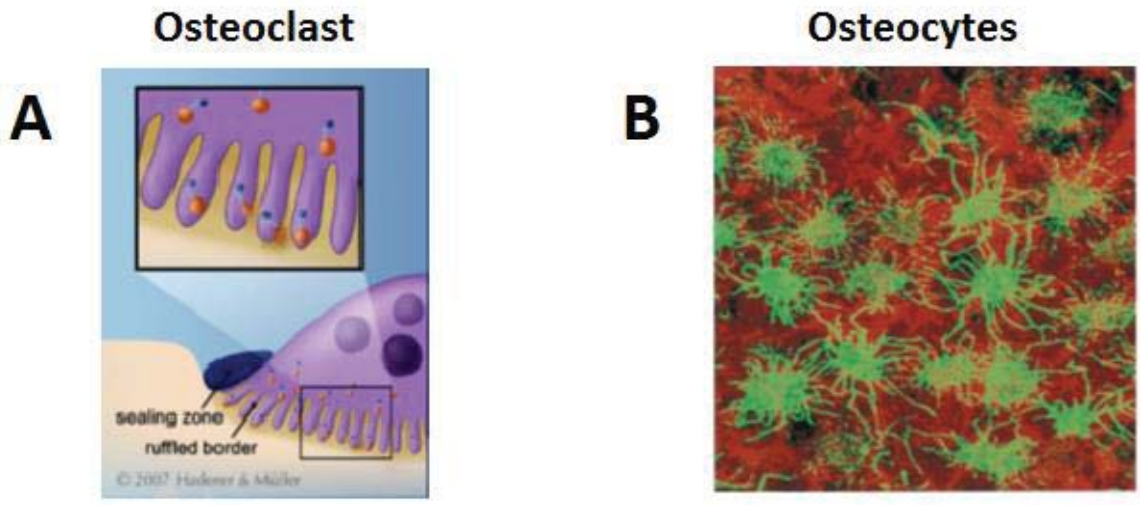

Figure 1.4. (A) Image of osteoclast, modified from Haderer & Muller (2007), reproduced with permission from IBMS BoneKEy. (B) Image of osteocytes, modified from Kamioka et al. (2001).

1.4. Bone Remodelling

Bone is an active tissue that is regularly remodelled throughout life by removing the old bone and replacing it with new tissue (Hadjidakis & Androulakis, 2006). This process is important to repair the structural damage and adapt to the different levels of mechanical load, help maintain serum calcium concentrations and in response to paracrine and endocrine influences (Robling et al., 2006; Sims & Gooi, 2008).

Bone remodelling is a complex process involving the coordinated actions of osteoclasts (resorption), osteoblasts (formation) and osteocytes (Sims & Gooi, 2008). The areas in the bone, where remodelling takes place, are known as “Bone Multicellular Units” (BMUs), which are found throughout the skeleton (Hadjidakis & Androulakis, 2006; Ott, 2010). The cellular activity in the BMU is balanced, thus the total of bone resorbed by osteoclasts is equal to the total bone modelled by osteoblasts (Sims & Gooi, 2008).

The remodelling process consists of subsequent stages: activation, resorption, reversal, formation and termination (Raggatt & Partridge, 2010). The first stage is activation. PTH, an endocrine remodelling signal and key regulator of calcium homeostasis, binds to the PTH receptor on osteoblast precursors (Raggatt & Partridge, 2010), resulting the activation of protein kinase A and C, and calcium intracellular signalling pathway in osteoblasts (Swarthout et al., 2002). The process modulates the secretion of molecules that recruit osteoclast precursors and promotes the activation and differentiation of osteoclasts, which lead to bone resorption.

The second phase is resorption. Osteoblasts release monocyte chemoattractant protein-1 (MCP-1) and recruit preosteoclasts to the remodelling site as the response to PTH-induced bone remodelling. The production of M-CSF and RANKL is increased while OPG expression is decreased to stimulate the proliferation, differentiation and maturation of osteoclasts. Mature osteoclasts establish a sealed zone to enable the degradation of mineralized bone matrix (Raggatt & Partridge, 2010).

After the completion of osteoclastic bone resorption, the reversal phase takes place. The mononuclear cells engulf and remove undigested collagen and prepare the bone surface for subsequent osteoblast bone formation (Hadjidakis & Androulakis, 2006; Matsuo & Irie, 2008). In the formation phase, the coupling signal, arise from bone resorbing osteoclasts, degraded bone matrix and reversal cells, stimulates the differentiation of preosteoblasts and activates bone formation in bone resorption lacunae. Whilst bone formation is activated, osteoclastic bone resorption discontinues and osteoclasts undergo apoptosis (Matsuo & Irie, 2008; Raggatt & Partridge, 2010).

Once the resorbed bone has been replaced, the remodelling cycle is completed. It is characterized by the expression of sclerostin, a soluble molecule that binds to the low density lipoprotein receptor-related protein-5/6 (LRP5/6) and inhibits Wnt signaling, an inducer of bone formation, and the termination of bone formation (Li et al., 2005; Raggatt & Partridge, 2010).

Each stage of the cycle has a different duration: resorption may take place one to two weeks, reversal stage probably last up to four or five weeks, whist formation may take for about four months until the new bone is completely formed (Hadjidakis & Androulakis, 2006). The bone remodelling process is illustrated in figure 1.5.

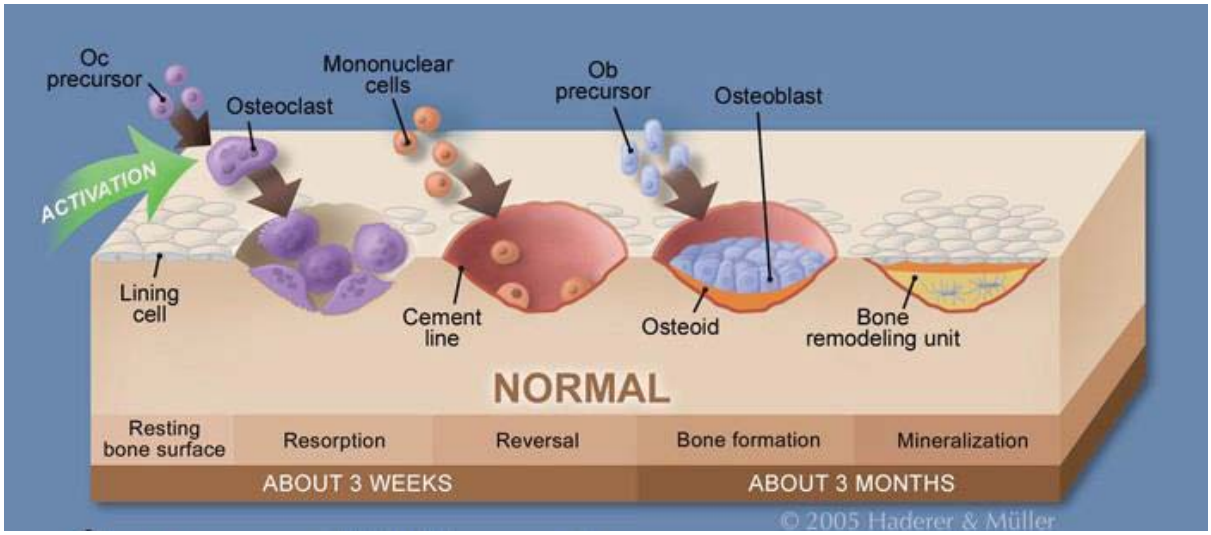

Figure 1. 5. Bone remodelling process involving subsequent stages: activation, resorption, reversal, formation and mineralization. Adapted from Haderer & Muller (2005), reproduced with permission from IBMS BoneKEy.

1.5. Regulation of bone metabolism

1.5.1. Hormonal factors

1. Vitamin D

Vitamin D is an essential component to support bone health and a regulator of calcium absorption (Bonjour et al., 2010). In addition, serum 25-hydroxyvitamin D_3 [25(OH)D_3] is a routine marker to measure vitamin D nutritional status (Sakuma et al., 2007).

Vitamin D is derived from two sources: the diet (D_2 or ergocalciferol) and endogenous production (D_3 or cholecalciferol). Vitamin D is not a common component in the majority of foods. The human body can produce vitamin D by subcutaneous reactions mediated by UVB radiation converting 7-dehydrocholesterol into pre-vitamin D_3. The next phase, pre-vitamin D_3 is isomerized in the skin to vitamin D_3, an inactive form of vitamin D (Heaney & Weaver, 2003).

Vitamin D absorbed from foods or produced in the skin is biologically inactive and needs two consecutive hydroxylations (Holick, 2009). In the liver, carbon 25 from vitamin D_2 or D_3 precursors is hydroxylated to form 25(OH)D_3 (calcidiol), catalysed by the enzyme vitamin D 25-hydroxylase (Prosser & Jones, 2004; Cheng et al., 2003; Cheng et al., 2004).

It has been identified that the vitamin D 25-hydroxylase enzyme contains at least four cytochrome P450 (CYP) isoforms: CYP27A1, CYP2J3, CYP2R1 and CYP3A4 (Prosser &

Jones, 2004). Of these four CYPs, CYP27A1 and CYP2R1 are considered to be the most viable candidates for enzyme 25-hydroxylase activity (Cheng et al., 2003).

The next hydroxylation on carbon 1α mainly occurs in the kidneys to produce the active form of vitamin D, 1,25-dihydroxyvitamin D_3 [$1,25(OH)_2D_3$] (calcitriol) catalysed by the 1α-hydroxylase enzyme that contains the cytochrome component CYP27B1. Several studies in both animals and humans revealed that 1α-hydroxylase containing CYP27B1 is located in the proximal renal tubules (Sawada et al., 2001; Miller & Portale, 2000).

A number of hormones and minerals including PTH, $1,25(OH)_2D_3$ itself, calcium and phosphorus tightly regulate renal $1,25\text{-}(OH)_2D_3$ synthesis through the activity of renal 1α-hydroxylase enzyme (Janssen et al., 2002). Activity of the 1α-hydroxylase enzyme has also been found in brain, placenta, prostate, osteoblasts, macrophages and keratinocytes (Miller & Portale, 2000). Furthermore, $1,25(OH)_2D$ regulates bone resorption, intestinal absorption and renal excretion of calcium (Wesseling et al., 2008)

Serum $25(OH)D_3$ is a routine marker of vitamin D status, rather than serum $1,25(OH)_2D_3$ which presents no information about vitamin D status and is normal or even high in secondary hyperparathyroidism related to vitamin D deficiency (Holick, 2009). The Institute of Medicine (IOM) recommends the normal serum $25(OH)D_3$ concentration is 50 nmol/L (20 ng/mL) (Ross et al., 2011). Some experts suggest the optimal serum $25(OH)D_3$ concentration for multiple health benefits starts at 75 nmol/L

(30 ng/mL) with the best benefits from 90 to 100 nmol/L (36-40 ng/mL) (Holick, 2009; Bischoff-Ferrari et al., 2006; Holick, 2006).

Sun exposure is the main source of vitamin D for individuals living in Australia and New Zealand (Diamond et al., 2005). Thus, serum concentration of $25(OH)D_3$, the indicator of vitamin D status, may have seasonal variation and is lower at the end of winter (Nowson & Margerison, 2002). People can receive between 10 and 50% more UV light in the summer which will lead to increased levels during summer (Rockell et al., 2006). In addition to seasonal variation, plasma $25(OH)D_3$ concentration in New Zealand women who live in the South was 6 nmol/L lower than those in the North Island due to 25% more UV light in North than those in South Island. In New Zealand, Pacific and Maori people, who have a darker skin than New Zealand Europeans tend to have a greater risk of low vitamin D status (Rockell et al., 2006).

New Zealanders do not usually eat foods high in vitamin D, such as organ meats and fatty fish (Russell et al., 1999) or the small number of margarines that are fortified (Rockell et al., 2006), and are at risk of low vitamin D status. In general, vitamin D status is maintained by sunlight exposure for most people. When the exposure to sunlight is adequate, vitamin D intake from diet can be considered unnecessary (Banke-Rasmussen et al., 2000; Holick, 2001; Ministry of Health, 2006). The recommendation is 5 µg vitamin D daily from diet required to maintain adequate levels of serum vitamin D in adults with limited sun exposure (Ministry of Health, 2006).

Inadequate sun exposure and low dietary vitamin D intake result in vitamin D deficiency which may be categorised into three stages: mild, moderate and severe. Mild vitamin D deficiency occurs when serum 25(OH)D levels are in the range 25-50 nmol/L. This results in an increased secretion of parathyroid hormone and high bone turnover (Diamond et al., 2005). Moderate vitamin D deficiency is defined as having serum 25(OH)D levels of 12.5-25 nmol/L (Lips, 2001). This level of deficiency has been related with increased serum PTH and high bone turnover (Lips, 2001), and higher risk of fracture in elderly (Weatherall, 2000). Severe vitamin D deficiency is diagnosed at a serum 25(OH)D of < 12.5 nmol/L resulting in osteomalacia (Diamond et al., 2005; Lips 2001). Severe deficiency is unusual in Australia and New Zealand (Diamond et al., 2005).

In the current study, isotope-dilution liquid chromatography–tandem mass spectrometry (ID-LC-MS/MS) with stable-isotope-labeled internal standard was used to measure plasma 25(OH)D_3. The assay is suitable for routine measures of plasma 25(OH)D_2 and 25(OH)D_3 (Maunsell et al., 2005). The present (ID-LC-MS/MS) assay offers increased specificity and sensitivity for the measurement of vitamin D_2 and D_3 metabolites. The technique can independently measure serum 25(OH)D_2 and D_3 without interruption from other dihydroxy metabolites of vitamin D due to different mass. Other benefits of using this method are the assay can be automated allowing up to 180 samples to be measured in a 24 hours period. Also, ID-LC-MS/MS has been fully validated (Maunsell et al., 2005).

2. Parathyroid Hormone

Parathyroid hormone is a polypeptide hormone produced by the parathyroid gland that contains around 84 amino acids in mammals. Together with the active form of vitamin D, $1,25(OH)_2D_3$, PTH is the main regulator of calcium homeostasis. It has a complex action on bone and particularly promotes the release of calcium and phosphate. In the kidney, it stimulates the reabsorption of calcium, but inhibits the reabsorption of phosphate (Brown & Juppner, 2006).

PTH has both catabolic (bone resorption) and anabolic (bone formation) actions on bone (Martin & Sims, 2005). The osteoblasts and osteoblast precursor cell, the marrow stromal cell, have key roles in mediating both actions of PTH (Brown & Juppner, 2006). The mechanisms in which PTH stimulates bone formation are not fully understood. PTH reduces pre-osteoblast and osteoblast apoptosis (Manolagas, 2000), resulting in an increased number of osteoblasts and osteoprogenitor cells (Aubin & Triffitt, 2002). It also increases the proliferation of osteoblasts, affecting the conversion of inactive bone lining cells to active osteoblasts (Li et al., 2002).

Parathyroid hormone also acts as catabolic agent for bone. Together with $1,25(OH)_2D_3$, PTH binds the osteoblast lineage cells to stimulate receptor activator of RANKL, decrease the soluble decoy molecule OPG, and therefore increase osteoclastic osteolysis. This action leads to increased mobilisation of calcium from bone, increased osteoclast activation and differentiation, and increased bone resorption (Goltzman, 2008; Silva et al., 2011).

3. Estrogen

Estrogen is the major sex steroid hormone influencing bone turnover (Riggs et al.,2002) and maintaining bone mass in men and women (Syed & Khosla, 2005). Estrogen has an anabolic action on bone. It indirectly causes a reduction of bone resorption by preventing osteoclastogenesis and can directly inhibit osteoclast function (Secreto et al., 2006). Estrogen acts directly on bone marrow cells, osteoblast lineage cells, and osteoclasts to inhibit the production of a number of cytokines including IL-1, IL-6, TNF-α, and M-CSF, and these cytokines are associated with estrogen reduction-induced bone loss (Syed et al., 2010; Lorenzo, 2008).

Estrogen deficiency, therefore, stimulates osteoclast differentiation, activation and maturation through the significant increase of proinflammatory cytokines including IL-6, granulocyte macrophage colony-stimulating factor (GM-CSF), and prostaglandin E2 (PGE2), a substance synthesized primarily from arachidonic acid which stimulates the effects of RANKL and inhibits the effects of OPG (Clowes et al., 2005). Also, increased cytokine production stimulates bone resorption during the bone remodelling process, increases the pool of early osteoclastic precursors in the bone marrow, and stimulates bone loss (Horowitz, 1993; Clowes et al., 2005; Pfeilschifter et al., 2002)

Several cytokines such as IL-6, PGE_2 and TNF-α have a key role in regulating the synthesis of estrogen. IL-6 is a potent stimulator of the activity of aromatase enzyme, a complex enzyme that converts androgen to estrogen in human body (Purohit et al., 2002; Reed & Purohit, 2002).

As a result, bone remodelling activity increases substantially among postmenopausal women and can result in bone loss of approximately 3% anually (Secreto et al., 2006). Thus, estrogen deficiency after menopause accelerates age-related bone loss, and bone mineral density can decrease significantly by 6 years after menopause (Ahlborg et al., 2003; Krall & Dawson-Hughes, 1993).

4. Thyroid hormone

Thyroid hormones (TH) are synthesized by the thyroid gland; both the inactive Tetraiodothyronine (T_4) and a small number of the active Triiodothyroxine (T_3). T_3 has a vital role in normal development of endochondral and intramembraneous bone processes and is important for the linear growth and the maintenance of bone mass in childhood (Duncan et al., 2003).

5. Insulin

Pancreatic β-cells produce insulin in response to increased levels of blood glucose after food intake (Fulzele & Clemens, 2012). Insulin also affects bone formation by its actions on osteoblasts (Ahdjoudj et al., 2001). Insulin may have important roles in osteoblast function by facilitating osteoblast differentiation and collagen synthesis (Kim et al., 2000). Individuals with insulin disorders, such as diabetes mellitus and insulin resistance, may develop early onset of osteoporosis, poor bone healing and have a higher risk of fragility fracture (Fulzele & Clemens, 2012; Hofbauer & Hamann, 2010).

6. Cytokines

Several inflammatory factors including prostaglandins (PGs), IL-1, IL-6, IL-11, TNF- α, and TNF-β, as well as RANKL, M-CSF, OPG, and interferon-ɣ (IFN-ɣ), a soluble cytokine and a potential RANKL inhibitor produced by T cells, are all stimulated in the first three days after fracture. Further, cytokines and PGs have vital roles in the initiation of bone repair (Gerstenfeld & Einhorn, 2006; Takayanagi, 2005).

IL-6, a pro-inflammatory cytokine, is involved in a number of processes in the body, including inducing bone resorption by increasing osteoclast formation and differentiation from precursors (Ishimi et al., 1990; Horowitz, 1993; Murray et al., 1997). It has been suggested that IL-6 promotes the production of RANKL and OPG mRNA in bone, likewise it increases prostaglandin production (Lorenzo, 2008).

7. Calcitonin

Calcitonin is a polypeptide hormone synthesized by thyroidal C cells in the thyroid gland (Huebner et al., 2008). Calcitonin acts to inhibit bone resoption and bone loss (Nieves et al., 1998) by decreasing osteoclast activity (Huebner et al., 2008). Disorders of calcitonin production are characterized by increased osteoclastic resorption such as osteoporosis and the hypercalcemia of malignancy (Deftos, 2006).

1. 5. 2. Lifestyle factors and the effects on bone

The interaction of hormonal, genetic, environmental, and dietary factors influence bone development, maintenance, and later loss (Cashman, 2002). Evidence suggests that genetics plays the major role in bone mass by contributing up to 80% to the bone mass change of individuals (Morrison et al., 1994). While lifestyle factors and diet seem to have a smaller effect on bone mass, these two components are vital for modulating the attainment of genetic potential peak bone mass (PBM) and reducing subsequent bone loss (Cashman, 2004; Rizzoli, 2008). Adequate intake of dietary components in utero, in infancy and during childhood and adolescence is essential to maximize peak bone mass and reduce later fracture risk (Goldberg, 2006).

During adolescence, bone mass increases significantly, and it may contribute to future reduction of fracture risk in the elderly. Repeated measurements of total body bone mineral content (BMC) and bone mass in longitudinal studies indicates that bone mass accrual during adolescence is very fast. By late adolescence, around age 18, both girls and boys will have attained 90% of their adult stature, 90% of PBM but only 57% of BMC (Nelson et al., 2006).

Peak bone mass is achieved through longitudinal growth, bone modelling and remodelling (Nelson et al., 2006). Rapid longitudinal growth occurs during childhood and adolescence which are the crucial periods for reaching optimum bone accrual, since bones undergo substantial and rapid changes in shape and size during the first two decades of life. Peak bone mass is reached by the end of the second or early in the third decade of life (Baxter-Jones et al., 2011). Bone health in later life is determined

by the amount of bone accrued during this period (Heaney et al., 2000). Factors such diet and physical activity influence bone mass accumulation during growth (Nelson et al., 2006; World Health Organization, 2003), and are discussed below.

1. Physical activity

Physical activity can promote bone growth and improve bone density (World Health Organization, 2003), increase mass and strength in childhood and adults, and reduce the risk of falling among elderly (New et al., 2000). Mechanical loading on bone from habitual physical activity increases bone mineral density, and the removal of mechanical loading leads to bone loss (Heaney et al., 2000). It is thought that mechanical loading stimulates cellular responses via loading-induced flow of the interstitial fluid of the canaliculi network of osteocytes. Subsequently, osteocytes which act as mechanosensors in this theory stimulate the osteoblasts and osteoclasts in their bone remodelling activities (Bakker et al., 2005).

Early moderate and vigorous physical activity benefits bone health during childhood (Janz et al., 2010). Slemenda et al (1991) found that weight bearing physical activity was significantly associated with hip and radius BMD in children.

In a longitudinal study, Baxter-Jones et al (2008) showed a positive relationship between physical activity and bone mineral content. They concluded that the bone mass advantages of physical activity during adolescence are sustained into young adulthood. A prospective study over 1 year of adolescent girls showed that a physical activity intervention resulted in increased BMD, but had no significant effect on bone

markers (Lehtonen-Veromaa et al., 2000). Further, Welten et al. (1995) found habitual weight-bearing physical activity in a young population was positively associated with lumbar BMD.

The mechanical loading of physical activity benefits the bone status of adult women as well. Exercise training in healthy premenopausal women was found to benefit the skeletal status of vertebrae and femoral neck (Kanders et al., 1988; Heinonen et al., 2012). Similar results were also illustrated in postmenopausal women (Nguyen et al., 2000). However, a randomized controlled trial (RCT) of an 18 months long exercise program found the benefit was not sustained at 3.5-year follow-up (Heinonen et al., 2012). Similarly, Mazess & Barden (1991) found no significant effect of physical activity on bone mass by 2 years follow up. In brief, physical activity positively influences BMD but the effects may not be long lasting once exercise ceases.

2. Smoking

Cigarette smoking stimulates earlier menopause, thus contributing to a reduced BMD during ageing (World Health Organization, 2003; Yoon et al., 2012). A cross sectional study of Australian women; 118 current smokers and 158 non-smokers found current smoking was associated with a 4-5% deficit of BMD at the lumbar spine, femoral neck and total body. The association was more evident in females having a body mass index (BMI) < 25 kg/m^2 (Jones & Scott, 1999). A 2-year longitudinal study of young women aged 20-39 years found that smokers had a lower BMD of the spine and a tendency for lower BMD of other sites compared to non-smokers (Mazess & Barden, 1991). Furthermore, studies reported that smoking increases the risk of fractures at all

skeletal sites (Baron et al., 2001; Hippisley-Cox & Coupland, 2009; Jenkins & Denison, 2008; Robbins et al., 2007) and decreases intestinal calcium absorption (Krall & Dawson-Hughes, 1999).

3. Alcohol

High levels of alcohol consumption may have adverse effects on bone health (World Health Organization, 2003). A prospective study found that alcohol intake was associated with a nonlinear increase in risk of hip and osteoporotic fractures. Alcohol intakes of 2 units or less per day had no increased risk; intake above this level was related to increased risk of osteoporotic and hip fractures (Kanis et al., 2005). Another study concluded that moderate alcohol intake has a U-shaped association with the risk of hip fracture, and a graded positive association with total hip BMD (Mukamal et al., 2007).

1. 5. 3. Nutrition

General nutrition and bone health

A number of nutrients and dietary components have been identified as being beneficial to bone health and metabolism (Bonjour et al., 2010; Cashman, 2002). Some nutrients that have advantageous effects on bone are inorganic minerals such as calcium, magnesium, zinc, copper, phosphorus, fluoride, potassium and other trace minerals (Goldberg, 2006). Over consumption of certain food components such as alcohol, caffeine, sodium, fluoride, phosphorus and n-6 fatty acids can potentially impair bone health (Cashman, 2007).

Nutritional factors may affect bone via several mechanisms involving endocrine and/or paracrine systems, alteration of bone composition, the ratio of bone formation to bone resorption, and calcium homeostasis (Cashman, 2007). The following review discusses nutrients that are supplemented in the current study including calcium, magnesium, zinc, and phosphorus.

1. Calcium

Calcium is a major mineral component of bones and teeth (Weaver, 2000). It contributes around 1200 g to the adult human body which is about 1-2% of the total body weight. Around 99% of calcium is found in mineralized tissues including bones and teeth as calcium phosphate (Cashman, 2002). The rest of calcium is found either in the plasma attached to plasma proteins or in the extra-cellular fluid as ionic calcium (Ca^{2+}). The latter, Ca^{2+}, is strongly regulated by vitamin D, calcitonin and PTH (Weaver & Heaney, 2006). In a combination with phosphate, calcium forms an insoluble salt, hydroxyapatite, which is the primary component of bone and teeth. These two minerals have an important role in bone strength by providing rigidity and structure (Nordin, 1997).

An adequate intake of calcium during childhood and adolescence optimises peak bone density (PBD), and therefore reduces osteoporotic fracture risk in old age (World Health Organization, 2003). Calcium requirements vary throughout the human lifecycle. The requirement increases during periods of growth; the rapid growth spurts in childhood and adolescence, during pregnancy and lactation, as well as during

periods of later life (Lanham-New, 2008; Cashman, 2002). The New Zealand and Australian nutrient reference value is 1000 mg/day of dietary calcium for adults and 1300 mg/day for adolescents and postmenopausal women (Ministry of Health, 2006).

The availability of dietary calcium is influenced by both the amount of dietary calcium and the absorption of calcium (Cashman, 2007). Calcium is required in relatively large amounts in the diet (Weaver & Heaney, 2006). Milk and dairy products provide a major proportion of dietary calcium (60-70%). Most foods are poor in calcium, except some green vegetables, bony fish, almonds and dried fruit (Horn, 2012; Heaney & Burckhardt, 1995). In addition, people may obtain calcium from mineral waters containing calcium bicarbonate and calcium sulfate (Bonjour et al., 2010).

This essential mineral is mainly absorbed in the small intestine, but the large intestine may become increasingly important once acidic fermentation occurs. Calcium must be released from food in a soluble and possibly ionized form to be absorbed (Cashman, 2007; Lanham-New, 2008). There are two absorptive mechanisms; saturable active (cell-mediated) and paracellular (passive) absorption (Favus el al., 2006). The saturable transcellular route is a complex process starting from the entry of luminal calcium into the enterocyte through the microvillae membranes. After calcium has passed the cytosol, there is an active extrusion from the enterocyte to lamina propria, and finally to the blood circulation.

It has been suggested that calbindin D_{9K}, a cytosolic vitamin D-dependent calcium-binding protein, facilitates the diffusion of Ca^{2+} by acting as an intracellular transporter

(Balmain, 1991; Cashman, 2007). Moreover, calcium transport across the intestinal cell is mediated by CaT1, a calcium transporter, followed by active extrusion into the lamina propria by CaATPase (Balesaria et al., 2009)

Significant evidence exists for the role of active vitamin D ($1,25[OH]_2D_3$) in promoting active calcium absorption (Abrams et al., 2005; Abrams et al., 2009). It has been reported that calcium absorption increases as serum 25-hydroxy vitamin D increases from around 20 to 80 nmol/L in adults and adolescents (Heaney et al., 2003a; Abrams et al., 2005).

The nonsaturable paracellular pathway is a passive calcium absorption process via the tight junction between mucosal cells in jejunum and ileum. This route is independent of physiological and nutritional regulation, and is concentration dependent (Figure 1.6). Passive transport is the main calcium absorption pathway when calcium intake is adequate and high; while the active vitamin D-dependent transcellular absorption is the primary transport when dietary calcium is low (Bronner & Pansu, 1999; Cashman, 2007).

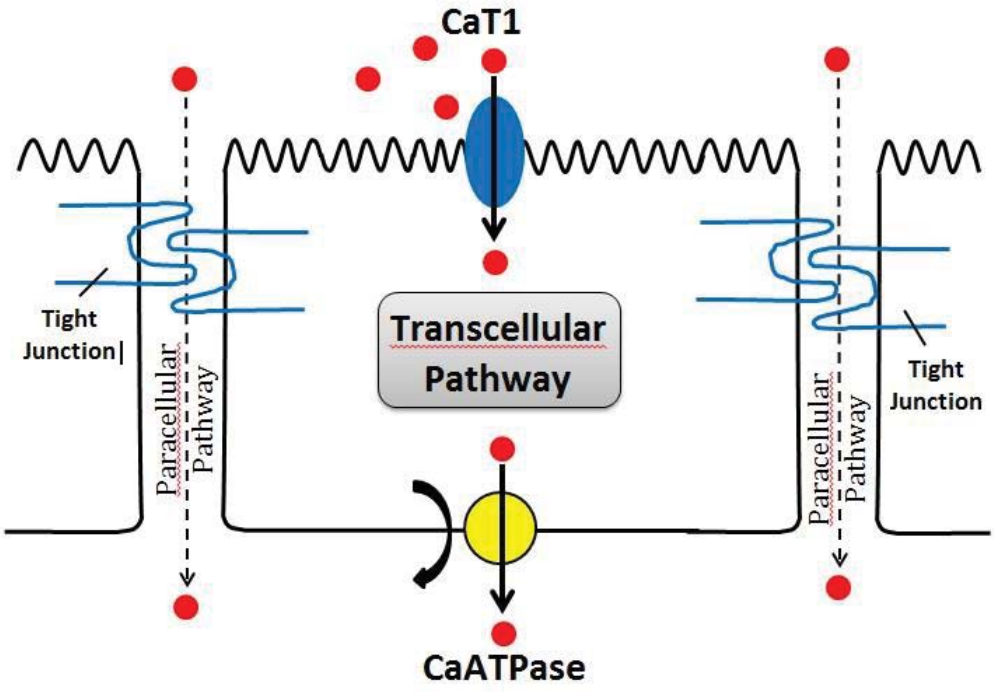

Figure 1.6. Calcium transport. Calcium is illustrated as red circle. In the transcellular pathway, calcium transport across the intestinal cell is mediated by CaT1, a calcium transporter, followed by active extrusion into the lamina propria by CaATPase. In the paracellular pathway, calcium is transported via the tight junction between mucosal cells.

Duodenum and jejunum are responsible for around 90% of calcium absorbed during adequate calcium intake (Favus et al., 2006). The increased requirement of calcium stimulates calcium active transport which mainly occurs in duodenum and ileum and a lesser amount in jejunum and all parts of colon to increase calcium absorption from 25-45% to 55-70% (Favus et al., 2006).

Body calcium maintenance is influenced by estrogen status. After menopause, intestinal absorption and renal tubular reabsorption of calcium decline. Thus, the age-related alteration in calcium metabolism in males and females has led to the

recommendation to increase calcium intake after the age of fifty (Heaney & Weaver, 2003).

2. Phosphorus

Phosphorus is an essential nutrient for bone mineralization and low serum phosphorus concentration may impair skeletal mineralization (Heaney et al., 2000; Favus et al., 2006). Around 80% from the total body phosphorus is stored in skeleton and teeth (Takeda et al., 2012). In the human body, around 85% of the total phosphate is found in the hydroxyapatite crystals in bone and 15% in soft tissues, and only 0.1% in the extracellular fluids (Favus et al., 2006).

Most dietary phosphate is found in the protein-rich foods such as milk, meat, cheese, fish, poultry and foods containing phosphate-based additives (Takeda et al., 2012). The New Zealand and Australian nutrient reference value is 1000 mg/day of dietary phosphorus intake for women aged 31-50 years old (Ministry of Health, 2006).

Phosphate homeostasis is maintained by the regulation of dietary intestinal absorption, renal reabsorption and excretion, bone formation, and intracellular storage pools (Marks et al., 2010). The absorption of phosphate is directly associated with dietary phosphate, which is dependent on both passive and active transport. Passive transport is influenced by luminal phosphate concentration, which reaches the highest level after a meal, and active (cell-mediated phosphate) transport is promoted by $1,25(OH)_2D_3$ (Favus et al., 2006). Dietary intake of phosphorus and PTH influence phosphate reabsorption. Around 85% of phosphate reabsorption occurs in the

proximal tubule of kidney, and PTH reduces proximal tubule phosphate reabsorption (Favus et al., 2006).

3. Magnesium

Magnesium, one of the macro elements of bone ash, ranks as the second most plentiful intracellular cation. Magnesium has important roles in bone including maintaining bone health, preventing osteoporosis and stimulating bone formation (Tucker, et al., 1999; Speich et al., 2001; Bonjour et al., 2010). Magnesium is also important in calcium metabolism and calcium balance (Tucker, et al., 1999).

Magnesium is mainly stored in bone and intracellularly in muscle and soft tissues, only around 1% of the total body magnesium is in blood circulation (Elin, 1994). This mineral is circulated in the blood in the same manner to calcium: 30% is bound to the protein (albumin) and not filtered by the glomerulus, 55% is free or ionized magnesium and the remaining 15% is complex magnesium. The latter two composites are filtered by the glomerulus (Vokes, 2006). Magnesium is attached to albumin and its affinity is influenced by pH (Vokes, 2006).

Green leafy vegetables, legumes, seeds, nuts and mineral water are considered good sources of dietary magnesium (Whitney et al., 2011). The New Zealand and Australian nutrient reference value is 320 mg/day of dietary magnesium intake for women aged 31-50 years old (Ministry of Health, 2006).

Low magnesium intake contributes to magnesium deficiency, but the symptoms rarely occur except with diseases (Whitney et al., 2011). Magnesium deficiency has been associated with a number of diseases from hypertension, asthma, migraines to osteoporosis (Quamme, 2008). In relation to bone health, deficiency of this essential mineral may cause disturbed calcium homeostasis and bone loss. The latter is therefore a risk factor for osteoporosis (Rude et al., 2010).

The alteration of magnesium homeostasis occurs primarily in the extracellular compartment since the levels of intracellular magnesium are tightly regulated and reserved (Vormann, 2003). Magnesium homeostasis is controlled by intestinal absorption and renal excretion. In the normal condition, reduced consumption of magnesium is balanced by increased intestinal absorption and decreased renal excretion of magnesium, thus adjustment is regulated by hormonal and metabolic influences (Konrad et al., 2004).

Intestinal magnesium absorption occurs via two different pathways; a non-saturable passive paracellular pathway (the majority of absorption) and a saturable active transcellular system (Konrad et al., 2004; Quamme, 2008). It is primarily absorbed in the small intestine, with a lesser amount absorbed in the large intestine (Konrad et al., 2004).

4. Zinc

Zinc, the multipurpose trace element, is a cofactor required by more than 300 enzymes. Most cells contain zinc and the highest concentrations are in bone and muscle which account for up to 85% of total body zinc (Tapiero & Tew, 2003).

It has been suggested that zinc promotes bone mineralization and formation. This essential mineral stimulates aminoacyl-tRNA synthetase in osteoblastic cells, and activates the production of cellular protein. Further, it inhibits bone resorption by preventing the formation of osteoclast-like cells in the bone marrow (Yamaguchi, 1998).

Protein-rich foods such as shellfish, poultry, meats, milk and cheese are good sources of zinc. Whole grains, legumes and cereal are also good sources of zinc when consumed in large amounts (Whitney et al., 2011). In New Zealand diets, phytate, present in grains, is typically not high enough to disturb zinc absorption (Whitney et al., 2011). The New Zealand and Australian nutrient reference value is 8 mg/day for young women aged 31-50 years old (Ministry of Health, 2006).

Several conditions including low zinc intake, low bioavailability, nutrient interactions, and losses through disease processes contribute to zinc depletion (Ma et al., 2007), and therefore can retard bone growth (Yamaguchi, 1998). The signs of zinc deficiency are not manifest until a significant reduction in plasma zinc concentration occurs (King et al., 2001). When acute zinc deficiency occurs, the levels of depletion varies in tissues, in which zinc concentrations in muscles, skin, hair and heart remain stable,

while zinc is extensively reduced in the plasma, bone, testes and liver (King et al., 2001).

Around 20-40% of dietary zinc is absorbed primarily as a complex in the small intestine. It is mainly regulated by the gastrointestinal tract. Likewise, the excretion of endogenous zinc by the gastrointestinal tract is important to maintain zinc balance (King et al., 2000).

1. 6. Bone assessment: Bone and bone markers

1. 6. 1. Bone mineral density (BMD)

Several factors such as genetics, calcium intake, hormonal status, physical activity and body weight influence bone mineral density, and in turn, osteoporosis and risk of fracture later in life (Krall & Dawson-Hughes, 1993; Wardlaw, 1996).

The World Health Organization defined osteoporosis as a disorder characterized by a decline of bone mass and microarchitectural deterioration of bone tissue (Kanis et al., 1994). In New Zealand, the occurrence of osteoporosis increases gradually. Based on data from the 2006/07 New Zealand Health Survey, one in 34 adults (2.9%) was identified as having osteoporosis by their clinicians. This equates to around 90,000 adults. After adjustment for age, the incidence of osteoporosis was higher in women than for men in New Zealand (Gerritsen et al., 2008).

Bone densitometry assessment is central in measuring bone mass in order to diagnose osteoporosis (Cummings et al., 2002). Bone strength and the risk of fracture can be predicted by identifying a BMD value (Nguyen et al., 2005). BMD measurements can be derived from two techniques, dual energy X-ray absorptiometry (DEXA) and quantitative ultrasound (QUS).

Dual energy X-ray absorptiometry is a tool measuring BMD, BMC, lean tissue and body fat, and the differences in density and composition in each different compartment (Ellis, 2000). DEXA is the gold standard and the most widely validated method for measuring the change of BMD over time and diagnosing osteoporosis (World Health Organization, 2003; Lewiecki, 2005). DEXA measurements are obtained at the spine, hip and forearm, in which BMD is calculated using the BMC and the bone area. BMD is expressed as gram per square cm (Lunt et al., 1997).

A T-score is derived from the BMD value in comparison to a young adult-female aged 20-29 years. Based on the WHO criteria for the bone status, osteoporosis is diagnosed as a T-score of less than 2.5 standard deviations (SD) (the T-score < -2.5 SD), osteopenia as a T-score from -1.0 SD and -2.5 SD below the average of young women, and the normal as within 1.0 SD (+1 or −1) (World Health Organization, 2003).

Another method to measure BMD is quantitative ultrasound (QUS) of the calcaneus, which reveals bone structure and BMD of the calcaneus (Moayyeri et al., 2009). This method, which was reported for the first time in 1984, is considered non-invasive, low-cost, easy to use and free from radiation (Knapp et al., 2001; Dane & Dane, 2012).

However, evidence indicates that the sensitivity and specificity of QUS are not adequately high for QUS to be used as a substitute to DEXA (Dane et al., 2008). The other limitation in the clinical use of QUS is there is no consensus criteria for osteoporosis using this method as WHO criteria for osteoporosis is based on DEXA measurement and direct use of the criteria to QUS is inappropriate (Pais et al., 2010).

There is increasing evidence for the use of QUS measurement of the heel for predicting fracture risk. Studies in elderly people reported that QUS measurement of the calcaneus can predict the risk of hip fracture and other fractures (Bauer et al., 1997; Pluijm et al., 1999). For instance, in a study involving more than 7000 elderly women in Switzerland, Krieg et al (2006) confirmed that heel QUS techniques (the water-bath heel QUS Achilles+ and the dry system heel QUS Sahara) can predict the risk of hip fracture.

1. 6. 2. Bone markers

Bone turnover markers are the biochemical markers that reflect the rate of bone formation and bone resorption (Lenora et al., 2007) including serum osteocalcin (OC), serum bone-specific alkaline phosphatase (B-ALP), serum C-propeptide of type I collagen (PICP), the cross-linked C telopeptide of type I collagen, and N-telopeptide of type I collagen (NTx) (Garnero et al., 2009).

1. Osteocalcin

Osteocalcin (OC), the vitamin-K dependent protein of bone, is produced by the osteoblast. The osteoblast produces three particular Gla-proteins: osteocalcin, protein S, and matrix Gla-protein (Vermeer et al, 1995; Kindblom et al., 2009). Part of the newly synthesized OC is secreted into the blood circulation; this might be used as diagnostic marker of bone formation (Vermeer et al., 1995; Seibel, 2003). It has been suggested that OC has an important role in bone mineralization (Pepmueller et al., 1996; Kanazawa et al., 2009). But the specific function of osteocalcin remains unclear (Vermeer et al., 1995).

Around 20% of the newly synthesized OC is free in the circulation (Pepmueller et al., 1996; Kanazawa et al., 2009). Circulating osteocalcin has a brief half-time and is promptly filtered and excreted by the kidney (Garnero & Delmas, 1998). The functionality of osteocalcin is influenced by vitamin K intake and the function of osteocalcin in human bone can be compromised by the vitamin K nutritional status (Vermeer at al., 1995). Vitamin K stimulates the ɣ-carboxylation of uncarboxylated osteocalcin into carboxylated osteocalcin. A high level of uncarboxylated osteocalcin indicates vitamin K depletion (Shea et al., 2009). Several factors affect the circulating osteocalcin levels: osteocalcin synthesis by the osteoblast, content of Gla in the protein, and 1,25 dihydroxyvitamin D levels (Risteli & Risteli, 1993).

Serum OC shows a circadian rhythm in a small range, a nightly greatest value at 4 am and a lowest point at 5 am (Garnero & Delmas, 1998). Serum OC is related to bone turnover (Luukinen et al., 2000). Researchers found that there was a significant

increase in OC during the postmenopausal period. It suggests that OC is related to the current bone mass and estimate the future bone loss (Lofman et al., 2005). OC concentration increases with increasing age, and is negatively associated with bone mineral density (Garnero et al., 1996b; Melton III et al., 1997). Moreover, osteoporotic women have high bone turnover including high levels of serum osteocalcin (Melton III et al., 1997).

2. The cross-linked C telopeptide of type I collagen (CTx)

Type I collagen contributes around 90% to the organic matrix of mineralized bone and is the most abundant collagen type as the supporting structures of soft tissues (Risteli et al., 1993). CTx is a peptide fragment produced by collagen degradation (Rosen et al., 2000) which is released during bone resorption and one of the bone resorption markers (Garnero et al., 1996a; Prouteau et al., 2006).

CTx is also considered as one of the bone markers in predicting the risk of fracture (Johnell et al., 2002), since increased bone turnover has been proposed to increase the risk of osteoporotic fracture (Garnero et al., 1996a). Eastell et al (2003) confirmed that decreased urinary CTx (median, 60%) and NTx (51%) were significantly associated with decreased vertebral fracture risk. Women with a low BMD as well as a high CTx concentration are at a higher risk of hip fracture than those with only low BMD or high bone resorption (Garnero et al., 1996a). Studies of osteoporosis treatment reported that bone turnover markers are more strongly associated with the reduction of fracture risk than BMD. These findings support the use of biochemical markers of bone

turnover as surrogates for the reduction of fracture risk, possibly even more than BMD (Eastell & Hannon, 2008).

Another study showed that menopause caused a 79-97% increase in the levels of bone resorption markers (CTx and NTx) (Garnero et al., 1996b). Furthermore, increased degradation of type I collagen in bone is associated with several diseases including multiple myeloma and rheumatoid arthritis (Risteli et al., 1993).

In a study of premenopausal women, it was found that bone turnover markers including CTx significantly decreased with increasing age, and is associated with the changes of serum phosphate and body weight (Adami et al., 2008). Another study comparing the levels of bone turnover markers including CTx between oral contraceptive pill users (CP) and non-users in premenopausal female suggested that the levels of CTx in CP are lower compare to those of non-users (de Papp, et al., 2007).

SUMMARY

Bone has several functions: providing structural support, protecting vital organs, movement, maintaining acid-base balance and electrolyte, and providing blood cells by hematopoitic tissue in the red bone marrow. In general there are two types of bone: long and flat bone, and two forms of bone: cortical (compact) and trabecular (spongy) bone. Bone consists of organic and inorganic matrix, and bone cells (osteogenic cells, osteoblasts, osteoclasts, and osteocytes). Bone is an active tissue that regularly removes and renews the tissue through bone resorption and formation processes. Several factors involve in the regulation of bone metabolism such as hormonal factors:

vitamin D, parathyroid hormone, estrogen, thyroid hormone, insulin, cytokines and calcitonin; lifestyle factors: physical activity, smoking, and alcohol; nutritional factor such as intake of calcium, magnesium, phosphorus and zinc. Bone mass and bone strength can be predicted by identifying BMD values. DEXA and QUS are two techniques that are commonly used to measure BMD. The rate of bone formation and resorption can be measured by biochemical markers, such as osteocalcin (bone formation) and CTx (bone resorption).

Section 2. Studies on calcium and milk supplementation, and physical activity

A meta-analysis in 1990 showed that there was a positive relationship between calcium intake and bone mass at all ages (Graham Cumming, 1990). However, a more recent meta-analysis of randomized controlled trials of healthy children revealed that calcium supplementation had only a small effect on bone mineral density of the upper limb (Winzenberg et al., 2006a).

In calcium supplementation studies, both intervention and cross-sectional studies have confirmed a positive effect of calcium intake on bone mass, particularly of young females (Johnston et al., 1992; Kanders et al., 1988). A 4-year randomized control trial among young females aged 8-13 years old found that long-term calcium supplementation (1000 mg calcium citrate-malate supplementation daily, given in 4 pills) significantly increased BMD during the pubertal growth spurt compared to control (Matkovic et al., 2005).

Furthermore, most calcium supplementation studies reported a reduction in bone turnover markers. A cohort study of adolescent females reported that high calcium intake caused changes in bone turnover markers indicating decreased bone resorption. They found that increased calcium retention was due to increased calcium absorption and decreased bone resorption (Wastney et al., 2000).

Data from a study of 15 healthy participants (eight females and seven males) aged 30 years old shows that short-term increase of dietary calcium in both male and female subjects from approximately 500 mg to 1600 mg daily significantly reduced bone resorption markers (Shapses et al., 1995). Most studies found that increased calcium intake significantly reduced bone turnover markers, especially bone resorption markers.

Reserachers found that vitamin D deficiency among healthy premenopausal women is associated with low spine BMD (Adami et al., 2009). A study of 608 healthy premenopausal Italian women showed that vitamin D levels had a significant positive association with spine BMD (Adami et al., 2009). Similarly, a study of Indian women found that vitamin D deficiency coexisted with low bone mineral density in both reproductive and postmenopausal women (Harinarayan et al., 2011). More recently it has been suggested that the approach to maintain bone health in premenopausal women should include increased consumption of bone-supplement components including calcium, vitamin D and protein (Josse et al., 2012).

It has been suggested that the alternative way of micronutrient supplementation is by adding the substance to food products (Allen, 2006). A number of studies supplementing milk and milk products were carried out in children, adolescents and premenopausal women. A randomized double-blind controlled trial supplementing calcium-enriched foods containing 850 mg of calcium over 48 weeks was done in Caucasian pre-pubertal girls aged 7.9 years. Calcium derived from a milk extract was used to enrich several food products such as yoghurts, cakes, fruit juices, biscuits and

chocolate bars. They found significant differences in BMD of the radial metaphysis and diaphysis, and femoral trochanter and diaphysis between those in the intervention and the placebo groups. They concluded that girls in the intervention group had a significant increase in bone mass accrual compared to those in the placebo group (Bonjour et al., 1997).

In a randomized study of adolescent girls aged 12.2 years; 300 mL milk supplementation per day had no significant effect on bone markers, however it increased BMC and BMD after 18 months compared to control (Cadogan et al., 1997). The largest RCT involved 757 girls and was carried out on Chinese school girls aged 10 years from nine primary schools in Beijing over 2 years. Participants were randomized into three groups: the first group received 330 mL milk fortified with calcium, and the second group received the same quality of milk with the additional vitamin D on school days over 2 years, and the third group served as control. The fortified milk consumption resulted in significant increases in height, body weight, BMC and BMD in the groups receiving milk with or without vitamin D, suggesting that school-milk programs may improve bone growth during childhood (Du et al., 2004). The inclusion of vitamin D in the fortified milk resulted in significantly increased vitamin D status (Du et al., 2004). Furthermore, Zhu et al (2005) found that milk supplementation lead to higher increases in periosteal diameter and cortical thickness measured in metacarpal bone, and higher level of IGF-I in both milk groups.

Another study was done on Chinese children aged 9-10 years supplementing milk powder (equivalent to 1300 mg and 650 mg calcium) for 18 months (Lau et al., 2004).

Significantly greater increases in BMD at both total hip and spine were observed in the 1300 mg-milk powder group compared to the control group. In addition, a significant increase of the total body BMD and smaller increases in BMD at the total hip and spine were observed in 650 mg-milk powder group compared to the control group. Results indicate that milk powder supplementation in Chinese children enhances bone accretion (Lau et al., 2004).

The four studies mentioned above (Cadogan et al., 1997; Du et al., 2004; Zhu et al., 2005; Lau et al., 2004) were performed during on-going bone accrual in childhood. The studies did not include physical exercise as the variable measured in the study and did not follow-up the findings after the supplementation stops. The usual calcium intake of young females in China (356 mg/d) differs from those in New Zealander (778 mg/d) (Russell et al., 1999; Du et al., 2002). Moreover, some studies have not measured the markers of bone turnover (Du et al., 2004; Zhu et al., 2005; Lau et al., 2004).

Merrilees et al. (2000) carried out a milk supplementation study in teenagers aged 15-18 years old. The study included 2 years intervention and one year follow up. The intervention was dairy food products such as milk, flavoured milk, cheese, dairy dessert and yoghurt containing about 1160 mg calcium daily. Significant increases of BMD at the trochanter, lumbar spine and femoral neck were observed in the intervention group. Increases of BMC at trochanter and lumbar spine were observed in intervention group but the increases were not statistically significant.

Woo et al. (2007) performed a milk supplementation study among 442 young Chinese females aged 20-35 years. The intervention was two sachets of milk powder containing 1000 mg calcium, 80 µg vitamin K_1 and 5 µg vitamin D_3 daily over 24 months. An increase in BMD and a decrease in markers of bone turnover were observed over time in both intervention and control groups. They concluded that lack of compliance and age-related bone metabolism most likely justify the lack of change in BMD or bone turnover markers among this study population (Woo et al., 2007).

A study in the UK showed that daily intake of calcium-fortified ice cream containing 96, 244, 459 or 676 mg calcium among premenopausal females with low intake of calcium resulted in a significant reduction of the marker of bone resorption serum CTx, without influencing weight gain (Ferrar et al., 2011).

In premenopausal women, Kruger et al. (2006) carried out a short-term study of young Caucasian women in New Zealand by supplementing fortified milk (1000 mg calcium and 80 µg vitamin K1) over 16 weeks. They concluded that high calcium milk supplementation had significantly decreased bone turnover markers, and the additional phylloquinone (vitamin K1) fortification markedly improved vitamin K status in the premenopausal women over 16 weeks.

In conclusion, milk supplementation positively increased BMD, BMC, and bone accretion in children and adolescents. A significant increase of BMD was also observed in adolescents supplemented with dairy food products. A similar study to the present study on not physically active premenopausal women found that milk

supplementation significantly reduced bone turnover markers (Kruger et al., 2006), but the effects may not be significant if there is lack of compliance (Woo et al., 2007). The summary of fortified milk studies is shown in table 1.1.

Table 1.1. The summary of fortified milk and milk product studies

Study	Intervention	Mean age (years)	Duration	Sex	Results
Bonjour et al., 1997	calcium-enriched foods containing 850 mg of calcium such as yoghurts, cakes, fruit juices, biscuits and chocolate bars	7.9	12 mo	F	Significant differences in BMD in the radial metaphysis and diaphysis, and femoral trochanter and diaphysis between those in intervention and placebo group
Cadogan et al., 1997	Milk (568 mL)	12.2	18 mo	F	Increased BMD and BMC in intervention group. No significant effects on BTM.
Du et al., 2004	Milk (330 mL)	10.1	24 mo	F	Significant increases in height, body weight, BMC and BMD in intervention group compared to control
Zhu et al., 2005	Milk (330 mL)	10.1	24 mo	F	Significant increases in periosteal diameter and cortical thickness of metacarpal bone, and increase in IGF-I concentration
Lau et al., 2004	Milk (equivalent to 1300 mg and 650 mg Ca)	10.0	18 mo	F/M	Significantly greater increases in BMD at both total hip and spine were observed in 1300 mg-milk powder group compared to the control group. A significant increase in the total body BMD and smaller increases in BMD at the total hip and spine in 650 mg-milk powder group compared to the control group
Merrilees et al. 2000	Dairy food products (milk, flavoured milk, cheese and yoghurt containing 1160	16	24 mo	F	Significant increases in BMD at trochanter, lumbar spine and femoral neck.

	mg Ca)				
Woo et al., 2007	Milk (equivalent to 1000 mg Ca, 80 µg vit K1 and 5 µg vit D3)	28.0	24 mo	F	An increase in BMD and a decrease in markers of bone turnover were observed over time in both intervention and control groups
Ferrar et al., 2011	Calcium-fortified ice cream containing 96, 244, 459 or 676 mg calcium	29.6	28 days	F	Significant reduction in the bone turnover serum CTx, without influencing weight gain
Kruger et al. 2006	Milk (equivalent to 1000 mg Ca & 80 µg vit K1)	27.2	4 mo	F	Significantly decreased bone turnover markers in intervention group, and the additional vitamin K1 fortification markedly improved vitamin K status.

It remains unclear whether calcium intake and physical activity act synergistically in enhancing bone health. A review reported that weight-bearing PA and adequate calcium intake of around 1,000 mg may benefit bone health; PA may influence bone strength as it directly affects bone mass (via mechanical loading), while nutrition seems to have indirect effects on bone mass (Specker & Vukovich, 2007). Additionally, in a review of 17 published trials, Specker (1996) reported that PA benefits BMD at high calcium intakes; no effect was found at mean calcium intake less than 1000 mg per day.

A double-blind randomized trial found that a combination of milk supplementation containing 800 mg of calcium and physical exercise over 1 year significantly improved BMD acquisition in several bone sites such as lumbar spine, femoral neck, trochanter, ward's, mid-radius and ultra-distal radius in healthy female children aged 8 to 13 years old (Courteix et al., 2005). A similar finding was reported by Specker & Binkley (2003)

in a randomized study of calcium supplementation in children aged 3-5 years. It was found that the difference in leg BMC in the higher PA group and lower PA group was more prominent in children receiving the calcium supplement than the placebo. It was suggested that the intake of calcium modifies the bone response to physical activity in young children (Specker & Binkley, 2003).

In a cross sectional study of premenopausal and postmenopausal women evaluating the effects of long-term physical activity and calcium intake, Uusi-Rasi et al (2002) reported that the influence of physical activity did not become evident until older age. They found that PA was negatively associated with radius BMC in premenopausal women, and the situation was opposite for postmenopausal subjects (Uusi-Rasi et al., 2002).

The majority of studies on fortified milk have measured vitamin D status, biochemical markers of bone turnover, and bone density in young girls, during the continuing bone accrual period, and postmenopausal women. Most studies evaluating the effects of calcium or milk supplementation and physical activity have examined children and adolescents, and while these studies confirmed significant improvement in BMD and BMC, the impact of calcium intake and physical activity in adults is unclear. Very few milk supplementation studies that include evaluation of vitamin D status and bone turnover markers were done in premenopausal women, particularly those who are physically active.

Section 3. Objectives

Objectives

The main objective of this study was to evaluate the effects of 12 weeks supplementation with calcium and vitamin D fortified milk on bone turnover markers: osteocalcin and C-telopeptide of type 1 collagen, and vitamin D status of active, healthy premenopausal women aged 30-45 years old in Palmerston North, New Zealand.

Hypothesis

The hypothesis is that calcium and vitamin D supplementation will improve vitamin D status and reduce bone resorption of healthy active premenopausal subjects.

Chapter 2

Methods

2.1. Overview of study

This study was a repeat measure design and was performed in Palmerston North, New Zealand. Participants were asked to visit Medlab, Palmerston North, and the Human Nutrition Laboratory (Institute of Food, Nutrition and Human Health, Massey University) for a total of six visits to complete all assessments. The calcium and vitamin D fortified milk powder (Anlene^{TM}) was obtained from Fonterra Cooperative Ltd (New Zealand). A schedule of all measurements is shown in the figure 2.1.

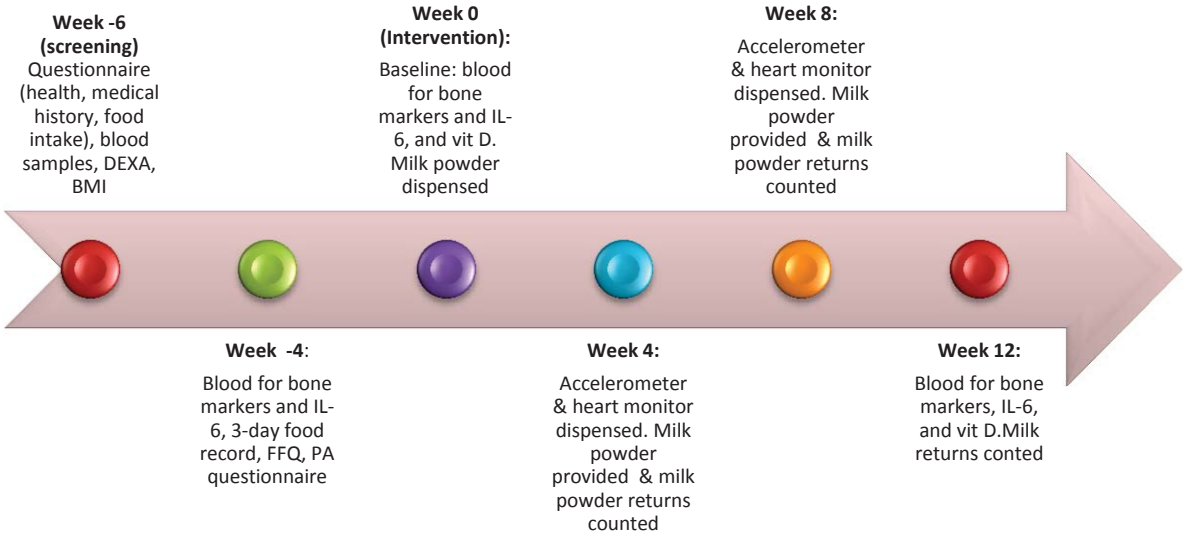

Figure 2.1. The schedule of measurement from week -6 until week 12.

Prior to beginning the study, women were screened for inclusion criteria. The current study assessed a group of active women over an 18-week period. Before the intervention, dietary calcium and vitamin D intakes were assessed by dietary recall and

food frequency questionnaire (FFQ). Habitual PA was assessed using a questionnaire. The women supplemented their diet with daily fortified milk over 12 weeks. Bone turnover markers and IL-6 were measured 3 times over the course of the study (week -4, 0 and 12). Vitamin D status was assessed from blood collection at week 0 and week 12. During the study, PA was objectively measured using accelerometers. Compliance of fortified milk consumption was assessed by measuring the amount of unused milk powder at monthly over the intervention period.

2.2. Intervention

A commercially available high calcium and vitamin D fortified milk powder was used and the formulation of calcium, magnesium, zinc and vitamin D is shown in table 2.1. The fortified milk powder provided 1200 mg calcium, 96 mg magnesium, 2.4 mg zinc and 10 µg vitamin D per day.

Table 2.1. Composition of the calcium and vitamin D fortified milk, composition given as per 100 g and 60 g serve.

Nutrient	Per 100 g	Per 60 g
Energy (kJ)	1565	940
Protein (g)	21.3	12.8
Carbohydrate (g)	63.3	38
Fat (g)	2.66	1.6
Calcium (mg)	1998	1200
Magnesium (mg)	159.8	96
Zinc (mg)	4	2.4
Vitamin D (µg)	16.67	10

Participants received two servings (2 x 30 gram) of milk powder with a scoop to measure the right amount of powder to be mixed in 200 mL of potable water in each serving for 12 weeks. The milk was taken in the morning at breakfast and at night after dinner. Participants were allowed to have smaller doses of milk, as long as they take the full 400 mL for each day.

All participants were asked to follow their normal diet. The fortified milk was not to replace the common milk taken in coffee or tea and regular consumption of dairy products was not to be stopped. They were asked to complete a diary of their daily intake to note when they took the milk, and to provide any comments about the acceptability of the milk powder such as taste, smell, body sensations, stomach upset and convinience. Figure 2.1 shows a schedule of all measurements.

2.3. Participants

2.3.1. Inclusion criteria

Participants were healthy premenopausal women aged 30-45 years old. They were physically active and exercised at least 3 days/week for 30 minutes. Their BMI was between 17 - 30 kg/m^2.

2.3.2. Exclusion criteria:

Women who were currently pregnant or breastfeeding were excluded from the study. Those who had a history of metabolic bone diseases affecting bone density and any hormonal disorders or disturbances such as diabetes mellitus, thyroid and parathyroid

disease, either diagnosed or based on blood result at screening were excluded from the study. Women who had kidney impairment, liver diseases, and any bone fractures in the last 6 months were also excluded from the study. Those who were smokers, consumed 2 units of alcohol or more per day, used any medication influencing bone mass, and used supplementations such as calcium, vitamin D, and multivitamin on a regular basis up to 2 months before the commencement of the trial were excluded. Women who had a milk allergy, or lactose intolerance, or were unable to confirm to consume 2 glasses of milk per day during the intervention period were also excluded. Also, those who had an irregular menstrual cycle, and had T-score of hip or spine for BMD less than -2.5 SD were excluded from the study.

2.3.3. Recruitment process

The study was advertised through local newspapers, posters in the local gyms and at Massey University, and in an email to Massey University students and staff.

2.3.4. Participants involved in the study

Forty three healthy premenopausal women living in Palmerston North were recruited in the study. Women who expressed interest in the study filled out a screening form confirming that they could drink two glasses of milk per day, and answered questions on their physical activity, health and medical history; including questions on smoking and alcohol habits. They were also asked to provide screening blood samples for full blood count, liver and kidney function, electrolytes such as sodium and potassium, total protein, albumin, glucose, insulin, and calcium analysis. The tests were performed by a local diagnostic laboratory using standardized methodology. The blood results had

to be normal before participants could be involved in the study. Screening measurements including DEXA scan of hip and spine were performed for each participant. The DEXA scan was carried out in the Human Nutrition Research Unit when the biochemical blood screening showed normal results.

2.4. Ethical approval and considerations

The study was reviewed and approved by the Massey University Human Ethics Committee: Southern A, Application 11/77, Massey University Human Ethics Committee: Southern A. Participation in the study was optional. Participants were provided with an information sheet and informed consent outlining the study procedures, time schedule and the participant's rights. The informed consents were signed at the first meeting before any data was collected. Confidentiality was guaranteed by assigning identification codes for the participants. Care was taken to minimize any discomfort to the participants during measurement procedures.

2.5. Data collection

2.5.1. Anthropometric measurements

Body weight and height were measured to calculate participant's BMI. Participants were asked to remove their shoes and outer clothes. A digital weighing scale was used to measure body weight to the nearest 0.1 kilograms and a stadiometer was used to measure standing height to the nearest 0.1 centimetres. BMI was calculated using the formula:

$$BMI = \frac{\text{Weight (kg)}}{\text{Height (m)}^2}$$

2.5.2. Dual energy X-ray Absorptiometry Measures

DEXA (Hologic, Discovery A, Bedford, MA, USA) was used to measure BMD and BMC. Quality control scans were carried out every day with a calibration block certified by the manufacturer based on the manufacturer's instruction. Clibration values were accepted if coefficient variation <0.5%. Participant's BMD (g/cm^2) and BMC (g) were measured at the femoral neck of the left hip and L1-L4 of the lumbar spine.

2.5.3. Blood samples

Participants were asked to visit the Human Nutrition Research Unit between 8 to 10 am after an overnight fast to have blood samples taken. There were total of four visits for blood sample. Plasma samples were taken for screening and measuring IL-6, plasma vitamin D and markers of bone formation and resorption: CTx and osteocalcin.

Plasma samples for $25(OH)D_3$, bone markers, and IL-6 were aliquoted and stored at temperature -70 to -80 degrees until the completion of the study. A small amount of blood samples was frozen (with the permission from participants) in case repeated analyses were required. Participants were informed that they could request any unused blood to be returned when the study was finished. Bone markers and plasma $25(OH)D_3$ were examined at Canterbury Health Laboratories, Christchurch, New Zealand (http://www.chl.co.nz/).

Plasma $25(OH)D_3$ was measured using isotope-dilution liquid chromatography–tandem mass spectrometry (ID-LC-MS/MS) (Maunsell et al., 2005). CTx and osteocalcin were

analysed by electrochemiluminescence immunoassay using the Roche COBAS® e411 system (Roche Diagnostics, Indianapolis, IN, USA). IL-6 was assessed using human IL-6 high sensitivity ELISA (BMS213HS, eBioscience, Vienna, Austria) at the Human Nutrition Cell Biology Laboratory. The eBioscience ELISA provides precise measurement below the conventional ELISA limits; the coefficient of variation (CV) intra-assay was 4.9% and inter-assay was 6.0%. The signal from the enzyme horseradish peroxidase (HRP) and the colorimetric-detectable substrate tetramethylbenzidin (TMB) were amplified using the Tyramide Signal Amplification (TSA) technology (Ebioscience, 2012).

2.5.4. Dietary Assessment

Dietary information was collected using an estimated 3-day food record and a FFQ. Participants were asked to complete an estimated 3-day food record over one weekend day and two weekdays to measure the intake of macronutrients and micronutrients. Instructions and an example were provided on how to fill out the food record. Participants were instructed to record the quantity of each food and drink consumed using household measures (e.g. cups, teaspoons and tablespoons), weight marked on the packages (e.g. 600 mL Coke, 32 g cereal bar and a slice of bread), food scale (e.g. grams), and comparisons (e.g. a pack of cards and a hen's egg).

All participants were encouraged to stay with their habitual diet. A self-addressed envelope was given to each participant to return the completed food record. All the food records were checked by researchers and any incomplete and vague information was revised by confirming with participants in person or by phone call. The average intake of macronutrients and micronutrients was assessed using FoodWorks 7 (2012

Xyris Software, Australia, Pty Ltd). Nutrient analysis was carried out on all complete food records.

A modification of the Quigley and Watts (1997) NNS FFQ was administered. The FFQ included questions on key food habits of participants (e.g. eating pattern and the frequency of several food groups) including:

1. dairy foods
2. bread and cereal foods
3. meat and fish
4. miscellaneous
5. vegetables
6. fresh fruits (in season)
7. other fruits
8. drinks

In the analysis, food containing ≥30 mg of calcium and 10 IU (0.25 μg) of vitamin D were considered sources of calcium and sources of vitamin D respectively. All the FFQs were checked by researchers and any incomplete and unclear information was revised by confirming with participants during the following meeting or by phone call. Both questionnaires were completed before the commencement of the intervention in order to measure participant's habitual intakes and to prevent inadvertent recording of intervention milk supplement intake.

2.5.5. Physical Activity

Usual physical activity levels were estimated using the Sport and Recreation New Zealand (SPARC) short-form New Zealand Physical Activity Questionnaires (NZPAQ) (McLean & Tobias, 2004) and 7-day accelerometer records in a subset of subjects (n= 25). The SPARC short-form NZPAQ was administered by face-to-face interview.

Participants were asked to wear the accelerometer (ActiGraph, LLC, Pensacola, FL, USA) and heart rate monitors for at least 10 hours each day over seven consecutive days. The heart rate monitor (the Polar WearLink®+ transmitter belt, Christchurch, New Zealand) was worn directly against the skin around the chest, in the middle of the diaphragm and below the chest muscles. It was attached to an elastic strap that may be adapted easily to make sure the monitor fit tightly and comfortably. The accelerometer was worn at the waist on the right hand side, over or under clothes, attached to an elastic strap or volunteers own belt (Figure 2.2).

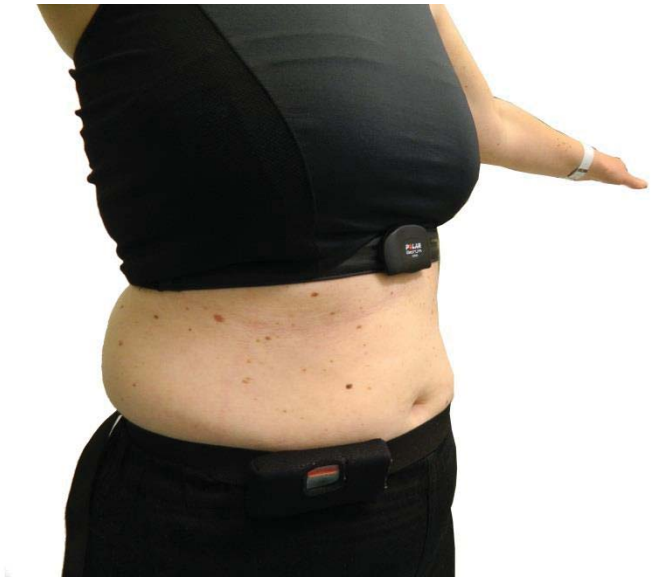

Figure 2.2. Placement of the heart rate monitor and accelerometer.

Face to face instruction and an illustrating figure from Moy et al. (2010) were given to participants on the placement of these devices. The accelerometer and the heart rate monitor were dispensed in week 4 and 8. An example of the output from the accelerometers is illustrated in figure 2.3. The intensity of physical activity was calculated using accelerometer software calculation (ActiLife 5, ActiGraph, LLC,Pensacola, FL, USA).

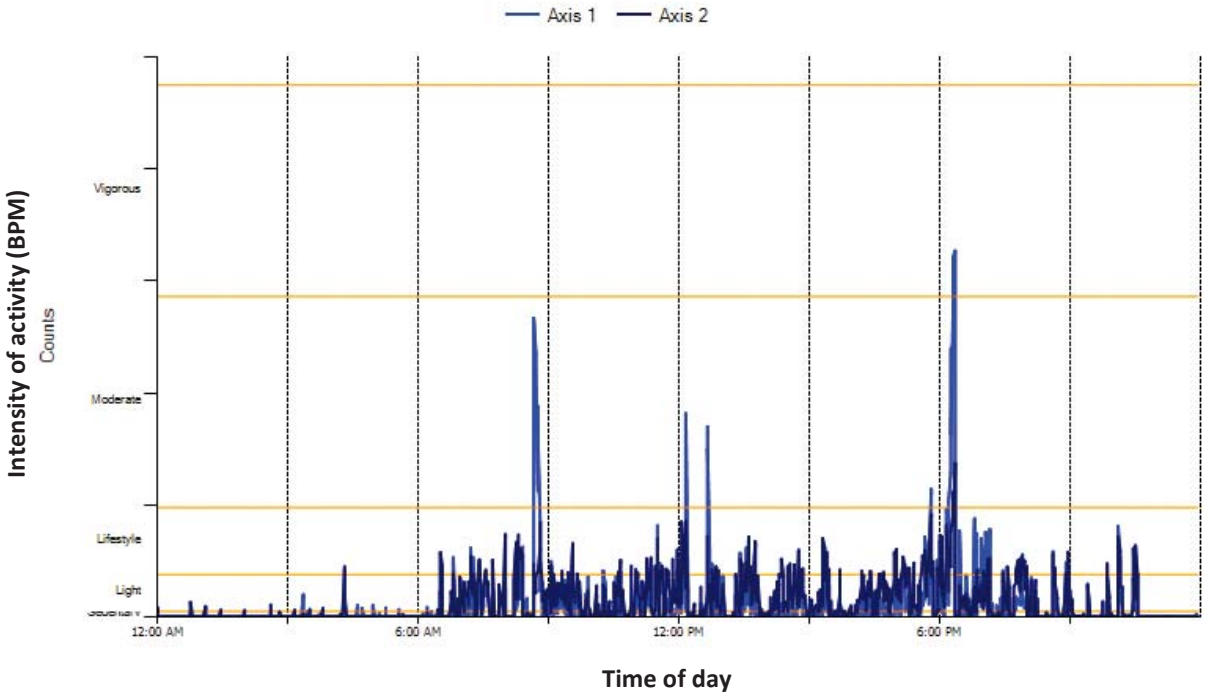

Figure 2.3. An example of a graph resulting from one day of accelerometer use. As shown, the levels of activity elevate and fall during the day. The highest peaks appeared to around 8.30 am and 6.30 pm. The intensity of activity was determined by heart rate (beats per minute [BPM]).

2.6. Compliance

The returned milk powder from the previous period was counted, medication records checked, at week 4, 8, and 12 to confirm compliance. Participants with compliance <80% were withdrawn from the study.

2.7. Statistical analysis:

SAS Version 9.2 (SAS Institute Inc. Cary, NC 27513, USA) was used for statistical analysis. Univariate analysis was used to measure the mean and standard deviation (SD) of each variable. Kolmogorov-Smirnov test was performed to test the normality of the data. Paired T-test was performed to measure the difference between plasma 25(OH)D_3, CTx and osteocalcin, and IL-6 levels at baseline and week 12. Multiple regression was used to measure the interaction between the levels of plasma vitamin D, calcium intake, and bone markers. The Pearson correlation was used to evaluate the relationship between the levels of physical activity in the questionnaire and accelerometer. The results were considered statistically significant if the *p*-value was <0.05.

Chapter 3

Results

Forty three participants were recruited in the current study, and all of them completed the PA questionnaire. Anthropometric measurements were made on 42 participants; blood samples collected from 40 participants were analysed for plasma $25(OH)D_3$; bone turnover markers and IL-6 analysed from 39; the estimated 3-day food record and FFQ were completed by 39; and the 7-day accelerometer measurements completed by 25 participants.

3.1. Anthropometric characteristics

The mean anthropometric characteristics and hip and spine BMD are represented in table 3.1.

Table 3.1. Anthropometric characteristics of 42 participants at baseline. Data is given on mean ± SD

Characteristic	Mean ± SD
Anthropometric Characteristic	
Age (years)	38.6 ± 5.0
Height (cm)	166.5 ± 5.7
Weight (kg)	70.3 ± 13.8
BMI (kg/m^2)	25 ± 4
Bone Characteristic	
Hip BMD (g/cm^2)	0.964 ± 0.102
Hip BMC (g)	31.725 ± 5.504
Hip T-score	0.1 ± 0.9
Hip Z-score	0.3 ± 0.9
Spine BMD (g/cm^2)	1.086 ± 0.080
Spine BMC (g)	65.339 ± 9.605

Spine T-score	0.4 ± 0.7
Spine Z-score	0.6 ± 0.8

At baseline, participants had a BMI of 25 ± 4 kg/m^2 which was in normal range and fit with the criteria. The BMDs and T-score of the women were within normal range for both hip and lumbar spine.

3.2. Blood analyses

3.2.1. Plasma 25(OH)D_3 levels

Figure 3.1 shows the comparison of the mean plasma 25(OH)D_3 levels at week 0 and week 12.

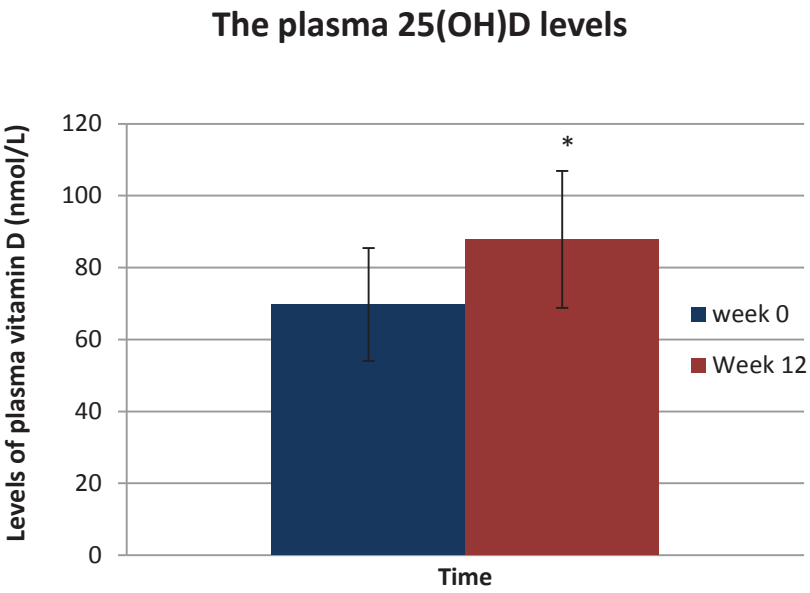

Figure 3.1. Mean ± SD of plasma vitamin D at week 0 (69.75 ± 15.87) and after supplementation, at week 12 (87.83 ± 19.06) of 40 participants. * = indicates statistical significant; *p*-value <.0001 for the mean.

The mean plasma vitamin D level in week 12 (87.83 ± 19.06) increased compared to week 0 (69.75 ± 15.87). Vitamin D status of the women at both baseline and week 12

were within the normal range based on the Institute of Medicine recommendation for 25(OH)D_3 level of 50 nmol/L).

3.2.2. Bone Markers (CTx and Osteocalcin)

The mean of CTx and osteocalcin at baseline and week 12 is shown in table 3.2.

Table 3.2. the mean CTX and osteocalcin at baseline and week 12

Bone markers	Time	n	Mean ± SD	Difference (*p*-value)
CTx (µg/L)	Baseline	39	0.31 ± 0.12	<.0001*
	Week 12	39	0.21 ± 0.09	
Osteocalcin (µg/L)	Baseline	39	22.63 ± 6.64	0.0003*
	Week 12	39	19.64 ± 6.25	

*indicates significant difference (*p*-value <0.05)

Significant differences were found in the levels of bone turnover markers (CTx and osteocalcin) after milk supplementation compared to the levels at baseline (*p*-value <0.05). Milk supplementation, therefore, reduced bone turnover markers significantly.

3.2. 3. Interaction between the levels of plasma vitamin D, calcium intake, and bone markers

The interaction between plasma vitamin D concentration, calcium intake and the levels of bone markers is shown table 3.3.

Table 3.3. The interaction between plasma 25(OH)D_3, calcium intake and bone markers

Variable	Intercept	Plasma vit D	Calcium	R^2
CTx	0.282	0.0005	-0.0001	0.21
(*p*-value)	(0.0006)	(0.485)	(0.005)*	
OC	24.078	-0.001	-0.005	0.11
(*p*-value)	(<.0001)	(0.9825)	0.052	

*indicates significant difference (*p*-value <0.05)

Only dietary calcium intake was a significant predictor of CTx level, plasma vitamin D was not. Both the level of plasma vitamin D and calcium intake were not significant predictors of the OC level.

3.2.4. Interleukin-6 (IL-6)

The mean IL-6 at baseline and week 12 is presented in table 3.4.

Table 3.4. The mean IL-6 at baseline and week 12

Markers	Time	n	Mean ± SD	Difference (*p*-value)
IL-6 (pg/mL)	Baseline	35	0.68 ± 0.46	0.0447*
	Week 12	35	0.81 ± 0.54	

*indicates significant difference (p-value <0.05)

As shown, there was an increased concentration of IL-6 at week 12 compared to baseline. IL-6 is a measure of proinflammatory cytokine.

3.3. Nutrient intakes

3.3.1. 3-day food record

Thirty nine participants completed estimated 3-day food records. The mean intakes of macronutrients are shown in table 3.5.

Table 3.5. Comparison of the mean intake of macronutrients and AMDR

Nutrient	Mean ± SD		AMDR
	Intake per day (g)	Percent of energy	
Protein	87 ± 21	17 ± 3 % of energy	15% - 25% of energy
Fat	80 ± 34	32 ± 6 % of energy	20% - 35% of energy
Saturated fat	32 ± 14	13 ± 3 % of energy	<10% of energy
Carbohydrate	252 ± 58	47 ± 6 % of energy	45% - 65% of energy

Table 3.5 illustrates that the participating women met adequate intakes of macronutrients based on Acceptable Macronutrient Distribution Ranges (AMDR) for macronutrients to reduce chronic disease from the Ministry of Health. They met the AMDR for protein, fat and carbohydrate, however, intake of saturated fat at 13% was higher than the AMDR.

The mean intakes of micronutrients and the percentages of the estimated Nutrient Reference Values (NRV) are shown in table 3.6. The New Zealand and Australian NRV is 1000 mg of calcium for women aged 31-50 years. In addition, they recommend 5 µg of dietary vitamin D based on intakes to maintain plasma vitamin D levels for adults with limited sun exposure (Ministry of Health, 2006).

Table 3.6. Comparison of the mean intake of micronutrients and NRV

Nutrient	Mean ± SD	%			
		EAR	RDI	AI	UL
Calcium (mg)*	1013 ± 367	120%	101%		
Vitamin D (μg)*	3.9 ± 2.1			78.4%	
Magnesium (mg)*	360 ± 109	136%	112%		
Zinc (mg)*	11 ± 2.9	169%	137%		
Phosphorus (mg)*	1553 ± 356	268%	155%		
Iron (mg)	13.25 ± 3.21	166%	74%		
Sodium (mg)	2816 ± 931			612%	122%
Iodine (μg)	62.25 ± 19.44	62%	42%		
Selenium (μg)	52.14 ± 17.90	104%	87%		
Dietary fibre (g)	28 ± 8			112%	
Water (L)	2598 ± 975			93%	
Total vitamin A equivalents (μg)	1043 ± 534	208%	149%		
Riboflavin (mg)	2.15 ± 0.65	239%	195%		
Niacin equivalents (mg)	35.95 ± 10.74	327%	257%		
Vitamin B6 (mg)	1.83 ± 0.56	166%	141%		
Vitamin B12 (μg)	3.80 ±1.59	190%	158%		
Folate (Dietary Folate Equivalents) (μg)	424.37 ± 192.33	133%	106%		
Vitamin C (mg)	124 ± 82	414%	276%		
Vitamin E (mg)	10.86 ± 3.84			155%	
Copper (mg)	1.53 ± 0.59			128%	
Manganese (μg)	4846 ± 1717			97%	
Potassium (mg)	3436 ± 818			123%	

*nutrients that are important for bone metabolism. EAR (Estimated Average Requirement): a daily nutrient level estimated to meet the requirements of half the healthy individuals in a particular life stage and gender group; RDI (Recommended Dietary Intake): the average daily nutrient intake that is sufficient to meet nutrient requirements of nearly all (97-98%) healthy individuals in a particular life stage and gender group; AI (Adequate Intake): the average daily nutrient intake level based on observed or experimentally-determined approximations or estimates of nutrient intake by a group or groups of apparently healthy people that are assumed to be adequate; UL (Upper Level of Intake): the highest average daily intake level likely to pose no adverse health effects to almost all individuals in the general population. As intake increase above the UL, the potential risk of adverse effects increases.

As shown, the dietary calcium intake of study participants met the RDI of 1000 mg daily. However, vitamin D intake was lower than the AI; participants consumed approximately 3.9 µg per day. Participants had adequate intakes for other minerals important for bone metabolism.

Of note, the intake of iron (13.25 mg/day) and selenium (52.14 µg/day) were above the EAR, but less than the RDI. Evaluating an intake that lies between the RDI and the EAR is difficult; the intake is possibly adequate, but there is a greater chance that it is inadequate (Whitney et al., 2011). Dietary sodium intake was 2816 mg/day, which exceeds the AI (460 mg/day) and the UL (2300 mg/day). Interestingly, intake of iodine at 62.25 µg/day, was less than both the EAR and the RDI; indicating intake is probably inadequate (Whitney et al., 2011).

3.3.2. Food frequency questionnaire

Forty participants completed the food frequency questionnaire. Table 3.7 demonstrates the frequency that calcium and vitamin D containing foods were consumed.

Table 3.7. FFQ of calcium and vitamin D food sources.

Food group	Frequency (weekly)
Calcium and Vitamin D containing foods	
Bread	11
Eggs	2
Milk as a drink	2
Colby, mild, tasty and cheddar cheese	2
Calcium containing foods	
Yoghurt (plain or flavoured)	3
Cheese: cottage, ricotta, mozzarella, feta, edam, brie, blue and cream cheese	2
Muesli bars	2
Green leafy vegetables (eg. kale, spinach, silver beet, puha, watercress, mustard greens, taro leaf (eg palusami), karengo (seaweed)	2
Nuts	2
Chocolate (including chocolate bars)	1
Peanut butter and other nut spreads	1
Dried fruit (eg. raisins, sultanas, currants, apricots, prunes, dates)	1
Milo and cocoa	1
Decaffeinated coffee	1
Flavoured milk (eg milk shake, iced coffee)	1
Vitamin D containing foods	
Bacon, ham, luncheon meats, salami or brawn, meat fillings in sandwiches	1
White fish dishes (eg cod, roughy)	1
Oily fish dishes (eg sardines, tuna, salmon)	1

A food was considered as a calcium source, if it contained ≥ 30 mg per 100 mg serving size, and it was considered a vitamin D source if it contained ≥ 0.25 μg (10 IU) per 100 mg serving size.

The participating women consumed a number of foods containing both calcium and vitamin D. On average, volunteers ate bread 11 times per week and consumed eggs, milk, colby and cheddar cheese twice per week. Participants consumed calcium

containing foods three times a week (yoghurt) and twice a week (cheese, muesli bar, green leafy vegetables, and nuts). Vitamin D containing foods generally included oily fish dishes (such as sardines, tuna or salmon), white fish dishes (cod and roughy), and processed meats which were commonly consumed once per week.

3.4. Physical activity measures

Habitual physical activity levels were estimated by physical activity questionnaire and 7-day accelerometer records. Forty three participants completed the questionnaires and accelerometer data were obtained from 25. Table 3.8 shows the mean time spent doing brisk walking, moderate and vigorous physical activity.

Table 3.8. The mean levels of physical activity from questionnaire NZPAQ-SF

Activity (weekly)	**Mean ± SD (n=43)**
Walking (minute)	139 ± 141
Moderate (minute)	239 ± 327
Vigorous (minute)	267 ± 232
Total (minute)	645 ± 388

As shown, in total participants spent 645 ± 388 minutes engaged in physical activity per week. Total physical activity was calculated according to the method of McLean & Tobias (2004); where each minute of vigorous activity is considered equal to two minutes of moderate activity, and then time spent in walking, moderate and vigorous activity is summed.

The intensity and duration profiles of physical activity recorded by 7-day accelerometer wear by 25 volunteers are shown in table 3.9.

Table 3.9. The mean levels of physical activity from 7-day accelerometer records

Activity	Mean ± SD (n=25)
Light PA (minute)	417 ± 303
Moderate (minute)	298 ± 139
Vigorous (minute)	57 ± 58
Very vigorous (minute)	5 ± 11

Participants spent most of their time in light activity and least time in very vigorous activity, as expected.

Table 3.10 shows the correlation of physical activity levels (light, moderate and vigorous) between PA questionnaires and 7-day accelerometer results. Pearson correlation analysis was performed in 22 participants after the exclusion of outliers.

Table 3.10. The correlation of physical activity levels between PA questionnaires and 7-day accelerometer measures

Activity	Mean ± SD		R^2	P-Value
	Accelerometer(n=22)	Questionnaire (n= 22)		
Light PA (minute)	415 ± 319	115 ± 74	0.024	0.4925
Moderate (minute)	289 ± 143	208 ± 225	0.232	0.0234*
Vigorous (minute)	59 ± 61	126 ± 130	0.003	0.7890

*indicates significant relationship (*p*-value <0.05)

A significant correlation was found between moderate physical activity (MPA) duration from the questionnaire and accelerometer (*p*-value <0.05). Participants reported less time spent on light physical activity LPA) in the questionnaire compared to that objectively measured by accelerometers. Participants reported more time spent on

physical activity at vigorous intensity (VPA) in the questionnaire than was assessed objectively by the accelerometer.

5. Compliance and adherence

On average, participants consumed 97% of the milk supplement provided over the duration of the study. Twenty eight per cent of participants had a 100% compliance (i.e. consumed every milk supplement sachet they were given over the duration of the study). One woman did not want to continue at week 2, one was not used to drinking milk, and another one did not attend week 12.

Chapter 4

Discussion and conclusions

4.1. Interpretation of the results

The current study was designed to measure the effects of calcium and vitamin D fortified milk on the bone markers: OC and plasma CTx, and vitamin D status of active, healthy premenopausal women aged 30-45 years old in Palmerston North, New Zealand over a period of 12 weeks. Calcium and vitamin D fortified milk significantly increased the level of plasma vitamin D and IL-6, and decreased the levels of bone turnover markers: OC and CTx.

The mean age of the study population was 38.6 ± 5.0 years old. Participants had a BMI of 25 ± 4 kg/m^2 which met the study inclusion criteria. In comparison with other bone marker studies of premenopausal women, the BMI of participants in the present study was slightly higher than the BMI of control-group women (26.9 ± 1.03 years) in Palmerston North, New Zealand 23.5 ± 0.51 kg/m^2 (Kruger et al., 2006), and the BMI of women (34.6 ± 2.8 years) from the United Kingdom, France, Belgium, and the United States 23.3 ± 2.81 kg/m^2 (Glover et al., 2009). The BMI was similar to that reported for similarly aged women (39.1 ± 4.3 years) in the United Kingdom 25.7 ± 5.42 kg/m^2 (Glover et al., 2008).

The T-scores of the hip and lumbar spine were 0.1 ± 0.9 and 0.4 ± 0.7, respectively. A T-score between +1 and -1 is considered normal (World Health Organization, 2003),

which indicates that our participants had normal bone density. Similar T-scores for premenopausal women aged 25-44 years were reported by Winzenberg et al. (2006b). These researchers reported a T-score of the femoral neck of 0.93 ± 0.14, and for the lumbar spine of 1.08 ± 0.12 (Winzenberg et al., 2006b)

4.1.1. Effects of high calcium and vitamin D fortified milk on vitamin D status

The current study was undertaken from autumn to winter periods (March – August). The mean plasma 25(OH)D_3 level at baseline was 69.75 nmol/L, which is higher than the value reported for New Zealand European and Others (NZEO) women (48 nmol/L) in the 1997 National Nutrition Survey (NNS97) database (Rockell et al., 2006). Furthermore, the 2008/09 New Zealand Adult Nutrition Survey reported the overall mean level of vitamin D for New Zealand adults aged 15 years and over was 63.0 nmol/L, and for women was 62.4 nmol/L (Ministry of Health, 2012). Thus, the vitamin D levels of our cohort of active women in Palmerston North were higher than previously reported.

One possible explanation for this result is that plasma 25(OH)D_3 concentration in New Zealand women who live in the North is in general 6 nmol/L higher than those in the South Island due to 25% greater UV light in the North than South Island (Rockell et al., 2006). It is not surprising therefore that the vitamin D level of our cohort women who live in North Island was higher the NNS97 Survey and the 2008/2009 New Zealand Adult Nutrition Survey, in which the vitamin D concentration of participating men and women from both islands was measured. In addition, plasma 25(OH)D_3 level is associated with

physical activity (Wicherts et al., 2007). Physical activity is an independent predictor of 25(OH)D_3 level, likely as a surrogate for outdoor activities which tend to have higher sun exposure (Giovannucci et al., 2006).

Over the three month supplementation period, in which an extra 1200 mg calcium and 10 µg vitamin D were provided per day, the vitamin D status of our cohort of women increased significantly. Similarly, in a study among Chinese girls, Du et al. (2004) found that their intervention group who were supplemented milk containing 560 mg calcium and 3.33 µg of vitamin D daily, for 24 months had significantly higher vitamin D status than those receiving only calcium and those in the control group.

In a study of women aged 55 years and over, Kruger et al. (2010) reported that 1200 mg calcium and 9.6 µg vitamin D fortified milk over 16 weeks significantly improved vitamin D status of South East Asian women. However, in a study of young premenopausal females in New Zealand, the supplementation of 5 µg vitamin D_3 daily had no effect on the vitamin D status of the intervention group; there was in fact a slight reduction of 25(OH)D_3 levels over 4 months (Kruger et al., 2006). This was postulated to be due to a seasonal influence; baseline measurements were taken in the summer and final measurements were during winter (Kruger et al., 2006). An alternate explanation comes from Heaney et al. (2003b) who suggest that in order to maintain serum 25(OH)D_3 level, 12.5 µg of oral vitamin D_3 or 500 IU is needed each day. Nonetheless, our supplemented level of 10 µg vitamin D per day was sufficient to improve vitamin D status. Vitamin D is important in promoting intestinal calcium

absorption, development of the osteoblast and synthesis of osteocalcin by the osteoblasts (Heaney, 1999; Heaney et al., 2003b).

4.1.2. Effects of high calcium and vitamin D fortified milk on bone turnover markers

The mean CTx at baseline in the current study was 0.31 μg/L, which is similar to the level of CTx of premenopausal women in the United Kingdom (0.30 ng/mL) (Glover et al., 2008), but it is lower than the mean CTx concentration reported previously in France (0.34 ng/mL), Belgium (40.2 ng/mL), the United States (33.7 ng/mL) and New Zealand (0.44-049 μg/L) (Glover, et al., 2009; Kruger et al., 2006). The low CTx levels indicate a low rate of bone resorption.

Several possible factors have been identified to have an effect on bone turnover markers. Firstly participation in sports or other physical activities influence bone turnover markers (Glover et al., 2009). A study reported that young premenopausal women who were in regular sports or physical activities had lower mean biochemical markers of bone formation and bone resorption (Glover et al., 2009). Different geographical locations could also contribute to the significantly different levels of bone turnover markers (Cohen et al., 1998), however this factor is still debated. BMI significantly influences the levels of bone turnover markers in premenopausal women (Glover et al., 2008). Glover et al. (2008) reported that premenopausal women with lower mean BMI had significantly higher bone turnover markers: CTx and OC.

The consumption of high calcium and vitamin D fortified milk significantly reduced the levels of CTx after 12 weeks in the current study. In a previous study, Kruger et al. (2006) found that high calcium skim milk supplementation over 16 weeks significantly reduced bone turnover markers (CTx, OC and type I amino terminal procollagen peptide [PINP]) of premenopausal New Zealand women. Additionally, in another study by the same researchers of women aged over 55 years, the authors reported that high calcium and vitamin D fortified milk which provided 1200 mg calcium and 9.6 µg vitamin D daily significantly reduced the concentration of CTx in South East Asian women over 4 months (Kruger et al., 2010)

The most likely explanation for the decreased CTx is the antiresorptive effect of high calcium intake (Matkovic et al., 2005; Rozen et al., 2003). Increased calcium intake stimulates increased calcium retention due to increased fractional intestinal calcium absorption, leading to suppression of bone resorption and remodelling, which in turn decreases the rate of bone turnover (Wastney et al., 2000; Slemenda et al., 1997).

Bone formation can be evaluated by measuring the biochemical marker OC (Szulc & Delmas, 2008). In comparison with other bone marker studies, the level of OC at baseline in the current study was 22.63 µg/L; which is higher than the value reported for healthy, young premenopausal females living in the United Kingdom (15.1 µg/L) (Glover, et al., 2008), and lower than those previously measured in New Zealand (28.2 µg/L) (Kruger et al., 2006). To date, no reference range of the bone turnover markers for young premenopausal women has been established (Glover, et al., 2008).

High calcium and vitamin D fortified milk resulted in a significant reduction in OC level in the current study. Plasma OC decreased by 13.2% after 12 weeks supplementation. The reduction of plasma OC was in agreement with the results of calcium and/or milk supplementation in children (Slemenda et al., 1997), premenopausal (Kruger et al., 2006) and postmenopausal women (Kruger et al., 2010). Specifically, a short-term study on calcium and vitamin D fortified milk intervention in premenopausal Caucasian New Zealand women, reported reduced serum osteocalcin over 4 months (Kruger et al., 2006). Under physiological circumstances, bone formation and resorption are coupled; the amount of bone formed at a BMU is equal to that removed by bone resorption, especially in young adults. Bone resorption is reduced with milk supplementation; bone formation markers are stable for several weeks and then decrease as osteoblasts fill in a smaller number of resorptive cavities (Need, 2006; Szulc & Delmas, 2008).

In the present study, bone turnover markers were used rather than bone density. Bone markers respond more quickly to interventions. Bone turnover markers are adequately sensitive in monitoring the acute changes of bone turnover. The changes of bone turnover markers can be measured after a few weeks or months, whist any significant changes of bone mineral density may be seen after at least two years of an intervention (Christenson, 1997; Delmas et al., 2000; Glover, et al., 2008; Kruger et al., 2010).

4.1.3. Effects of high calcium and vitamin D fortified milk on IL-6

Over the calcium and vitamin D fortified milk intervention period, the levels of IL-6 increased significantly. The production of cytokines such as IL-6, IL-1 and TNF are increased when serum estrogen decreases (Ross, 2006). In relation to bone metabolism, a study of 200 Scottish women involving 26 premenopausal and the remainder postmenopausal women, reported that variation at the IL-6 gene locus may affect the genetic regulation of bone mass (Murray et al., 1997). Another study of postmenopausal females found an association between the IL-6 polymorphism and osteoporosis in postmenopausal women (Czerny, B. et al., 2010). Serum levels of IL-6 are higher in osteoporosis (Bustamante et al., 2007) and it has been suggested that the role of IL-6 in osteoporosis is by promoting osteoclastic differentiation and activation (Edwards & Williams, 2010). As a proinflammatory cytokine, IL-6 is normally strongly regulated and expressed at low levels, except during trauma, infection, fever and stress (Ershler & Keller, 2000; Harden et al., 2011). In the present study we found a significant increase in IL-6. Some participants reported that they had a period of illness such as cold, fever, cough and influenza during the intervention period.

4.1.4. Nutrient intakes

The mean vitamin D intake was 3.9 ± 2.1 μg/day, which is 78% of the adequate intake recommendation. However, intake was higher than estimated population means in Australia and New Zealand (2.0–2.4 μg/day), data from a review where authors have taken from the Australia New Zealand Food Authority (ANZFA) (Nowson & Margerison, 2002).

Vitamin D intake in the present study was about the same as that previously found in women in the United Kingdom (3.7 μg/day) (Calvo et al., 2005). Like New Zealand, in the United Kingdom there is no mandatory fortification of staple foods with vitamin D (Calvo et al., 2005; Rockell et al., 2006; Nowson & Margerison, 2002). Vitamin D intake in the present study was lower than that found in the United States and Canada (7.33 μg/day), where there is mandatory fortification of staple foods with vitamin D.

Although Japan and Norway have little or no fortification of foods, these populations have higher vitamin D intakes (7.1 μg/day and 5.9 μg/day, respectively) than those of the present study; likely due to high fish consumption (Calvo et al., 2005). In the present study we found that the participating women had oily fish dishes such as sardines, tuna and salmon only once a week.

In summary, a number of factors may influence the intake of vitamin D including; mandatory food fortification, vitamin D supplementation, high fish consumption, a vegetarian diet and other dietary patterns (Calvo, et al., 2004; Calvo et al., 2005). In New Zealand, a small number of foods are fortified and the people do not generally

consume foods high in vitamin D such as fatty fish and organ meats (Russell et al., 1999).

The average intake of calcium in this study met the RDI (Ministry of Health, 2006) and exceeded the mean calcium intake of New Zealand females (691 mg/day) estimated from the NNS97 (Russell et al., 1999). In comparison with a similar age group, a study of Caucasian premenopausal women in Dunedin, New Zealand, reported a mean dietary calcium intake of 808.1 mg/day (Wilson & Horwath, 1996). A previous study of Caucasian premenopausal women in Palmerston North reported a calcium intake of 1034 mg/day (Kruger et al., 2006). Food choices may influence the demographic differences in calcium intake in the New Zealand population (Horwath et al., 2001). In the present study we had a small number of participants who were physically active, which indicates their interest in health. A study reported that participants with good health-related behaviour illustrated by higher intensities of physical activity, also had higher intakes of fruit and vegetables (Trudeau et al., 1998).

In comparison with another study of a similar age group, Mangano et al. (2011) reported mean dietary calcium intake of 730 mg/day in United States adult females aged 31-40 years old from NHANES 2003-2006 database. Thus, calcium intake in the present study was found to be in the same range or higher than that reported in other studies. Adequate lifetime calcium intake as well as adequate intake during adulthood are beneficial to bone health, including reducing the risk of osteoporosis in later life (Nicklas, 2003; Mangano et al., 2011).

Magnesium, zinc and phosphorus are also important for bone metabolism (Goldberg, 2006; Cashman, 2007). In the current study, these minerals were consumed in greater quantities than the usual intake of New Zealand women (Russell et al., 1999). The possible explanation might be due to activity levels of the current cohort, and therefore greater energy intakes (8,873 kJ) than NZEO women in the NNS97 (8,426 kJ) (Russell et al., 1999). Habitual physical activity of women in the current study might reflect other good health-related behaviours such as fruit and vegetable intake resulting in higher mineral intakes important to bone health (Trudeau et al., 1998).

The intake of iron (13.25 mg/day) and selenium (52 μg/day) met the EAR but were lower than the RDI. Women in the current study had a higher iron intake compared to those reported in the NNS97 (10.3 mg/day) (Russell et al., 1999). Selenium intake was higher than intake reported in the NNS97 (39 μg/day) (Russell et al., 1999) and Dunedin female residents (38 μg/day) (Thomson, 2004). In general, the New Zealand population has a low dietary selenium intake, and intake varies with eating habits and the geographical sources of foods (Thomson, 2004).

The intake of iodine in the present study (62.25 μg/day) was less than both EAR and RDI; indicating that intake is probably inadequate (Whitney et al., 2011). A previous study done in Palmerston North found a similar iodine intake in women of childbearing-age (65.4 μg/day) (Mohd-Shukri, 2011). It has been suggested that New Zealand women have low iodine intake due to low good food sources of iodine (Mohd-Shukri, 2011), despite the 2009 mandatory fortification of bread with iodine, the iodine intake remains low.

Dietary sodium intake was 2816 mg/day, which exceed the AI (460 mg/day) and the upper level for 2300 mg/day. The intake was also higher than the estimated sodium intake in adults by Food Standards Australia New Zealand (FSANZ) of 1900 to 2500 mg/day (Keogh et al., 2012). Of note, measuring dietary sodium intake and iodine from iodized salt consumption is extremely difficult due to difficulties of getting good serving size estimates. Such problems tend to result in inaccurate and inconsistent findings (Cook et al., 2007).

4.1.5. Physical activity

The levels of physical activity from accelerometers were 417 ± 303, 298 ± 139, 57 ± 58, and 5 ± 11 minutes of light, moderate, vigorous and very vigorous activity per week respectively. Total activity summed to 777 minutes per week. The Ministry of Health recommends that adults should be active every day by including at least 30 minutes of moderate activity at least five days a week (Ministry of Health, 2013), which approximates 150 minutes of total moderate intensity physical activity a week. The participants in the current study met the MOH recommendation, moderate physical activity averaged 298 minutes per week and around 67% of participants reported doing moderate intensity activity on 5 days or more.

4.2. Conclusions

4.2.1. Conclusions

In conclusion, calcium and vitamin D fortified milk supplementation increased vitamin D status and decreased bone turnover markers, CTx and osteocalcin, significantly in active premenopausal women aged 30-45 years old in Palmerston North, New Zealand over a period of 12 weeks.

Participants had normal ranges of BMI, and T-score of hip and lumbar spine at baseline. Participants had normal vitamin D status at baseline and after milk supplementation. The dietary intake of calcium, magnesium, zinc and phosphorus met the RDI, however dietary vitamin D intake was below the RDI. The intake of calcium was a significant predictor of CTx, but plasma $25(OH)D_3$ was not. Both calcium intake and the plasma $25(OH)D_3$ concentration were not significant predictors of OC concentration.

4.2.2 Implications

Limited studies evaluating the changes in biochemical markers of bone turnover have been performed on premenopausal women, particularly physically active participants, in response to fortified milk intervention. Adequate intake of calcium during childhood and adolescence should optimize peak bone mass. After peak bone mass has been reached, adequate dietary calcium intake should maintain bone density and reduce bone resorption. The results from the current study do indicate that resorption was

reduced significantly due to the milk intake in the cohort of young women. Long-term intake of milk and having sufficient calcium in the diet, combined with an adequate vitamin D status will help reduce bone loss with ageing and could reduce the risk of fracture in later life.

4.2.3. Limitations and suggestions for future improvements

The present study used a self-reported PA questionnaire, which has a potential to be misreporting due to an ambiguous understanding for the different levels of PA by participants. Although efforts were implemented to minimize the level of overestimation by providing written and verbal examples of intensities of physical activity. Self-reported questionnaires can also lead to inaccurate recall and significant overestimation of time spent on daily physical activity due to the fact that most daily activities are intermittent in nature (Trost et al., 2002; Fukukawa et al., 2004; Anderson et al., 2005; Slootmaker et al., 2009). Limited accelerometers were available in the present study. This caused a queue for accelerometer use and therefore the PA measurements using questionnaire and 7-day accelerometer were performed over different time periods.

The study was a short-term trial with a small sample size. Future studies, particularly long-term studies with a larger sample size, are needed to assess the effects of milk supplementation on vitamin D status and the changes of bone turnover markers and bone density in premenopausal women. To evaluate whether these is a dose response in bone turnover markers to increased calcium and vitamin D intake and different intensities of exercise, future studies are needed.

References

Abrams, S. A., Griffin, I. J., Hawthorne, K. M., Gunn, S. K., Gundberg, C. M., & Carpenter, T. O. (2005). Relationships among vitamin D levels, parathyroid hormone, and calcium absorption in young adolescents. *Journal of Clinical Endocrinology & Metabolism, 90*(10), 5576-5581.

Abrams, S. A., Hicks, P. D., & Hawthorne, K. M. (2009). Higher serum 25-hydroxyvitamin D levels in school-age children are inconsistently associated with increased calcium absorption. *Journal of Clinical Endocrinology & Metabolism, 94*(7), 2421-2427.

Adami, S., Bertoldo, F., Braga, V., Fracassi, E., Gatti, D., Gandolini, G., . . . Battista Rini, G. (2009). 25-hydroxy vitamin D levels in healthy premenopausal women: association with bone turnover markers and bone mineral density. *Bone, 45*(3), 423-426.

Adami, S., Bianchi, G., Brandi, M. L., Giannini, S., Ortolani, S., DiMunno, O., . . . Rossini, M. (2008). Determinants of bone turnover markers in healthy premenopausal women. *Calcified tissue international, 82*(5), 341-347.

Ahdjoudj, S., Lasmoles, F., Oyajobi, B. O., Lomri, A., Delannoy, P., & Marie, P. J. (2001). Reciprocal control of osteoblast/chondroblast and osteoblast/adipocyte differentiation of multipotential clonal human marrow stromal F/STRO-1(+) cells. *Journal of Cellular Biochemistry, 81*(1), 23-38.

Ahlborg, H. G., Johnell, O., Turner, C. H., Rannevik, G., & Karlsson, M. K. (2003). *Bone loss and bone size after menopause. New England journal of medicine, 349*(4), 327-334.

Allen, L., & World Health Organization (2006*). Guidelines on food fortification with micronutrients*: World Health Organization.

Anderson, C. B., Hagströmer, M., & Yngve, A. (2005). Validation of the PDPAR as an adolescent diary: effect of accelerometer cut points. *Medicine and science in sports and exercise, 37*(7), 1224.

Aubin, J. E., & Triffitt, J. T. (2002). Mesenchymal stem cells and osteoblast differentiation. *Principles of bone biology*, 1, 59-81.

Aubin, J. E., Lian, J. B., Stein, G. S. (2006). Bone formation: maturation and functional activities of osteoblast lineage cell. In M. J. Favus (Ed.), *Primer on the metabolic bone diseases and disorders of mineral metabolism* (pp. 20-27). Washington, DC: American Society for Bone and Mineral Research.

Australian Government Department of Health and Aging, New Zealand Ministry of Health and National Health and Medical Research Council. (2006). *Nutrient reference values for Australia and New Zealand.* Retrieved January 10, 2013 from http://www.nhmrc.gov.au/publications/synopses/_files/n27.pdf.

Bakker, A., Klein-Nulend, J., Tanck, E., Albers, G., Lips, P., & Burger, E. (2005). Additive effects of estrogen and mechanical stress on nitric oxide and prostaglandin E 2 production by bone cells from osteoporotic donors. *Osteoporosis International, 16*(8), 983-989.

Balesaria, S., Sangha, S., & Walters, J. R. F. (2009). Human duodenum responses to vitamin D metabolites of TRPV6 and other genes involved in calcium absorption. *American Journal of Physiology-Gastrointestinal and Liver Physiology, 297*(6), G1193-G1197.

Balmain, N. (1991). Calbindin-D9k. A vitamin-D-dependent, calcium-binding protein in mineralized tissues. *Clinical orthopaedics and related research*(265), 265.

Banke-Rasmussen, G. L. H., Ernst Hansen, Bente Koch, Leif Mosekilde, Christian Mølgaard, Ole Helmer Sørensen, Lars Ovesen, L. (2000). Vitamin D: should the supply in the Danish population be increased? *International journal of food sciences and nutrition, 51*(3), 209-215.

Baron, J. A., Farahmand, B. Y., Weiderpass, E., Michaelsson, K., Alberts, A., Persson, I., & Ljunghall, S. (2001). Cigarette smoking, alcohol consumption, and risk of hip fracture in women. *Archives of internal medicine, 161*(7), 983.

Bauer, D. C., Gluer, C. C., Cauley, J. A., Vogt, T. M., Ensrud, K. E., Genant, H. K., & Black, D. M. (1997). Broadband ultrasound attenuation predicts fractures strongly and independently of densitometry in older women: a prospective study. *Archives of internal medicine, 157*(6), 629.

Baxter-Jones, A. D. G., Faulkner, R. A., Forwood, M. R., Mirwald, R. L., & Bailey, D. A. (2011). Bone mineral accrual from 8 to 30 years of age: an estimation of peak bone mass. *Journal of Bone and Mineral Research, 26*(8), 1729-1739.

Baxter-Jones, A. D. G., Kontulainen, S. A., Faulkner, R. A., & Bailey, D. A. (2008). A longitudinal study of the relationship of physical activity to bone mineral accrual from adolescence to young adulthood. *Bone, 43*(6), 1101-1107.

Bell, N. H. (2003). RANK ligand and the regulation of skeletal remodeling. *Journal of Clinical Investigation, 111*(8), 1120-1121.

Bischoff-Ferrari, H. A., Giovannucci, E., Willett, W. C., Dietrich, T., & Dawson-Hughes, B. (2006). Estimation of optimal serum concentrations of 25-hydroxyvitamin D for multiple health outcomes. *The American journal of clinical nutrition, 84*(1), 18-28.

Blair, H. C., Zaidi, M., & Schlesinger, P. H. (2002). Mechanisms balancing skeletal matrix synthesis and degradation. *Biochemical Journal, 364*(Pt 2), 329.

Bonewald, L. F. (2007). Osteocytes as dynamic multifunctional cells. *Annals of the New York Academy of Sciences, 1116*(1), 281-290.

Bonjour, J. P., Carrie, A. L., Ferrari, S., Clavien, H., Slosman, D., Theintz, G., & Rizzoli, R. (1997). Calcium-enriched foods and bone mass growth in prepubertal girls: a randomized, double-blind, placebo-controlled trial. *Journal of Clinical Investigation, 99*(6), 1287.

Bonjour, J. P., Guéguen, L., Palacios, C., Shearer, M. J., & Weaver, C. M. (2010). Minerals and vitamins in bone health: the potential value of dietary enhancement. *British Journal of Nutrition, 101*(11), 1581.

Boyle, W. J., Simonet, W. S., & Lacey, D. L. (2003). Osteoclast differentiation and activation. *Nature, 423*(6937), 337-342.

Brodsky, B., & Persikov, A. V. (2005). Molecular structure of the collagen triple helix. *Advances in protein chemistry, 70*, 301-339.

Bronner, F., & Pansu, D. (1999). Nutritional aspects of calcium absorption. *The Journal of nutrition, 129*(1), 9-12.

Brown, E. M. & Juppner, H. (2006). Parathyroid hormone: Synthesis, secretion, and action. In M. J. Favus (Ed.), *Primer on the metabolic bone diseases and disorders of mineral metabolism* (pp. 90-99). Washington, DC: American Society for Bone and Mineral Research.

Bustamante, M., Nogues, X., Mellibovsky, L., Agueda, L., Jurado, S., Caceres, E., . . . Grinberg, D. (2007). Polymorphisms in the interleukin-6 receptor gene are associated with bone mineral density and body mass index in Spanish postmenopausal women. *European Journal of Endocrinology, 157*(5), 677-684.

Cadogan, J., Eastell, R., Jones, N., & Barker, M. E. (1997). Milk intake and bone mineral acquisition in adolescent girls: randomised, controlled intervention trial. *Bmj, 315*(7118), 1255-1260.

Calvo, M. S., Whiting, S. J., & Barton, C. N. (2004). Vitamin D fortification in the United States and Canada: current status and data needs. *The American journal of clinical nutrition, 80*(6), 1710S-1716S.

Calvo, M. S., Whiting, S. J., & Barton, C. N. (2005). Vitamin D intake: a global perspective of current status. *The Journal of nutrition, 135*(2), 310-316.

Cao, J. J. (2011). Effects of obesity on bone metabolism. *J Orthop Surg Res, 6*(1), 30-36.

Cashman, K. (2002). Calcium intake, calcium bioavailability and bone health. *British Journal of Nutrition, 87*(2), 169-178.

Cashman, K. (2004). Diet and control of osteoporosis. *Functional foods, ageing and degenerative disease*. Cambridge, UK: Woodhead Publishing Limited, 83-114.

Cashman, K. D. (2007). Diet, nutrition, and bone health. *The Journal of nutrition, 137*(11), 2507S-2512S.

Cheng, J. B., Levine, M. A., Bell, N. H., Mangelsdorf, D. J., & Russell, D. W. (2004). Genetic evidence that the human CYP2R1 enzyme is a key vitamin D 25-hydroxylase. *Proceedings of the National Academy of Sciences of the United States of America, 101*(20), 7711-7715.

Cheng, J. B., Motola, D. L., Mangelsdorf, D. J., & Russell, D. W. (2003). De-orphanization of Cytochrome P450 2R1 A MICROSOMAL VITAMIN D 25-HYDROXYLASE. *Journal of Biological Chemistry, 278*(39), 38084-38093.

Christenson, R. H. (1997). Biochemical markers of bone metabolism: an overview. *Clinical biochemistry, 30*(8), 573-593.

Clowes, J. A., Riggs, B. L., & Khosla, S. (2005). The role of the immune system in the pathophysiology of osteoporosis. *Immunological reviews, 208*(1), 207-227.

Cohen, F., Eckert, S., & Mitlak, B. (1998). Geographic differences in bone turnover: data from a multinational study in healthy postmenopausal women. *Calcified tissue international, 63*(4), 277-282.

Cook, N. R., Cutler, J. A., Obarzanek, E., Buring, J. E., Rexrode, K. M., Kumanyika, S. K., . . . Whelton, P. K. (2007). Long term effects of dietary sodium reduction on cardiovascular disease outcomes: observational follow-up of the trials of hypertension prevention (TOHP). *Bmj, 334*(7599), 885.

Cooper, C., & Eastell, R. (1993). Bone gain and loss in premenopausal women. *British Medical Journal, 306*(6889), 1357-1358.

Corral, D. A., Amling, M., Priemel, M., Loyer, E., Fuchs, S., Ducy, P., . . . Karsenty, G. (1998). Dissociation between bone resorption and bone formation in osteopenic transgenic mice. *Proceedings of the National Academy of Sciences, 95*(23), 13835-13840.

Costa, A. G., Cusano, N. E., Silva, B. C., Cremers, S., & Bilezikian, J. P. (2011). Cathepsin K: its skeletal actions and role as a therapeutic target in osteoporosis. *Nature Reviews Rheumatology, 7*(8), 447-456.

Courteix, D., Jaffre, C., Lespessailles, E., & Benhamou, L. (2005). Cumulative effects of calcium supplementation and physical activity on bone accretion in premenarchal children: a double-blind randomised placebo-controlled trial. *International journal of sports medicine, 26*(5), 332-338.

Cummings, S. R., Bates, D., & Black, D. M. (2002). Clinical use of bone densitometry. *JAMA: the journal of the American Medical Association, 288*(15), 1889-1897.

Czerny, B., Kaminski, A., Kurzawski, M., Kotrych, D., Safranow, K., Dziedziejko, V., …. Pawlik, A., (2010). The association of IL-1β, IL-2, and IL-6 gene polymorphisms with bone mineral density and osteoporosis in postmenopausal women. *European Journal of Obstetrics & Gynecology and Reproductive Biology 149*, 82–85.

Dane, C., & Dane, B. (2012). Which Method is More Efficient in Determining Osteoporosis, QUS or DEXA? *International Journal of Endocrinology and Metabolism, 10*(4), 642-643.

Dane, C., Dane, B., Cetin, A., & Erginbas, M. (2008). The role of quantitative ultrasound in predicting osteoporosis defined by dual-energy X-ray absorptiometry in pre-and postmenopausal women. *Climacteric, 11*(4), 296-303.

de Papp, A. E., Bone, H. G., Caulfield, M. P., Kagan, R., Buinewicz, A., Chen, E., . . . Reitz, R. E. (2007). A cross-sectional study of bone turnover markers in healthy premenopausal women. *Bone, 40*(5), 1222-1230.

Deftos, L. J. (2006). Calcitonin. In M. J. Favus (Ed.), *Primer on the metabolic bone diseases and disorders of mineral metabolism* (pp. 115-122). Washington, DC: American Society for Bone and Mineral Research.

Delmas, P. D., Eastell, R., Garnero, P., Seibel, M., & Stepan, J. (2000). The use of biochemical markers of bone turnover in osteoporosis. *Osteoporosis international, 11*(18), 2-17.

Dempster, D. W. (2006). Anatomy and functions of the adult skeleton. In M. J. Favus (Ed.), *Primer on the metabolic bone diseases and disorders of mineral metabolism* (pp. 7-11). Washington, DC: American Society for Bone and Mineral Research.

Diamond, T. H., Eisman, J. A., Mason, R. S., Nowson, C. A., Pasco, J. A., Sambrook, P. N., . . . Endocrine Soc Aust Osteo, A. (2005). Vitamin D and adult bone health in Australia and New Zealand: a position statement. *Medical Journal of Australia, 182*(6), 281-285.

Dibba, B., Prentice, A., Ceesay, M., Stirling, D. M., Cole, T. J., & Poskitt, E. M. E. (2000). Effect of calcium supplementation on bone mineral accretion in Gambian children accustomed to a low-calcium diet. *The American journal of clinical nutrition, 71*(2), 544-549.

Du, X., Greenfield, H., Fraser, D., Ge, K., Liu, Z., & He, W. (2002). Milk consumption and bone mineral content in Chinese adolescent girls. *Bone, 30*(3), 521-528.

Du, X., Zhu, K., Trube, A., Zhang, Q., Ma, G., Hu, X., . . . Greenfield, H. (2004). School-milk intervention trial enhances growth and bone mineral accretion in Chinese girls aged 10–12 years in Beijing. *British Journal of Nutrition, 92*(01), 159-168.

Ducy, P., Schinke, T., & Karsenty, G. (2000). The osteoblast: a sophisticated fibroblast under central surveillance. *Science, 289*(5484), 1501-1504.

Duncan Bassett, J., & Williams, G. R. (2003). The molecular actions of thyroid hormone in bone. *Trends in Endocrinology & Metabolism, 14*(8), 356-364.

Eastell, R., & Hannon, R. A. (2008). Biomarkers of bone health and osteoporosis risk. *Proceedings of the Nutrition Society, 67*, 157–162.

Eastell, R., Barton, I., Hannon, R., Chines, A., Garnero, P., & Delmas, P. (2003). Relationship of early changes in bone resorption to the reduction in fracture risk with risedronate. *Journal of Bone and Mineral Research, 18*(6), 1051-1056.

Ebioscience. (2012). *Human IL-6 high sensitivity ELISA*. Retrieved December 4, 2012, from http://www.ebioscience.com/human-il-6-high-sensitivity-elisa-kit.htm.

Edwards, C., & Williams, E. (2010). The role of interleukin-6 in rheumatoid arthritis-associated osteoporosis. *Osteoporosis international, 21*(8), 1287-1293.

Eghbali-Fatourechi, G., Khosla, S., Sanyal, A., Boyle, W. J., Lacey, D. L., & Riggs, B. L. (2003). Role of RANK ligand in mediating increased bone resorption in early postmenopausal women. *Journal of Clinical Investigation, 111*(8), 1221-1230.

Elin, R. (1994). Magnesium: the fifth but forgotten electrolyte. *American journal of clinical pathology, 102*(5), 616.

Ellis, K. J. (2000). Human body composition: in vivo methods. *Physiological Reviews, 80*(2), 649-680.

Ershler, W. B., & Keller, E. T. (2000). Age-associated increased interleukin-6 gene expression, late-life diseases, and frailty. *Annual review of medicine, 51*(1), 245-270.

Favus, M. J., Bushinsky, D. A., Lemann, J. (2006). Regulation of calcium, magnesium, and phosphate metabolism. In M. J. Favus (Ed.), *Primer on the metabolic bone diseases and disorders of mineral metabolism* (pp. 76-83). Washington, DC: American Society for Bone and Mineral Research.

Ferrar, L., van der Hee, R., Berry, M., Watson, C., Miret, S., Wilkinson, J., . . . Eastell, R. (2011). Effects of calcium-fortified ice cream on markers of bone health. *Osteoporosis international, 22*(10), 2721-2731.

Feskanich, D., Willett, W. C., Stampfer, M. J., & Colditz, G. A. (1997). Milk, dietary calcium, and bone fractures in women: a 12-year prospective study. *American journal of public health, 87*(6), 992-997.

Fukukawa, Y., Nakashima, C., Tsuboi, S., Kozakai, R., Doyo, W., Niino, N., . . . Shimokata, H. (2004). *Age differences in the effect of physical activity on depressive symptoms. Psychology and Aging, 19*(2), 346.

Fulzele, K., & Clemens, T. L. (2012). Novel functions for insulin in bone. *Bone, 50,* 452-456.

Garnero, P., & Delmas, P. D. (1998). BIOCHEMICAL MARKERS OF BONE TURNOVER:: Applications For Osteoporosis. *Endocrinology &Metabolism Clinics of North America, 27*(2), 303-323.

Garnero, P., Hausherr, E., Chapuy, M. C., Marcelli, C., Grandjean, H., Muller, C., . . . Delmas, P. D. (1996a). Markers of bone resorption predict hip fracture in elderly women: the EPIDOS Prospective Study. *Journal of Bone and Mineral Research, 11*(10), 1531-1538.

Garnero, P., Sornay-Rendu, E., Chapuy, M. C., & Delmas, P. D. (1996b). Increased bone turnover in late postmenopausal women is a major determinant of osteoporosis. *Journal of Bone and Mineral Research, 11*(3), 337-349.

Garnero, P., Sornay-Rendu, E., Chapuy, M. C., & Delmas, P. D. (2009). Increased bone turnover in late postmenopausal women is a major determinant of osteoporosis. *Journal of Bone and Mineral Research, 11*(3), 337-349.

Gerritsen, S., Stefanogiannis, N., & Galloway, Y. (2008). *A Portrait of Health: Key Results of the 2006/07 New Zealand Health Survey*: Ministry of Health.

Gerstenfeld, L. C., & Einhorn, T. A. (2006). Fracture healing: The biology of bone repair and regeneration. In M. J. Favus (Ed.), *Primer on the metabolic bone diseases and disorders of mineral metabolism* (pp. 42-48). Washington, DC: American Society for Bone and Mineral Research.

Giovannucci, E., Liu, Y., Rimm, E. B., Hollis, B. W., Fuchs, C. S., Stampfer, M. J., & Willett, W. C. (2006). Prospective study of predictors of vitamin D status and cancer incidence and mortality in men. *Journal of the National Cancer Institute, 98*(7), 451-459.

Glover, S. J., Gall, M., Schoenborn-Kellenberger, O., Wagener, M., Garnero, P., Boonen, S., . . . Eastell, R. (2009). Establishing a reference interval for bone turnover markers in 637 healthy, young, premenopausal women from the United Kingdom, France, Belgium, and the United States. *Journal of Bone and Mineral Research, 24*(3), 389-397.

Glover, S., Garnero, P., Naylor, K., Rogers, A., & Eastell, R. (2008). Establishing a reference range for bone turnover markers in young, healthy women. *Bone, 42*(4), 623-630.

Goldberg, G. (2006). Nutrition and bone. *Women's Health Medicine, 3*(4), 157-159.

Goltzman, D. (2008). Studies on the mechanisms of the skeletal anabolic action of endogenous and exogenous *parathyroid hormone. Archives of biochemistry and biophysics, 473*(2), 218-224.

Gori, F., Hofbauer, L. C., Dunstan, C. R., Spelsberg, T. C., Khosla, S., & Riggs, B. L. (2000). The expression of osteoprotegerin and RANK ligand and the support of osteoclast formation by stromal-osteoblast lineage cells is developmentally regulated. *Endocrinology, 141*(12), 4768-4776.

Graham Cumming, R. (1990). Calcium intake and bone mass: a quantitative review of the evidence. *Calcified tissue international, 47*(4), 194-201.

Hadjidakis, D. J., & Androulakis, I. I. (2006). Bone remodeling. *Annals of the New York Academy of Sciences, 1092*(1), 385-396.

Halioua, L., & Anderson, J. (1989). Lifetime calcium intake and physical activity habits: independent and combined effects on the radial bone of healthy premenopausal Caucasian women. *The American journal of clinical nutrition, 49*(3), 534-541.

Harden, L. M., du Plessis, I., Roth, J., Loram, L. C., Poole, S., & Laburn, H. P. (2011). Differences in the relative involvement of peripherally released interleukin (IL)-6, brain IL-1β and prostanoids in mediating lipopolysaccharide-induced fever and sickness behavior. *Psychoneuroendocrinology, 36*(5), 608-622.

Harinarayan, C., Sachan, A., Reddy, P. A., Satish, K., Prasad, U., & Srivani, P. (2011). Vitamin D status and Bone Mineral Density in Women of Reproductive and Postmenopausal age groups: A cross-sectional study from South India. *JAPI, 59*, 699.

Heaney, R. P. (1999). Lessons for nutritional science from vitamin D. *The American journal of clinical nutrition, 69*(5), 825-826.

Heaney, R. P., & Burckhardt, P. (1995). *Nutrition and bone health*. Paper presented at the Nutritional aspects of osteoporosis '94. Challenges of modern medicine. Ares-Serono symposia series: challenges of modern medicine.

Heaney, R. P., & Weaver, C. M. (2003). Calcium and vitamin D. *Endocrinology and metabolism clinics of North America, 32*(1), 181

Heaney, R. P., Davies, K. M., Chen, T. C., Holick, M. F., & Barger-Lux, M. J. (2003b). Human serum 25-hydroxycholecalciferol response to extended oral dosing with cholecalciferol. *The American journal of clinical nutrition, 77*(1), 204-210.

Heaney, R. P., Dowell, M. S., Hale, C. A., & Bendich, A. (2003a). Calcium absorption varies within the reference range for serum 25-hydroxyvitamin D. *Journal of the American College of Nutrition, 22*(2), 142-146.

Heaney, R., Abrams, S., Dawson-Hughes, B., Looker, A., Marcus, R., Matkovic, V., & Weaver, C. (2000). Peak bone mass. *Osteoporosis international, 11*(12), 985-1009.

Heinonen, A., Mantynen, J., Kannus, P., Uusi-Rasi, K., Nikander, R., Kontulainen, S., & Sievanen, H. (2012). Effects of High-Impact Training and Detraining on Femoral Neck Structure in Premenopausal Women: A Hip Structural Analysis of an 18-Month Randomized

Controlled Exercise Intervention with 3.5-Year Follow-Up. *Physiotherapy Canada, 64*(1), 98-105.

Hill, P. A., Murphy, G., Docherty, A. J. P., Hembry, R. M., Millican, T. A., Reynolds, J. J., & Meikle, M. C. (1994). The effects of selective inhibitors of matrix metalloproteinases (mmps) on bone-resorption and the identification of mmps and timp-1 in isolated osteoclasts. *Journal of cell science, 107*, 3055-3064.

Hind, K., & Burrows, M. (2007). Weight-bearing exercise and bone mineral accrual in children and adolescents: a review of controlled trials. *Bone, 40*(1), 14-27.

Hippisley-Cox, J., & Coupland, C. (2009). Predicting risk of osteoporotic fracture in men and women in England and Wales: prospective derivation and validation of QFractureScores. *Bmj, 339*, b4229.

Hofbauer, L. C., & Hamann, C. (2010). Diabetes Mellitus and Osteoporosis. *Nutritional Influences on Bone Health*, 103-108.

Hofbauer, L. C., Khosla, S., Dunstan, C. R., Lacey, D. L., Boyle, W. J., & Riggs, B. L. (2000). The roles of osteoprotegerin and osteoprotegerin ligand in the paracrine regulation of bone resorption. *Journal of Bone and Mineral Research, 15*(1), 2-12.

Holick, M. F. (2001). Sunlight "D" ilemma: risk of skin cancer or bone disease and muscle weakness. *The Lancet, 357*(9249), 4-6.

Holick, M. F. (2006). Resurrection of vitamin D deficiency and rickets. *Journal of Clinical Investigation, 116*(8), 2062.

Holick, M. F. (2009). Vitamin D status: measurement, interpretation, and clinical application. *Annals of epidemiology, 19*(2), 73-78.

Horn, G. (2012). Sources of dietary calcium in patients attending an osteoporosis clinic. *Nutrition, 4*, 55-60.

Horowitz, M. C. (1993). Cytokines and estrogen in bone: anti-osteoporotic effects. *Science, 260*(5108), 626.

Horwath, C., Parnell, W., Wilson, N., & Russell, D. (2001). Attaining optimal bone status: lessons from the 1997 National Nutrition Survey. *New Zealand medical journal, 114*, 138-140.

Hsu, H., Lacey, D. L., Dunstan, C. R., Solovyev, I., Colombero, A., Timms, E., . . . Sarosi, I. (1999). Tumor necrosis factor receptor family member RANK mediates osteoclast differentiation and activation induced by osteoprotegerin ligand. *Proceedings of the National Academy of Sciences, 96*(7), 3540-3545.

Huebner, A. K., Keller, J., Catala-Lehnen, P., Perkovic, S., Streichert, T., Emeson, R. B., . . . Schinke, T. (2008). The role of calcitonin and α-calcitonin gene-related peptide in bone formation. *Archives of biochemistry and biophysics, 473*(2), 210-217.

IBMS BoneKey. (2012). *An Introduction to Clinical Osteoporosis*. Retrieved September 1, 2012 from http://www.nature.com/bonekey/index.html

Ishimi, Y., Miyaura, C., Jin, C. H., Akatsu, T., Abe, E., Nakamura, Y., . . . Hirano, T. (1990). IL-6 is produced by osteoblasts and induces bone resorption. *The Journal of Immunology, 145*(10), 3297.

Janssen, H. C. J. P., Samson, M. M., & Verhaar, H. J. J. (2002). Vitamin D deficiency, muscle function, and falls in elderly people. *The American journal of clinical nutrition, 75*(4), 611-615.

Janz, K. F., Letuchy, E. M., Gilmore, J. M. E., Burns, T. L., Torner, J. C., Willing, M. C., & Levy, S. M. (2010). Early physical activity provides sustained bone health benefits later in childhood. *Medicine and science in sports and exercise, 42*(6), 1072.

Jenkins, M. R., & Denison, A. V. (2008). Smoking status as a predictor of hip fracture risk in postmenopausal women of northwest Texas. *Preventing chronic disease, 5*(1), A09.

Johnell, O., Oden, A., De Laet, C., Garnero, P., Delmas, P., & Kanis, J. (2002). Biochemical indices of bone turnover and the assessment of fracture probability. *Osteoporosis international, 13*(7), 523-526.

Johnston Jr, C. C., Miller, J. Z., Slemenda, C. W., Reister, T. K., Hui, S., Christian, J. C., & Peacock, M. (1992). Calcium supplementation and increases in bone mineral density in children. *New England journal of medicine, 327*(2), 82-87.

Jones, G., & Scott, F. S. (1999). A Cross-Sectional Study of Smoking and Bone Mineral Density in Premenopausal Parous Women: Effect of Body Mass Index, Breastfeeding, and Sports Participation. *Journal of Bone and Mineral Research, 14*(9), 1628-1633.

Kaiser, J., Lemaire, T., Naili, S., Sansalone, V., & Komarova, S. (2012). Do calcium fluxes within cortical bone affect osteocyte mechanosensitivity? *Journal of Theoretical Biology, 303*, 75-86.

Kamioka, H., Honjo, T., & Takano-Yamamoto, T. (2001). A three-dimensional distribution of osteocyte processes revealed by the combination of confocal laser scanning microscopy and differential interference contrast microscopy. *Bone, 28*(2), 145-149.

Kanazawa, I., Yamaguchi, T., Yamamoto, M., Yamauchi, M., Kurioka, S., Yano, S., & Sugimoto, T. (2009). Serum osteocalcin level is associated with glucose metabolism and atherosclerosis parameters in type 2 diabetes mellitus. *Journal of Clinical Endocrinology & Metabolism, 94*(1), 45-49.

Kanders, B., Dempster, D. W., & Lindsay, R. (1988). Interaction of calcium nutrition and physical activity on bone mass in young women. *Journal of Bone and Mineral Research, 3(*2), 145-149.

Kanis, J. A., Johansson, H., Johnell, O., Oden, A., De Laet, C., Eisman, J. A., . . . Tenenhouse, A. (2005). Alcohol intake as a risk factor for fracture. *Osteoporosis international, 16*(7), 737-742.

Kanis, J. A., Melton III, L. J., Christiansen, C., Johnston, C. C., & Khaltaev, N. (1994). The diagnosis of osteoporosis. *Journal of Bone and Mineral Research, 9*(8), 1137-1141.

Karsenty, G. (2008). Transcriptional control of skeletogenesis. *Annu. Rev. Genomics Hum. Genet., 9*, 183-196.

Keogh, J. B., Lange, K., Hogarth, R., & Clifton, P. M. (2012). Foods contributing to sodium intake and urinary sodium excretion in a group of Australian women. *Public health nutrition, 1*(1), 1-6.

Khosla, S. (2001). Minireview: The opg/rankl/rank system. *Endocrinology, 142*(12), 5050-5055.

Kim, S. J., Chun, J. Y., & Kim, M. S. (2000). Insulin stimulates production of nitric oxide via ERK in osteoblast cells. *Biochemical and Biophysical Research Communications, 278*(3), 712-718.

Kindblom, J. M., Ohlsson, C., Ljunggren, Ö., Karlsson, M. K., Tivesten, Å., Smith, U., & Mellström, D. (2009). Plasma osteocalcin is inversely related to fat mass and plasma glucose in elderly Swedish men. *Journal of Bone and Mineral Research, 24*(5), 785-791.

King, J. C., Shames, D. M., & Woodhouse, L. R. (2000). Zinc homeostasis in humans. *The Journal of nutrition, 130*(5), 1360S-1366S.

King, J. C., Shames, D. M., Lowe, N. M., Woodhouse, L. R., Sutherland, B., Abrams, S. A., . . . Jackson, M. J. (2001). Effect of acute zinc depletion on zinc homeostasis and plasma zinc kinetics in men. *The American journal of clinical nutrition, 74*(1), 116-124.

Kleinnulend, J., Semeins, C., Ajubi, N., Nijweide, P., & Burger, E. (1995). Pulsating fluid flow increases nitric oxide (NO) synthesis by osteocytes but not periosteal fibroblasts-correlation with prostaglandin upregulation. *Biochemical and Biophysical Research Communications, 217*(2), 640-648.

Knapp, K., Blake, G., Spector, T., & Fogelman, I. (2001). Multisite quantitative ultrasound: precision, age-and menopause-related changes, fracture discrimination, and T-score equivalence with dual-energy X-ray absorptiometry. *Osteoporosis international, 12*(6), 456-464.

Kong, Y. Y., Feige, U., Sarosi, I., Bolon, B., Tafuri, A., Morony, S., . . . McCabe, S. (1999). Activated T cells regulate bone loss and joint destruction in adjuvant arthritis through osteoprotegerin ligand. *Nature, 402*, 43-47.

Konrad, M., Schlingmann, K. P., & Gudermann, T. (2004). Insights into the molecular nature of magnesium homeostasis. *American Journal of Physiology-Renal Physiology, 286*(4), F599-F605.

Krall, E. A., & Dawson-Hughes, B. (1993). Heritable and life-style determinants of bone mineral density. *Journal of Bone and Mineral Research, 8*(1), 1-9.

Krall, E. A., & Dawson-Hughes, B. (1999). Smoking increases bone loss and decreases intestinal calcium absorption. *Journal of Bone and Mineral Research, 14*(2), 215-220.

Krieg, M. A., Cornuz, J., Ruffieux, C., Van Melle, G., Büche, D., Dambacher, M. A., . . . Kraenzlin, M. (2006). Prediction of hip fracture risk by quantitative ultrasound in more than 7000 Swiss women≥ 70 years of age: comparison of three technologically different bone ultrasound devices in the SEMOF study. *Journal of Bone and Mineral Research, 21*(9), 1457-1463.

Kruger, M. C., Booth, C. L., Coad, J., Schollum, L. M., Kuhn-Sherlock, B., & Shearer, M. J. (2006). Effect of calcium fortified milk supplementation with or without vitamin K on biochemical markers of bone turnover in premenopausal women. *Nutrition, 22*(11), 1120-1128.

Kruger, M. C., Schollum, L. M., Kuhn-Sherlock, B., Hestiantoro, A., Wijanto, P., Li-Yu, J., . . . Eastell, R. (2010). The effect of a fortified milk drink on vitamin D status and bone turnover in post-menopausal women from South East Asia. *Bone, 46*(3), 759-767.

Lanham-New, S. A. (2008). Importance of calcium, vitamin D and vitamin K for osteoporosis prevention and treatment. *Proceedings of the Nutrition Society, 67*(2), 163-176.

Lau, E. M., Lynn, H., Chan, Y. H., Lau, W.,Woo, J. (2004). Benefits of milk powder supplementation on bone accretion in Chinese children. *Osteoporosis International, 15*, 654–8.

Lehtonen-Veromaa, M., Möttönen, T., Irjala, K., Nuotio, I., Leino, A., & Viikari, J. (2000). A 1-year prospective study on the relationship between physical activity, markers of bone metabolism, and bone acquisition in peripubertal girls. *Journal of Clinical Endocrinology & Metabolism, 85*(10), 3726-3732.

Lenora, J., Ivaska, K., Obrant, K., & Gerdhem, P. (2007). Prediction of bone loss using biochemical markers of bone turnover. *Osteoporosis international, 18*(9), 1297-1305.

Lewiecki, E. (2005). Clinical applications of bone density testing for osteoporosis. Minerva medica, 96(5), 317.

Li, X., Zhang, Y., Kang, H., Liu, W., Liu, P., Zhang, J., . . . Wu, D. (2005). Sclerostin binds to LRP5/6 and antagonizes canonical Wnt signaling. *Journal of Biological Chemistry, 280*(20), 19883-19887.

Li, Y. C., Kong, J., Wei, M., Chen, Z. F., Liu, S. Q., & Cao, L. P. (2002). 1, 25-Dihydroxyvitamin D3 is a negative endocrine regulator of the renin-angiotensin system. *Journal of Clinical Investigation, 110*(2), 229-238.

Li, Y., Xi, B., Li, K., & Wang, C. (2012). Association between vitamin D receptor gene polymorphisms and bone mineral density in Chinese women. *Molecular biology reports*, 1-9.

Lips, P. (2001). Vitamin D deficiency and secondary hyperparathyroidism in the elderly: consequences for bone loss and fractures and therapeutic implications. *Endocrine reviews, 22*(4), 477-501.

Lofman, O., Magnusson, P., Toss, G., & Larsson, L. (2005). Common biochemical markers of bone turnover predict future bone loss: A 5-year follow-up study. *Clinica Chimica Acta, 356*(1-2), 67-75.

Lorenzo, J., Horowitz, M., & Choi, Y. (2008). Osteoimmunology: interactions of the bone and immune system. *Endocrine reviews, 29*(4), 403-440.

Lunt, M., Felsenberg, D., Adams, J., Benevolenskaya, L., Cannata, J., Dequeker, J., . . . Khaw, K. (1997). Population-based geographic variations in DXA bone density in Europe: the EVOS Study. *Osteoporosis international, 7*(3), 175-189.

Luukinen, H., Käkönen, S. M., Pettersson, K., Koski, K., Laippala, P., Lövgren, T., . . . Väänänen, H. (2000). Strong prediction of fractures among older adults by the ratio of carboxylated to total serum osteocalcin. *Journal of Bone and Mineral Research, 15*(12), 2473-2478.

Ma, G., Li, Y., Jin, Y., Du, S., Kok, F. J., & Yang, X. (2007). Assessment of intake inadequacy and food sources of zinc of people in China. *Public health nutrition, 10*(8), 848-854.

Mangano, K. M., Walsh, S. J., Insogna, K. L., Kenny, A. M., & Kerstetter, J. E. (2011). Calcium intake in the United States from dietary and supplemental sources across adult age groups: New Estimates from the National Health and Nutrition Examination Survey 2003-2006. *Journal of the American Dietetic Association, 111*(5), 687-695.

Manolagas, S. C. (2000). Birth and death of bone cells: basic regulatory mechanisms and implications for the pathogenesis and treatment of osteoporosis. *Endocrine reviews*, *21*(2), 115-137.

Marieb, E. N., & Hoehn, K. (2006). *Human Anatomy & Physiology: seventh edition*. Retrieved January 16, 2013 from http://www.trimble.k12.ky.us/tchsweb/teachers/DGRIFFIN/Anatomy%20and%20Physiology/chapter%206/HAP7_ch06-a_files/frame.htm#slide0025.htm

Marks, J., Debnam, E. S., & Unwin, R. J. (2010). Phosphate homeostasis and the renal-gastrointestinal axis. *American Journal of Physiology-Renal Physiology, 299*(2), F285-F296.

Martin, T. J., & Sims, N. A. (2005). Osteoclast-derived activity in the coupling of bone formation to resorption. *Trends in molecular medicine*, *11*(2), 76-81.

Matkovic, V., Goel, P. K., Badenhop-Stevens, N. E., Landoll, J. D., Li, B., Ilich, J. Z., . . . Ha, E. J. (2005). Calcium supplementation and bone mineral density in females from childhood to young adulthood: a randomized controlled trial. *The American journal of clinical nutrition, 81*(1), 175-188.

Matsuo, K., & Irie, N. (2008). Osteoclast–osteoblast communication. *Archives of biochemistry and biophysics, 473*(2), 201-209.

Maunsell, Z., Wright, D. J., & Rainbow, S. J. (2005). Routine isotope-dilution liquid chromatography–tandem mass spectrometry assay for simultaneous measurement of the 25-hydroxy metabolites of vitamins D2 and D3. *Clinical Chemistry, 51*(9), 1683-1690.

Mazess, R. B., & Barden, H. S. (1991). Bone density in premenopausal women: effects of age, dietary intake, physical activity, smoking, and birth-control pills. *The American journal of clinical nutrition, 53*(1), 132-142.

McLean, G., & Tobias, M. (2004). *The New Zealand Physical Activity Questionnaires: Report on the validation and use of the NZPAQ-LF and NZPAQ-SF self-report physical activity survey instruments*. SPARC, Wellington, New Zealand.

Melton III, L. J., Khosla, S., Atkinson, E. J., O'Fallon, W. M., & Riggs, B. L. (1997). Relationship of bone turnover to bone density and fractures. *Journal of Bone and Mineral Research, 12*(7), 1083-1091.

Merrilees, M., Smart, E., Gilchrist, N., Frampton, C., Turner, J., Hooke, E., . . . Maguire, P. (2000). Effects of dairy food supplements on bone mineral density in teenage girls. *European Journal of Nutrition, 39*(6), 256-262.

Mikuni-Takagaki, Y., Kakai, Y., Satoyoshi, M., Kawano, E., Suzuki, Y., Kawase, T., & Saito, S. (2009). Matrix mineralization and the differentiation of osteocyte-like cells in culture. *Journal of Bone and Mineral Research, 10*(2), 231-242.

Miller, W. L., & Portale, A. A. (2000). Vitamin D 1 alpha-hydroxylase. *Trends in Endocrinology and Metabolism, 11*(8), 315-319.

Ministry of Health and Cancer Society of New Zealand. (2012). *Consensus Statement on Vitamin D and Sun Exposure in New Zealand*. Wellington: Ministry of Health.

Ministry of Health. (2012). *Vitamin D Status of New Zealand Adults: Findings from the 2008/09 New Zealand Adult Nutrition Survey*. Wellington: Ministry of Health.

Ministry of Health. (2013). *How much activity is recommended?*. Retrieved January 7, 2013 from http://www.health.govt.nz/yourhealth-topics/physical-activity/how-much-activity-recommended.

Moayyeri, A., Kaptoge, S., Dalzell, N., Bingham, S., Luben, R. N., Wareham, N. J., . . . Khaw, K. T. (2009). Is QUS or DXA Better for Predicting the 10-Year Absolute Risk of Fracture? *Journal of Bone and Mineral Research, 24*(7), 1319-1325.

Mohd-Shukri, N. H. (2011). *Iodine and selenium status and uptake, and iodine nutritional knowledge of women of childbearing-age in Palmerston North, New Zealand.* Massey University, Palmerston North, New Zealand.

Morrison, NA, Qi JC, Tokita A, Kelly PJ, Crofts L, Nguyen TV, Sambrook PN & Eisman JA. (1994) Prediction of bone density from vitamin D receptor alleles. *Nature, 367*, 284–287

Moy, K. L., Sallis, J. F., & Tanjasiri, S. P. (2010). Culturally-specific Physical Activity Measures for Native Hawaiian and Pacific Islanders. *Hawaii Medical Journal, 69*(5 suppl 2), 21.

Mukamal, K., Robbins, J., Cauley, J., Kern, L., & Siscovick, D. (2007). Alcohol consumption, bone density, and hip fracture among older adults: the cardiovascular health study. *Osteoporosis international, 18*(5), 593-602.

Murray, R., McGuigan, F., Grant, S., Reid, D., & Ralston, S. (1997). Polymorphisms of the interleukin-6 gene are associated with bone mineral density. *Bone, 21*(1), 89-92.

Need, A. G. (2006). Bone resorption markers in vitamin D insufficiency. *Clinica Chimica Acta, 368*(1), 48-52.

Nelson, D. A., Norris, S. A., & Gilsanz, V. (2006). Childhood and adolescence. In M. J. Favus (Ed.), *Primer on the metabolic bone diseases and disorders of mineral metabolism* (pp. 55-63). Washington, DC: American Society for Bone and Mineral Research.

Neville-Webbe, H., Cross, N., Eaton, C., Nyambo, R., Evans, C., Coleman, R., & Holen, I. (2004). Osteoprotegerin (OPG) produced by bone marrow stromal cells protects breast cancer cells from TRAIL-induced apoptosis. *Breast cancer research and treatment, 86*(3), 271-282.

New, S. A., Robins, S. P., Campbell, M. K., Martin, J. C., Garton, M. J., Bolton-Smith, C., . . . Reid, D. M. (2000). Dietary influences on bone mass and bone metabolism: further evidence of a positive link between fruit and vegetable consumption and bone health? 1. *The American journal of clinical nutrition, 71*(1), 142-151.

Nguyen, N. D., Pongchaiyakul, C., Center, J. R., Eisman, J. A., & Nguyen, T. V. (2005). Abdominal fat and hip fracture risk in the elderly: The Dubbo Osteoporosis Epidemiology Study. *BMC musculoskeletal disorders, 6*(1), 11.

Nguyen, T., Center, J., & Eisman, J. (2000). Osteoporosis in elderly men and women: effects of dietary calcium, physical activity, and body mass index. *Journal of Bone and Mineral Research, 15*(2), 322-331.

Nicklas, T. A. (2003). Calcium intake trends and health consequences from childhood through adulthood. *Journal of the American College of Nutrition, 22*(5), 340-356.

Nieves, J. W., Komar, L., Cosman, F., & Lindsay, R. (1998). Calcium potentiates the effect of estrogen and calcitonin on bone mass: review and analysis. *The American journal of clinical nutrition, 67*(1), 18-24.

Nordin, B. (1997). Calcium in health and disease. *Alimentation, Nutrition et Agriculture*.

Nowson, C. A., & Margerison, C. (2002). Vitamin D intake and vitamin D status of Australians. *Medical Journal of Australia, 177*(3), 149-152.

Ott, S. (2010). Bisphosphonates and BMU birth rate. *Osteoporosis International, 21*(5), 887-887.

Owens, J., & Chambers, T. J. (1993). Macrophage colony stimulating factor (M-CSF) is essential for osteoclast formation *in vitro*. *Biochemical and Biophysical Research Communications, 195*(3), 1401-1407.

Pais, R., Campean, R., Simon, S. P., Bolosiu, C. R., Muntean, L., & Bolosiu, H. D. (2010). Accuracy of quantitative ultrasound parameters in the diagnosis of osteoporosis. *Central European Journal of Medicine, 5*(4), 478-485.

Pepmueller, P. H., Cassidy, J. T., Allen, S. H., & Hillman, L. S. (1996). Bone mineralization and bone mineral metabolism in children with juvenile rheumatoid arthritis. *Arthritis & Rheumatism, 39*(5), 746-757.

Pfeilschifter, J., Köditz, R., Pfohl, M., & Schatz, H. (2002). Changes in proinflammatory cytokine activity after menopause. *Endocrine reviews, 23*(1), 90-119.

Pluijm, S., Graafmans, W., Bouter, L., & Lips, P. (1999). Ultrasound measurements for the prediction of osteoporotic fractures in elderly people. *Osteoporosis international, 9*(6), 550-556.

Pocock, G., & Richards, C. D. (2006). The control of growth, *Human Physiology: The basic of medicine* (pp. 495-510). Oxford: Oxford University Press.

Prosser, D. E., & Jones, G. (2004). Enzymes involved in the activation and inactivation of vitamin D. *Trends in biochemical sciences, 29*(12), 664-673.

Prouteau, S., Pelle, A., Collomp, K., Benhamou, L., & Courteix, D. (2006). Bone density in elite judoists and effects of weight cycling on bone metabolic balance. *Medicine and science in sports and exercise, 38*(4), 694.

Purohit, A., Newman, S. P., & Reed, M. J. (2002). The role of cytokines in regulating estrogen synthesis: implications for the etiology of breast cancer. *Breast Cancer Research, 4*(2), 65-69.

Quamme, G. A. (2008). Recent developments in intestinal magnesium absorption. *Current opinion in gastroenterology, 24*(2), 230.

Quigley, R. J., & Watts, C. (1997). *Food comes first: methodologies for the National Nutrition Survey of New Zealand: Public Health Group*, Ministry of Health.

Raggatt, L. J., & Partridge, N. C. (2010). Cellular and molecular mechanisms of bone remodeling. *Journal of Biological Chemistry, 285*(33), 25103-25108.

Reed, M., & Purohit, A. (2002). Aromatase regulation and breast cancer. *Clinical Endocrinology, 54*(5), 563-571.

Riggs, B. L., Khosla, S., & Melton, L. J. (2002). Sex steroids and the construction and conservation of the adult skeleton. *Endocrine reviews, 23*(3), 279-302.

Risteli, J., Elomaa, I., Niemi, S., Novamo, A., & Risteli, L. (1993). Radioimmunoassay for the pyridinoline cross-linked carboxy-terminal telopeptide of type I collagen: a new serum marker of bone collagen degradation. *Clinical Chemistry, 39*(4), 635-640.

Risteli, L., & Risteli, J. (1993). Biochemical markers of bone metabolism. *Annals of Medicine, 25*(4), 385-393.

Rizzoli, R. (2008). Nutrition: its role in bone health. *Best Practice & Research Clinical Endocrinology & Metabolism, 22*(5), 813-829.

Robbins, J., Aragaki, A. K., Kooperberg, C., Watts, N., Wactawski-Wende, J., Jackson, R. D., . . . Stefanick, M. L. (2007). Factors associated with 5-year risk of hip fracture in postmenopausal women. *JAMA: the journal of the American Medical Association, 298*(20), 2389-2398.

Robey, P. G. & Boskey, A. L., (2006). Extracellular matrix and biomineralization of bone. In M. J. Favus (Ed.), *Primer on the metabolic bone diseases and disorders of mineral metabolism* (pp. 12-19). Washington, DC: American Society for Bone and Mineral Research.

Robling, A. G., Castillo, A. B., & Turner, C. H. (2006). Biomechanical and molecular regulation of bone remodeling. *Annu. Rev. Biomed. Eng., 8*, 455-498.

Rockell, J. E. P., Skeaff, C., Williams, S., & Green, T. (2006). Serum 25-hydroxyvitamin D concentrations of New Zealanders aged 15 years and older. *Osteoporosis international, 17*(9), 1382-1389.

Rosen, H., Moses, A., Garber, J., Iloputaife, I., Ross, D., Lee, S., & Greenspan, S. (2000). Serum CTX: a new marker of bone resorption that shows treatment effect more often than other markers because of low coefficient of variability and large changes with bisphosphonate therapy. *Calcified tissue international, 66*(2), 100-103.

Ross, A. C., Manson, J. A. E., Abrams, S. A., Aloia, J. F., Brannon, P. M., Clinton, S. K., . . . Jones, G. (2011). The 2011 report on dietary reference intakes for calcium and vitamin D from the Institute of Medicine: what clinicians need to know. *Journal of Clinical Endocrinology & Metabolism, 96*(1), 53-58.

Ross, F. P. (2006).Osteoclast biology and bone resorption. In M. J. Favus (Ed.), *Primer on the metabolic bone diseases and disorders of mineral metabolism* (pp. 30-34). Washington, DC: American Society for Bone and Mineral Research.

Rozen, G. S., Rennert, G., Dodiuk-Gad, R. P., Rennert, H. S., Ish-Shalom, N., Diab, G., . . . Ish-Shalom, S. (2003). Calcium supplementation provides an extended window of opportunity for bone mass accretion after menarche. *The American journal of clinical nutrition, 78*(5), 993-998.

Rude, R. K., Singer, F. R., & Gruber, H. E. (2010). Skeletal and hormonal effects of magnesium deficiency. *Journal of the American College of Nutrition, 28*(2), 131.

Russell, D., Parnell, W., Wilson, N., Faed, J., Ferguson, E., Herbison, P., . . . Walker, R. (1999). *NZ food: NZ people. Key results of the 1997 National Nutrition Survey*. Wellington: Ministry of Health.

Sakuma, M., Endo, N., & Oinuma, T. (2007). Serum 25-OHD insufficiency as a risk factor for hip fracture. *Journal of bone and mineral metabolism, 25*(3), 147-150.

Saladin, K. S. (2007). Bone tissue. *Anatomy and physiology: The unity of form and function* (pp. 213-240). New York: McGraw-Hill Companies, Inc.

Sawada, N., Sakaki, T., Kitanaka, S., Takeyama, K., Kato, S., & Inouye, K. (2001). Enzymatic properties of human 25-hydroxyvitamin D3 1α-hydroxylase. *European Journal of Biochemistry, 265*(3), 950-956.

Secreto, F. J., David G. Monroe., and Spelsberg, T. C. (2006). Gonadal steroids and receptors. In M. J. Favus (Ed.), *Primer on the metabolic bone diseases and disorders of mineral metabolism* (pp. 84-89). Washington, DC: American Society for Bone and Mineral Research.

Seeman, E. (2002). Pathogenesis of bone fragility in women and men. *The Lancet, 359*(9320), 1841-1850.

Seibel, M. J. (2003). Biochemical markers of bone remodeling. *Endocrinology and metabolism clinics of North America, 32*(1), 83.

Shapses, S. A., Robins, S. P., Schwartz, E. I., & Chowdhury, H. (1995). Short-term changes in calcium but not protein intake alter the rate of bone resorption in healthy subjects as assessed by urinary pyridinium cross-link excretion. *The Journal of nutrition, 125*(11), 2814.

Shea, M. K., Gundberg, C. M., Meigs, J. B., Dallal, G. E., Saltzman, E., Yoshida, M., . . . Booth, S. L. (2009). γ-Carboxylation of osteocalcin and insulin resistance in older men and women. *The American journal of clinical nutrition, 90*(5), 1230-1235.

Silva, B., Costa, A., Cusano, N., Kousteni, S., & Bilezikian, J. (2011). Catabolic and anabolic actions of parathyroid hormone on the skeleton. *Journal of endocrinological investigation, 34*(10), 801.

Simonet, W., Lacey, D., Dunstan, C., Kelley, M., Chang, M. S., Lüthy, R., . . . Boone, T. (1997). Osteoprotegerin: a novel secreted protein involved in the regulation of bone density. *Cell, 89*(2), 309-319.

Sims, N. A., & Gooi, J. H. (2008). Bone remodeling: multiple cellular interactions required for coupling of bone formation and resorption. *Seminars in Cell & Developmental Biology, 19,* 444–451.

Slemenda, C. W., Miller, J. Z., Hui, S. L., Reister, T. K., & Johnston, C. C. (1991). Role of physical activity in the development of skeletal mass in children. *Journal of Bone and Mineral Research, 6*(11), 1227-1233.

Slemenda, C. W., Peacock, M., Hui, S., Zhou, L., & Johnston, C. C. (1997). Reduced rates of skeletal remodeling are associated with increased bone mineral density during the development of peak skeletal mass. *Journal of Bone and Mineral Research, 12*(4), 676-682.

Slootmaker, S. M., Schuit, A. J., Chinapaw, M. J. M., Seidell, J. C., & Van Mechelen, W. (2009). Disagreement in physical activity assessed by accelerometer and self-report in subgroups of age, gender, education and weight status. *International Journal of Behavioral Nutrition and Physical Activity, 6*(1), 17.

Specker, B. L. (1996). Evidence for an interaction between calcium intake and physical activity on changes in bone mineral density. *Journal of Bone and Mineral Research, 11*(10), 1539-1544.

Specker, B., & Binkley, T. (2003). Randomized trial of physical activity and calcium supplementation on bone mineral content in 3-to 5-year-old children. *Journal of Bone and Mineral Research, 18*(5), 885-892.

Specker, B., & Vukovich, M. (2007). Evidence for an interaction between exercise and nutrition for improved bone health during growth. *Medical Sport Science, 51,* 50-63.

Speich, M., Pineau, A., & Ballereau, F. (2001). Minerals, trace elements and related biological variables in athletes and during physical activity. *Clinica Chimica Acta, 312*(1), 1-11.

Stenbeck, G. (2002). Formation and function of the ruffled border in osteoclasts. *Seminars in Cell & Developmental Biology, 13*(4), 285-292.

Suda, T., Nakamura, I., Jimi, E., & Takahashi, N. (1997). Regulation of osteoclast function. *Journal of Bone and Mineral Research, 12*(6), 869-879.

Swarthout, J. T., D'Alonzo, R. C., Selvamurugan, N., & Partridge, N. C. (2002). Parathyroid hormone-dependent signaling pathways regulating genes in bone cells. *Gene, 282*(1-2), 1.

Syed, F. A., Mödder, U. I. L., Roforth, M., Hensen, I., Fraser, D. G., Peterson, J. M., . . . Khosla, S. (2010). Effects of chronic estrogen treatment on modulating age-related bone loss in female mice. *Journal of Bone and Mineral Research, 25*(11), 2438-2446.

Syed, F., & Khosla, S. (2005). Mechanisms of sex steroid effects on bone. *Biochemical and Biophysical Research Communications, 328*(3), 688-696.

Szulc, P., & Delmas, P. (2008). Biochemical markers of bone turnover: potential use in the investigation and management of postmenopausal osteoporosis. *Osteoporosis International, 19*(12), 1683-1704.

Takayanagi, H. (2005). Mechanistic insight into osteoclast differentiation in osteoimmunology. *Journal of molecular medicine, 83*(3), 170-179.

Takeda, E., Yamamoto, H., Yamanaka-Okumura, H., & Taketani, Y. (2012). Dietary phosphorus in bone health and quality of life. *Nutrition reviews, 70*(6), 311-321.

Tapiero, H., & Tew, K. D. (2003). Trace elements in human physiology and pathology: zinc and metallothioneins. *Biomedecine & Pharmacotherapy, 57*(9), 399-411.

Teitelbaum, S. L. (2000). Bone resorption by osteoclasts. *Science, 289*(5484), 1504-1508.

Teitelbaum, S. L. (2007). Osteoclasts: what do they do and how do they do it? *The American journal of pathology, 170*(2), 427-435.

Theoleyre, S., Wittrant, Y., Tat, S. K., Fortun, Y., Redini, F., & Heymann, D. (2004). The molecular triad OPG/RANK/RANKL: involvement in the orchestration of pathophysiological bone remodeling. *Cytokine & growth factor reviews, 15*(6), 457.

Thomson, C. D. (2004). Selenium and iodine intakes and status in New Zealand and Australia. *British Journal of Nutrition, 91*(5), 661-672.

Trost, S. G., Pate, R. R., Sallis, J. F., Freedson, P. S., Taylor, W. C., Dowda, M., & Sirard, J. (2002). Age and gender differences in objectively measured physical activity in youth. *Medicine and science in sports and exercise, 34*(2), 350.

Trudeau, E., Kristal, A. R., Li, S., & Patterson, R. E. (1998). Demographic and psychosocial predictors of fruit and vegetable intakes differ: implications for dietary interventions. *Journal of the American Dietetic Association, 98*(12), 1412-1417.

Tucker, K. L., Hannan, M. T., Chen, H., Cupples, L. A., Wilson, P. W. F., & Kiel, D. P. (1999). Potassium, magnesium, and fruit and vegetable intakes are associated with greater bone mineral density in elderly men and women. *The American journal of clinical nutrition, 69*(4), 727-736.

Udagawa, N., Takahashi, N., Akatsu, T., Tanaka, H., Sasaki, T., Nishihara, T., . . . Suda, T. (1990). Origin of osteoclasts: mature monocytes and macrophages are capable of differentiating into osteoclasts under a suitable microenvironment prepared by bone marrow-derived stromal cells. *Proceedings of the National Academy of Sciences, 87*(18), 7260-7264.

Uusi-Rasi, K., Sievänen, H., Pasanen, M., Oja, P., & Vuori, I. (2002). Associations of calcium intake and physical activity with bone density and size in premenopausal and postmenopausal women: a peripheral quantitative computed tomography study. *Journal of Bone and Mineral Research, 17*(3), 544-552.

Vaananen, H. K., & Horton, M. (1995). The osteoclast clear zone is a specialized cell-extracellular matrix adhesion structure. *Journal of cell science, 108*, 2729-2732.

Vaananen, H. K., & Laitala-Leinonen, T. (2008). Osteoclast lineage and function. *Archives of biochemistry and biophysics, 473*(2), 132-138.

Vaananen, H., Zhao, H., Mulari, M., & Halleen, J. M. (2000). The cell biology of osteoclast function. *Journal of cell science, 113*(3), 377-381.

Vermeer, C., Jie, K. S. G., & Knapen, M. (1995). Role of vitamin K in bone metabolism. *Annual review of nutrition, 15*(1), 1-21.

Vokes, T. J. (2006). Blood calcium, phosphate, and magnesium. In M. J. Favus (Ed.), *Primer on the metabolic bone diseases and disorders of mineral metabolism* (pp. 123-127). Washington, DC: American Society for Bone and Mineral Research.

Vormann, J. (2003). Magnesium: nutrition and metabolism. *Molecular Aspects of Medicine, 24*(1-3), 27-37.

Wardlaw, G. M. (1996). Putting body weight and osteoporosis into perspective. *The American journal of clinical nutrition, 63*(3), 433S-436S.

Wastney, M., Martin, B., Peacock, M., Smith, D., Jiang, X. Y., Jackman, L., & Weaver, C. (2000). Changes in calcium kinetics in adolescent girls induced by high calcium intake. *Journal of Clinical Endocrinology & Metabolism, 85*(12), 4470-4475.

Weatherall, M. (2000). A meta-analysis of 25 hydroxyvitamin D in older people with fracture of the proximal femur. *New Zealand medical journal, 113*(1108), 137-140.

Weaver, C. M. (2000). The growing years and prevention of osteoporosis in later life. *Proceedings of the Nutrition Society, 59*(02), 303-306.

Weaver, C., & Heaney, R. P. (2006). *Calcium in human health*: Humana Pr Inc.

Welten, D., Kemper, H., Post, G., Van Mechelen, W., Twisk, J., Lips, P., & Teule, G. (1995). Weight-bearing activity during youth is a more important factor for peak bone mass than calcium intake. *Journal of Bone and Mineral Research, 9*(7), 1089-1096.

Wesseling, K., Bakkaloglu, S., & Salusky, I. (2008). Chronic kidney disease mineral and bone disorder in children. *Pediatric Nephrology, 23*(2), 195-207.

Whitney, E., Rolfes, S. R., Crowe, T., Cameron-Smith, D., & Walsh, A. (2011). *Understanding nutrition: Australian and New Zealand edition*: Cengage Learning Australia Pty Ltd.

Wicherts, I. S., van Schoor, N. M., Boeke, A. J. P., Visser, M., Deeg, D. J. H., Smit, J., . . . Lips, P. (2007). Vitamin D status predicts physical performance and its decline in older persons. *Journal of Clinical Endocrinology & Metabolism, 92*(6), 2058-2065.

Wilson, P., & Horwath, C. (1996). Validation of a short food frequency questionnaire for assessment of dietary calcium intake in women. *European Journal of Clinical Nutrition, 50*(4), 220-228.

Winzenberg, T., Oldenburg, B., Frendin, S., De Wit, L., Riley, M., & Jones, G. (2006b). The effect on behavior and bone mineral density of individualized bone mineral density feedback and educational interventions in premenopausal women: a randomized controlled trial [NCT00273260]. *BMC public health, 6*(1), 12.

Winzenberg, T., Shaw, K., Fryer, J., & Jones, G. (2006a). Effects of calcium supplementation on bone density in healthy children: meta-analysis of randomised controlled trials. *Bmj, 333*(7572), 775.

Woo, J., Lau, W., Xu, L., Lam, C. W. K., Zhao, X., Yu, W., . . . Pocock, N. (2007). Milk supplementation and bone health in young adult Chinese women. *Journal of Women's Health, 16*(5), 692-702.

World Health Organization. (2003). *Prevention and management of osteoporosis: report of a WHO scientific group* (Vol. 921): World Health Organization.

Yamaguchi, M. (1998). Role of zinc in bone formation and bone resorption. *The Journal of Trace Elements in Experimental Medicine, 11*(2-3), 119-135.

Yang, S., Zhang, Y., Rodriguiz, R., Ries, W., & Key, L. (1996). Functions of the M-CSF receptor on osteoclasts. *Bone, 18*(4), 355-360.

Yasuda, H., Shima, N., Nakagawa, N., Yamaguchi, K., Kinosaki, M., Mochizuki, S., . . . Murakami, A. (1998). Osteoclast differentiation factor is a ligand for osteoprotegerin/osteoclastogenesis-inhibitory factor and is identical to TRANCE/RANKL. *Proceedings of the National Academy of Sciences, 95*(7), 3597-3602.

Yoon, V., Maalouf, N., & Sakhaee, K. (2012). The effects of smoking on bone metabolism. *Osteoporosis international*, 1-12.

Ypey, D. L., Weidema, A. F., Höld, K. M., Van Der Laarse, A., Ravesloot, J. H., Van Der Plas, A., & Nijweide, P. J. (1992). Voltage, calcium, and stretch activated ionic channels and intracellular calcium in bone cells. *Journal of Bone and Mineral Research, 7*(S2), S377-S387.

Zhu, K., Du, X., Cowell, C. T., Greenfield, H., Blades, B., Dobbins, T. A., . . . Fraser, D. R. (2005). Effects of school milk intervention on cortical bone accretion and indicators relevant to bone metabolism in Chinese girls aged 10–12 y in Beijing. *The American journal of clinical nutrition, 81*(5), 1168-1175.

Appendices 1 – 5

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Appendix 1

Participant information Sheet

MASSEY UNIVERSITY

Does a supplemented milk drink affect markers of osteoarthritis?

INFORMATION SHEET

Who are we?
The Institute of Food, Nutrition and Human Health is part of Massey University. Our role is to carry out research into the links between food and human health and in the development of new food products. **This study is funded by Fonterra Brands AME Ltd.**

Why are we doing this trial?
Osteoarthritis (OA) is the most common type of arthritis with degenerative changes of the joint and is a leading cause of chronic disability. Although OA usually affects adults from middle age, people that subject their joints to repetitive impact and torsional loading increase the risk of articular cartilage degeneration, which may result in the development of OA.

To date, there is no cure for OA. The only available treatments aim at reducing symptoms, such as pain and inflammation, maintaining joint mobility and limiting the loss of function. Progression of OA can be assessed using one of a number of pain and function scoring systems. Recently biomarkers in the blood and urine, thought to pick up markers originating from cartilage, bone and synovial breakdown, are being used to assess joint health. These biomarkers have also been used in studies to assess whether supplementation with over the counter products for joint health, such as Glucosamine, has an effect on OA.

In this study we want to assess in a small group of **active** younger women, whether the use of cartilage & bone biomarkers, is an effective way of assessing joint health. We want to compare the effects of daily milk drinks supplemented with two different doses of glucosamine, either 0.5g or 1.5g, or a dose of 2g of collagen on cartilage and bone biomarker responses and mobility scores.

Glucosamine is an amino sugar and used for synthesis of glycosylated proteins and lipids. Glucosamine may prevent loss of cartilage in the knee joints as osteoarthritis develops and is mildly anti-inflammatory. In the US it is one of the most common non-vitamin, non-mineral, dietary supplements used by adults. Glucosamine is marketed to support the structure and function of joints and the marketing is targeted to people suffering from osteoarthritis. Collagen hydrolysate is produced by enzymatic breakdown of collagenous tissue from raw materials such as fish or animal skins. Studies using cell culture have shown that collagen fragments, such as those contained in collagen hydrolysate, may help maintain cartilage and protect the joints. The source of collagen for this study is fish skin.

The milk we give you can be used to replace milk you usually have in your breakfast, but not in your tea or coffee.

This study will have 45 participants. You will be asked to come into the Human Nutrition Lab at Massey before 10am, after fasting since 9pm the night before, to have blood samples taken on

6 occasions, and to answer questionnaires on 4 occasions over an 18 week period. You may also be asked if you would wear an accelerometer for 7 days which will give an indication of intensity of your activity.

Would you like to take part

We would like to invite 45 Caucasian females, aged 30 to 45 years to join our study. To fit in to our study you should:

- Be active, run or walk for 30 minutes at least three times per week
- Be a non smoker
- Be premenopausal
- Have no history of metabolic bone disease or any systemic disease that may affect bone density
- Not have had formal diagnosis of osteoarthritis or rheumatoid arthritis
- Not have diabetes mellitus, either diagnosed or based on an elevated glucose level at screening
- Not have kidney impairment
- Not have any liver impairment
- Have not had a bone fracture in the last 6 months
- Not be taking any antacids, colostrum, vitamin or mineral supplements, or supplements associated specifically with joint health eg green lipped muscle, omega 3, glucoamine or condroitin
- Not exceed a moderate alcohol intake (2 units of alcohol per day)
- Not have an endocrine disease
- Not be taking any medication which will affect the study, not have a milk allergy, be intolerant to milk or be unable to confirm that they can take 2 glasses of milk per day

When the study is complete we will send you a summary of the findings of our research, if you would like one.

Will I get any financial compensation?

We will give you retail vouchers of $200 to compensate you for your inconvenience and time.

If you are interested in taking part please contact Chris Booth who will be happy to discuss the project and answer your questions.

Contact details:

Mrs Chris Booth
Human Nutrition Studies Laboratory
Institute of Food, Nutrition and Human Health
Massey University
Private Bag 11222
Palmerston North
Telephone: 06-350-5901 (or 0800 0800 28)
Fax: 06-350-5446
e-mail: **c.l.booth@massey.ac.nz**

Professor Marlena Kruger
Institute of Food, Nutrition and Human Health
Massey University
Private Bag 11222
Palmerston North
Telephone: 06-350-5905
Fax: 06-350-5446
e-mail: **m.c.kruger@massey.ac.nz**

What is involved?

We will ask you to go to Medlab to have a screening blood sample before we start the study and to fill in questionnaires about your health and the foods you eat. We will also ask you to come into the Human Nutrition Laboratory to have a Dexa scan of your bones and some anthropometric measurements. The whole of the study period is for 16 weeks after the initial screening.

When the study starts we will ask you to come to our lab between 8.00am and 10am to have blood samples taken six times. These visits will fortnightly for the first 6 weeks and then every fourth week for the next twelve weeks. There will be a total of six visits. You will also be asked

to complete a food frequency questionnaire to assess your calcium and vitamin D intake, to complete a 3 day food recall and to complete a physical activity questionnaire. You can complete these questionnaires at home in your own time.

On each of your visits a trained nurse will take a blood sample via venepuncture from a vein in your forearm.
The total amount of your time that the whole trial will take is approximately 8 hours. In regard of the time and commitment involved in this study we will give you vouchers to the value of $200.

A schedule of measurements is given below:

Time	Measurements
Week -6 Screening **Medlab visit for fasting blood sample** **Visit Human Nutrition laboratory, Massey University for Dexa scan, anthropometric measurements and completion of 24 hour food recall and health questionnaires.**	 Screening blood sample taken Dexa scan
Week - 4 **Visit Human Nutrition laboratory**	Blood and urine samples collected
Week - 2 **Visit Human Nutrition laboratory**	Blood and urine samples collected
Week zero **Visit Human Nutrition laboratory**	Food frequency to assess calcium and vitamin D intake, a 3 day food recall as well as physical activity questionnaire. Blood and urine samples collected Milk powder dispensed
Week + 4 **Visit Human Nutrition laboratory**	Blood and urine samples collected Milk powder dispensed and returns counted
Week + 8 **Visit Human Nutrition laboratory**	Blood and urine samples collected Milk powder dispensed and returns counted
Week + 12 **Visit Human Nutrition laboratory**	Blood and urine samples collected Milk powder returns counted 24 hour food recall Anthropometric measurements will be taken

In addition to the above, you will be asked if you would volunteer to wear an accelerometer to the intensity of your physical activity.

What are we going to measure?
Medical assessment before you start the study**:**
We will ask you questions about your health and current medications. The health screening blood sample taken by Medlab will be analysed for full blood count, liver function, kidney function, electrolytes, total protein, serum albumin, glucose, insulin, and serum calcium.

Body measurements:
We will measure your height and weight to estimate your body mass index. These measurements will be conducted in private. Body weight will be measured using ordinary weighing scales (you will be asked to remove your shoes and outer clothes) and standing height will be measured using a wall measure. We will also measure your waist and hip circumference using a tape measure.

Blood & Urine
During each of your lab visits we will take 15 mL of blood.
We will ask you to collect a urine sample when you visit the lab.

We shall use the blood to assess the levels of the cartilage markers; Collagen type II cleavage, Procollagen II C-peptide, and Cartilage Oligomeric Matrix protein, bone markers; C- terminal telopeptide of type I collagen and Osteocalcin, **25 (OH) D3** and an inflammation marker, interleukin-6. The urine will be analysed by Elisa for C- terminal telopeptide of type II collagen and creatinine. The creatinine and bone markers will be analysed at Canterbury Health Laboratories with the balance being analysed in our laboratory using commercial Elisa kits. Not all the blood will be used for the tests above. We will freeze a small amount in case we need to repeat any of these tests. We will ask for your permission before we do this. You can request that any unused blood is returned to you when the study is completed.

Intensity of physical activity

You may be asked to volunteer that the intensity of your activity be assessed. An Accelerometer will be used in conjunction with heart rate monitor to monitor physical activity over 7 days. You may be asked to wear the activity and heart rate monitors for at least 10 h per day over 7 consecutive days. The accelerometer is worn at the waist, over or under clothing, attached to an elastic strap or can easily be attached to the participants own belt. The heart rate monitor is worn directly against the skin around the chest, just below the chest muscles. The heart rate monitor is attached to an elastic strap that may be adjusted so that the monitor fits snugly and comfortably. You will be given individual instruction on the placement of these devices. The accelerometer will give an indication of your activity along with your heart rate which will indicate the intensity of your activity over 10 hour days.

Figure of how the accelerometer is fitted to the body.

Are any of the procedures harmful or painful?

Having a blood sample taken via venepuncture can be painful and to minimise this we employ a trained nurse who is experienced in this procedure and follows best practice guidelines. There is a small chance of bruising, haematoma, or sepsis around the site of placement following the venepuncture. Volunteers also have access to the City Doctors if they develop problems from the testing or have side effects. The costs for such a visit will be covered by the researchers.

Who will see the information about me?
When you join the trial you will be given a number and thereafter all information will be filed with the code number, and stored in a locked filing cabinet accessed by the research team only. When information from all the volunteers has been pooled, and made anonymous, it will be used in presentations to academic societies, scientific publications and reports to the funders, Fonterra Ingredients NZ Ltd. No names will be used, just the designated numbers. All personal

data will be destroyed at the end of the trial. Scientific data, filed on paper, will be shredded and electronic data will be deleted from our computer records and databases after 10 years. For the first 5 years it will be stored in a locked filing cupboard within a locked office. For the last 5 years it will be stored in a secure archive where all data is stored in boxes labelled by barcode only. It is accessible by nominated staff only, who require pin numbers for ID.

If anything untoward is found in your tests you will be contacted by Prof Marlena Kruger, informed of the results and asked whether you would like the results to be given to your medical practitioner or sent directly to you.

Who is funding this research?
This research is funded by Fonterra Brands AME Ltd.

Compensation for Injury

If physical injury results from your participation in this study, you should visit a treatment provider to make a claim to ACC as soon as possible. ACC cover and entitlements are not automatic and your claim will be assessed by ACC in accordance with the Accident Compensation Act 2001. If your claim is accepted, ACC must inform you of your entitlements, and must help you access those entitlements. Entitlements may include, but not be limited to, treatment costs, travel costs for rehabilitation, loss of earnings, and/or lump sum for permanent impairment. Compensation for mental trauma may also be included, but only if this is incurred as a result of physical injury.

If your ACC claim is not accepted you should immediately contact the researcher. The researcher will initiate processes to ensure you receive compensation equivalent to that to which you would have been entitled had ACC accepted your claim from Massey University.

What are my rights?
You are under no obligation to accept this invitation. If you decide to participate, you have the right to:

- *decline to answer any particular question;*
- *withdraw from the study (specify timeframe);*
- *ask any questions about the study at any time during participation;*
- *provide information on the understanding that your name will not be used unless you give permission to the researcher;*
- *be given access to a summary of the project findings when it is concluded.*

If you would like to participate in this study please call Chris Booth on 0800 0800 28

This project has been reviewed and approved by the Massey University Human Ethics Committee:Southern A, Application (11/77). If you have any concerns about the conduct of this research, please contact A/Prof Hugh Morton, Chair, Massey University Human Ethics Committee: Southern A, telephone 06 350 5799, 4265.
email humanethicsoutha@massey.ac.nz

Appendix 2

3-day food record

MASSEY UNIVERSITY

3 Day Food Record

Thank you very much for taking part in the Study. We are extremely grateful for your time, effort and commitment!

If you have any questions, please contact Rifana Cholidah on 022-1210780 or email

rifanacholidah@yahoo.com

All information in this diary will be treated with the strictest confidence. No one outside the study will have access to this.

Please bring this diary with you when you return for your next appointment

3 day food diary - What to do?

- Record all that you eat and drink on the following dates:

_____

_____

_____

- If possible record food at the time of eating or just after – try to avoid doing it from memory at the end of the day.

- Include all meals, snacks, and drinks, even tap water.

- Include anything you have added to foods such as sauces, gravies, spreads, dressings, etc.

- Write down any information that might indicate size or weight of the food to identify the portion size eaten.

- Use a new line for each food and drink. You can use more than one line for a food or drink. See the examples given.

- Use as many pages of the booklet as you need.

Describing Food and Drink

- Provide as much detail as possible about the type of food eaten. For example **brand names and varieties / types** of food.

General description	**Food record description**
Breakfast example – cereal, milk, sugar	1 cup Sanitarium Natural Muesli 1 cup Pam's whole milk 1 tsp Chelsea white sugar
Coffee	1 tsp Gregg's instant coffee 1 x 200ml cup of water 2 Tbsp Meadow fresh light green milk
Pasta	1 cup San Remo whole grain pasta

	spirals (boiled)
Pie	Big Ben Classic Mince and Cheese Pie (170g)

- Give details of all the **cooking methods** used. For example, fried (sort of oil/fat used), grilled, baked, poached, boiled…

General description	**Food record description**
2 eggs	2 size 7 eggs fried in 2tsp canola oil 2 size 6 eggs (soft boiled)
Fish	100g salmon (no skin) poached in 1 cup of water for 10 minutes

- When using foods that are cooked (eg. pasta, rice, meat, vegetables, etc), please record the **cooked portion** of food.

General description	**Food record description**
Rice	1 cup cooked Jasmine rice (cooked on stove top)
Meat	90g lean T-bone steak (fat and bone removed)
Vegetables	½ cup cooked mixed vegetables (Wattie's peas, corn, carrots)

- Please specify the **actual amount of food eaten** (eg. for leftovers, foods where there is waste)

General description	**Food record description**
Apple	1 x 120g Granny Smith Apple (peeled, core not eaten – core equated to ¼ of the apple)
Fried chicken drumstick	100g chicken drumstick (100g includes skin and bone); fried in 3 Tbsp Fern leaf semi-soft butter

- Because we are especially interested in your calcium intake, please take care to list **all** the milk you consume, and record what type of milk it was.

General description	Food record description
hot chocolate	I x cup hot chocolate made with Cadbury's powder and 150 mls Anchor Calcitrim milk, 100 ml hot water. No sugar

- **Record recipes** of home prepared dishes where possible: record how many the recipe fed and the proportion of the dish you ate. There are blank pages for you to add recipes or additional information.

Recording the amounts of food you eat

It is important to also record the quantity of each food and drink consumed. This can be done in several ways.

- By using household measures – for example, cups, teaspoons and tablespoons. Eg. 1 cup frozen peas, 1 heaped teaspoon of sugar.

- By weight marked on the packages – eg. a 425g tin of baked beans, a 32g cereal bar, 600ml Coke

- Weighing the food – this is an ideal way to get an accurate idea of the quantity of food eaten, in particular for foods such as meat, fruits, vegetables and cheese.

- For bread – describe the size of the slices of bread (eg. sandwich, medium, toast) – also include brand and variety.

- Using comparisons – eg. Meat equal to the size of a pack of cards, a scoop of ice cream equal to the size of a hen's egg.

- Use the food record instructions provided to help describe portion sizes.

General description	**Food record description**
Cheese (Edam)	1 heaped tablespoon of grated cheese 1 slice cheese (8.5 x 2.5 x 2mm) 1 cube cheese, match box size Grated cheese, size 10B

- If you go out for meals, describe the food eaten in as much detail as possible.

- ***Please eat as normally as possible - don't adjust what you would normally eat just because you are keeping a diet record and be honest! Your food record will be identified with a number rather than your name.***

Example day

Time food was eaten	Complete description of food (food and beverage name, brand, variety, preparation method)	Amount consumed (units, measures, weight)
Example7: 55am	Sanitarium weetbix	2 weetbix
" "	Anchor Blue Top milk	150ml
" "	Chelsea white sugar	2 heaped teaspoons
" "	Orange juice (Citrus Tree with added calcium – nutrition label attached)	1 glass (275 ml)
10.00am	Raw Apple (gala)	Ate all of apple except the core, whole apple was 125g (core was ¼ of whole apple)
12.00pm	Home made pizza (recipe attached)	1 slice (similar size to 1 slice of sandwich bread, 2 Tbsp tomato paste, 4 olives, 2 rashers bacon (fat removed), 1 Tbsp chopped spring onion, 3 Tbsp mozzarella cheese)
1.00pm	Water	500ml plain tap water
3.00pm	Biscuits	6 x chocolate covered Girl Guide biscuits (standard size)
6.00pm	Lasagne	½ cup cooked mince, 1 cup cooked Budget lasagne shaped pasta , ½ cup Wattie's creamy mushroom and herb pasta sauce, ½ cup mixed vegetables (Pam's carrots, peas and corn), 4 Tbsp grated Edam cheese
6.30pm	Banana cake with chocolate icing (homemade, recipe attached)	1/8 of a cake (22cm diameter, 8 cm high), 2 Tbsp chocolate icing
" "	Tip Top Cookies and Cream ice cream	1 cup (250g)
7.30pm	Coffee	1 tsp Gregg's instant coffee 1 x 300ml cup of water 2 Tbsp Meadow fresh blue top milk 2 tsp sugar

Date: **DAY 1**

Time food was eaten	Complete description of food (food and beverage name, brand, variety, preparation method)	Amount consumed

Date: **Day 1 continued**

Time food was eaten	Complete description of food (food and beverage name, brand, variety, preparation method)	Amount consumed

Recipes (Day 1)

Date: **DAY 2**

Time food was eaten	Complete description of food (food and beverage name, brand, variety, preparation method)	Amount consumed

Date: **Day 2 Continued**

Time food was eaten	Complete description of food (food and beverage name, brand, variety, preparation method)	Amount consumed

Recipes (Day 2)

Date: **DAY 3**

Time food was eaten	Complete description of food (food and beverage name, brand, variety, preparation method)	Amount consumed

Date: **Day 3 continued**

Time food was eaten	Complete description of food (food and beverage name, brand, variety, preparation method)	Amount consumed

Recipes (Day 3)

Appendix 3

Food Frequency Questionnaire

FOOD FREQUENCY QUESTIONNAIRE

Massey University

Institute of Food, Nutrition & Human Health

EATING PATTERN & FOOD FREQUENCY QUESTIONNAIRE

1. Please describe your **usual** eating pattern (please mark one only):

	EATING PATTERN	Please tick one
	Eat a variety of foods, including animal products	
	Eat eggs, dairy products, fish and chicken but avoid other meats	
	Eat eggs and dairy products but avoid all meats and fish	
	Eat eggs but avoid dairy products, all meats and fish	
	Eat dairy products but avoid eggs, all meats and fish	
	Eat no animal products	
	Other – please specify:	

2. **On average**, how many servings of <u>fruit</u> (fresh, frozen, canned or stewed) do you eat per day?

   Do not include fruit juice or dried fruit.

(a "serving" = 1 medium piece or 2 small pieces or ½ cup of stewed fruit)

	FRUIT Per Day	Please tick one
	I don't eat fruit	
	Less than 1 per day	

	1 serving	
	2 servings	
	3 servings	
	4 or more servings	

3. **On average**, how many servings of <u>vegetables</u> (fresh, frozen, canned) do you eat per day?

   Do not include vegetable juices.

   (a "serving" = 1 medium potato/kumara **or** ½ cup of cooked vegetables **or** 1 cup of salad vegetables)

	VEGETABLES Per Day	Please tick one
	I don't eat vegetables	
	Less than 1 per day	
	1 serving	
	2 servings	
	3 servings	
	4 or more servings	

4. **On average**, how many slices or rolls of <u>bread or toast</u> do you eat per day?

	BREADS Per Day	Please tick one
	I don't eat bread or toast ***Go to question 6***	
	Less than 1 per day	
	1 – 2	
	3 – 4	
	5 – 6	

	7 or more	

5. What type(s) of bread, rolls or toast do you eat most often?

(please only mark those you usually eat)

	BREADS Usual types	Please tick one
	White	
	White – high fibre	
	Wholemeal or wholegrain	
	Other – please specify:	

6. Do you put butter or margarine on bread or crackers?

If **no, go to question 7.**

If yes, what type(s) do you use most often?

		Please tick
	Butter	
	Unsalted butter	
	Butter and margarine blend	
	Low salt margarine	
	Polyunsaturated margarine eg “Miracle”, “Sunflower” etc	
	Reduced fat margarine eg “Slimarine”	
	“Praise” or “Olivio” margarine	
	Other – please specify:	

7. Do you usually eat breakfast cereal?

If **no – please go to question 8**

If yes – what breakfast cereal(s) do you have most often?

		Please tick one
	Weetbix	
	Cornflakes or rice bubbles	
	Toasted muesli	
	Untoasted muesli	
	Special K	
	Ricies	
	All-Bran, San-Bran, Bran flakes, of Weetbix “Hi-Bran”	
	Puffed Wheat or mini wheats	
	Porridge	
	Other – please specify:	

8. Milk - do you use or drink any type of milk? Yes/No

If **no – please go to question 9.**

If yes, what type do you have most often?

	MILK	Please tick
	Standard, homogenised milk (dark blue top)	
	Trim milk (green top)	
	Super Trim	
	Skim milk or low fat milk powder	
	Semi-skimmed milk (light blue top)	
	Whole or powdered whole milk (silver top)	
	'Calci-Trim' (yellow top)	
	'Slim and Fit'	
	Soy milk	
	Other – please specify:	

9. Do you eat eggs?

If **no – please go to question 10.**

If yes – how many eggs do you usually eat **per week** (do not count eggs used in baking etc).

	EGGS Per Week	Please tick one
	Less than 1 per week	
	1 egg	
	2 eggs	
	3 eggs	
	4 eggs	
	5 or more eggs per week	

10. DAIRY FOODS

How often do you usually eat these foods or drinks?

		Never	Less than once a month	1-3 times a month	Once a week	3-4 times a week	5-6 times a week	Once a day	2 or more times a day	
	Milk as a drink									
	Milk on breakfast cereals									
	Milk added to hot beverages made with water (eg coffee, tea, Milo)									
	Hot beverages made with milk (eg Milo, cocoa, hot chocolate drinks)									
	Flavoured milk (eg milkshake, iced coffee)									
	Cream or sour cream									
	Ice cream									
	Custard or milk based sauces									
	Yoghurt, plain or flavoured (including fromage frais)									
	Milk puddings (eg rice, semolina, instant)									
	Cream cheese									
	Cottage or ricotta cheese									
	Mozzarella, feta or camembert									
	Edam or gouda cheese									
	Colby, mild, tasty cheese, Cheddar									
	Brie, blue and other specialty cheeses									

11. BREADS & CEREAL FOOD

How often do you usually eat these foods?

		Never	Less than once a month	1-3 times a month	Once a week	3-4 times a week	5-6 times a week	Once a day	2 or more times a day	
	Focaccia, bagel, pita or other specialty bread									
	Crumpet, croissant, waffle, doughnut, fruit- or iced-bun, scone, pikelet, muffin, slice of cake or similar									
	Savoury or dry biscuits, crispbread or crackers									
	Rice (brown or white)									
	Pasta eg spaghetti, ravioli, macaroni, noodles									

12. MEAT & FISH

How often do you usually eat these foods?

		Never	Less than once a month	1-3 times a month	Once a week	3-4 times a week	5-6 times a week	Once a day	2 or more times a day	
	Beef, veal, hogget, lamb or pork dishes *include sausages, mince dishes (eg shepherds pie), mixed dishes (eg casserole, stir fry) and food eaten out (eg MacDonalds and takeaways)*									
	Chicken and other poultry dishes (eg turkey or duck)									
	Other meat eg venison, mutton bird									
	Bacon or ham, luncheon meats, salami or brawn, meat fillings in sandwiches									
	Liver (including pate)									
	Other offal (eg kidneys)									
	White fish dishes eg cod, roughy, etc *include battered fish, fish fingers, fish cakes etc*									
	Oily fish dishes eg sardine, tunas and salmon									
	Canned fish: please specify:									
	Shellfish and other sea food (eg mussels, oyster, paua, kina, pipis) or crab or prawns									

13. VEGETABLES (including fresh, frozen, canned)

How often do you usually eat these foods?

		Never	Less than once a month	1-3 times a month	Once a week	3-4 times a week	5-6 times a week	Once a day	2 or more times a day	
	Root vegetables eg potato, kumara, turnip, swede, parsnips, carrots, beetroot – *boiled, mashed, baked, roasted or as chips or fries*									
	Peas, beans, sweets corn, mixed veg, lentils, baked beans									
	Green leafy vegetables eg kale, spinach, silver beet, puha, watercress, mustard greens, Taro leaf (eg palusami), Whitloof, Karengo (seaweed)									
	Brussels sprouts, cabbage, cauliflower, coleslaw, broccoli, broccoflower									
	Lettuce or green salad									
	Onions or leeks									
	Mushrooms									
	Tomatoes									
	Sprouts (eg alfalfa, mung)									
	Soybeans, tofu, Taro									
	Green bananas (plantain)									
	Courgette/zucchini, marrow, cucumber, eggplant, squash, kamo kamo									
	Capsicum or peppers,									

14. FRUIT (fresh, frozen, stewed or canned)

How often do you usually eat these foods?

		Never	Less than once a month	1-3 times a month	Once a week	3-4 times a week	5-6 times a week	Once a day	2 or more times a day	
	Banana									
	Apple or pear									
	Oranges, mandarins, tangelos or other citrus fruit									
	Kiwifruit									
	Nectarine, peach, plum or apricot									
	Blackcurrants									
	Strawberries or other berries or cherries									
	Grapes									
	Raisins, sultanas, currants or other dried fruit (eg apricots, prunes, dates)									
	Mango, paw-paw, persimmon, feijoa, tamarillo, melon, pineapple or other fruit									

15. MISCELLANEOUS

How often do you usually eat these foods?

		Never	Less than once a month	1-3 times a month	Once a week	3-4 times a week	5-6 times a week	Once a day	2 or more times a day	
	Sweet pies or sweet pastries									
	Other puddings or desserts (not milk-based)									
	Biscuits -plain sweet, cream-filled or chocolate biscuits									
	Canned or packet soup (in winter)									
	Home-made soup (in winter)									
	Pizza									
	Muesli bars									
	Chocolate (including chocolate bars eg Moro bars)									
	Other confectionery									
	Jam, honey, marmalade or syrup									
	Peanut butter, other nut spreads									
	Vegemite or marmite									
	Nuts									
	Potato crisps, corn chips, Twisties etc									

16. DRINKS – *Note that the possible categories have changed*

		Never or rarely	3 times a month or less	1-2 times per week	3-6 times per week	1-2 times per day	3-5 times per day	6 or more times per day	
	Fruit juice eg fresh orange juice, Just Juice, Fresh-up, Robinson's, Rio Gold etc								
	Fortified juice: please specify type								
	Vegetable juice (eg tomato juice)								
	Fruit drink eg Choice, Rio Splice etc								
	Powdered drink (eg Raro, Vita-fresh)								
	Cordials/squashes including low calorie cordials								
	Carbonated drinks including low calorie drinks (eg Coke, lemonade, diet Sprite)								
	Sport's drinks (eg Gatorade, Powderade)								
	'Energy' drinks eg V, E2, Red Bull								
	Water (including unflavoured mineral water, soda water, tap water)								
	Coffee								
	Coffee – decaffeinated								
	Coffee substitute (eg Inka)								
	Milo, Cocoa, koko								
	Tea								
	Herbal tea								
	Soy beverages								
	Beer – low alcohol								
	Beer – ordinary								
	Red wine								
	White wine or champagne/sparkling wine								
	Wine cooler								
	Sparkling grape juice								
	Sherry or port								
	Spirits, liqueurs								

Appendix 4

NZPAQ-Short Form

New Zealand Physical Activity Questionnaire – Short Form (Version 1)

'I am going to ask you about the time you spent being physically active in the last 7 days, from last xxx to yesterday. Do not include activity undertaken today.

By 'active' I mean doing anything using your muscles.

'Think about activities at work, school or home, getting from place to place, and any activities you did for exercise, sport, recreation or leisure.

'I will ask you separately about brisk walking, moderate activities, and vigorous activities.'

Start Time:

Ask questions 1–7 (8 is optional)

Walking

1. During the last 7 days, on how many days did you ***walk at a brisk pace*** – a brisk pace is a pace at which you are breathing harder than normal? This includes walking at work or school, while getting from place to place, at home and at any activities that you did solely for recreation, sport, exercise or leisure.

   Think *only* about brisk walking done for at least 10 minutes at a time.

   ☐ _____ days per week (GO TO 2)

   ☐ None (GO TO 3)

2. How much time did you typically spend walking at a brisk pace on **each** of those days?

   ☐ _____ hours _____ minutes

Moderate physical activity

3. During the last 7 days, on how many days did you do **moderate** physical activities? 'Moderate' activities make you breathe harder than normal, **but only a little** – like carrying light loads, bicycling at a regular pace, or other activities like those on this card ***(Showcard 1 – Moderate Physical Activity)***. Do not include walking of any kind.

   Think *only* about those physical activities done for at least 10 minutes at a time.

   ☐ _____ days per week (GO TO 4)

   ☐ None (GO TO 5)

4. How much time did you typically spend on **each** of those days doing moderate physical activities?

   ☐ _____ hours _____ minutes

Vigorous physical activity

5. During the last 7 days, on how many days did you do **vigorous** physical activities? 'Vigorous' activities make you breathe **a lot** harder than normal ('huff and puff') – like heavy lifting, digging, aerobics, fast bicycling, or other activities like those shown on this card ***(Showcard 2 – Vigorous Physical Activity)***?

   Think only about those physical activities done for at least 10 minutes at a time.

   ☐ _____ days per week (GO TO 6)

   ☐ None (GO TO 7)

6. How much time did you typically spend on **each** of those days doing vigorous physical activities?

   ☐ _____ hours _____ minutes

Frequency of Activity

7. Thinking about all your activities over the last 7 days (including brisk walking), on how many days did you engage in:
   - At least 30 minutes of moderate activity (including brisk walking) that made you breathe a little harder than normal, OR
   - At least 15 minutes of vigorous activity that made you breathe a lot harder than normal ('huff and puff')?

   ☐ _____ days per week

   ☐ None

Stage of Change

Note: This question is optional

8. Describe your regular physical activity over the past six months. Regular physical activity means at least 15 minutes of vigorous activity (makes you 'huff and puff') or 30 minutes of moderate activity (makes you breathe slightly harder than normal) each day for 5 or more days each week. Include brisk walking.

   ☐ I am not regularly physically active and do not intend to be so in the next 6 months

   ☐ I am not regularly physically active but am thinking about starting in the next 6 months

   ☐ I do some physical activity but not enough to meet the description of regular physical activity

   ☐ I am regularly physically active but only began in the last 6 months

   ☐ I am regularly physically active and have been so for longer than 6 months

Finish Time:

Notes:

NZPAQ - Short Form Showcards

Showcard 1: Moderate Physical Activity

Carrying light loads	
Electrical work	Badminton (social)
Farming	Ballroom dancing
Heavy gardening (digging, weeding, raking, planting, pruning, clearing section)	Bowls (indoor, outdoor/lawn)
Heavy cleaning (sweeping, cleaning windows, moving furniture)	Cricket (outdoors – batting and bowling)
House renovation	Cycling (recreational – less than 15 km/hr – not mountain biking)
Machine tooling (operating lathe, punch press, drilling, welding)	Deer hunting
Lawn mowing (manual mower)	Doubles tennis
Plastering	Exercising at home (not gym)
Plumbing	Golf
	Horse riding/equestrian
Kapa haka practice	Kayaking – slow
Waiata-a-ringa	Skate boarding
	Surfing/body boarding
	Yachting/sailing/dingy sailing

Showcard 2: Vigorous Physical Activity

Carrying heavy loads	Boxing
Forestry	Aerobics
Heavy construction	Kayaking – fast
Digging ditches	Athletics (track and field)
Chopping or sawing wood	Aquarobics
	Skiing
Taiaha	Badminton (competitive)
Haka	Basketball
	Mountain biking
Soccer	Cricket – indoors (batting and bowling)
Rowing	Cycling – competitive
Rubgy League	Cycling – recreational (not mountain biking) – more than 15 km/hr
Rugby Union	Rock climbing
Hockey	Exercise classes / going to the gym (other than for aerobics) / weight training
Race walking	Netball
Running/jogging/cross country	Judo, karate, other martial arts
Table tennis (competitive)	Softball (running and pitching only)
Singles tennis	Squash
Touch rugby	Surf life saving
Tramping	Swimming – competitive
Triathlon	Waterpolo
Volleyball	

Appendix 5

Compliance and medical diary

MASSEY UNIVERSITY

ID	day	date	medication taken	taste / flavour	test drink morning	test drink night
Week 1	Monday					
Week 1	Tuesday					
Week 1	Wednesday					
Week 1	Thusday					
Week 1	Friday					
Week 1	Saturday					
Week 1	Sunday					
Week 2	Monday					
Week 2	Tuesday					
Week 2	Wednesday					
Week 2	Thusday					
Week 2	Friday					
Week 2	Saturday					
Week 2	Sunday					
Week 3	Monday					
Week 3	Tuesday					
Week 3	Wednesday					
Week 3	Thusday					
Week 3	Friday					
Week 3	Saturday					
Week 3	Sunday					
Week 4	Monday					
Week 4	Tuesday					
Week 4	Wednesday					
Week 4	Thusday					
Week 4	Friday					
Week 4	Saturday					
Week 4	Sunday					

MASSEY UNIVERSITY

ID	day	date	medication taken	taste / flavour	test drnk morning	test drink night
Week 5	Monday					
Week 5	Tuesday					
Week 5	Wednesday					
Week 5	Thusday					
Week 5	Friday					
Week 5	Saturday					
Week 5	Sunday					
Week 6	Monday					
Week 6	Tuesday					
Week 6	Wednesday					
Week 6	Thusday					
Week 6	Friday					
Week 6	Saturday					
Week 6	Sunday					
Week 7	Monday					
Week 7	Tuesday					
Week 7	Wednesday					
Week 7	Thusday					
Week 7	Friday					
Week 7	Saturday					
Week 7	Sunday					
Week 8	Monday					
Week 8	Tuesday					
Week 8	Wednesday					
Week 8	Thusday					
Week 8	Friday					
Week 8	Saturday					
Week 8	Sunday					

MASSEY UNIVERSITY

ID	day	date	medication taken	taste / flavour	test drink morning	test drink night
Week 9	Monday					
Week 9	Tuesday					
Week 9	Wednesday					
Week 9	Thusday					
Week 9	Friday					
Week 9	Saturday					
Week 9	Sunday					
Week 10	Monday					
Week 10	Tuesday					
Week 10	Wednesday					
Week 10	Thusday					
Week 10	Friday					
Week 10	Saturday					
Week 10	Sunday					
Week 11	Monday					
Week 11	Tuesday					
Week 11	Wednesday					
Week 11	Thusday					
Week 11	Friday					
Week 11	Saturday					
Week 11	Sunday					
Week 12	Monday					
Week 12	Tuesday					
Week 12	Wednesday					
Week 12	Thusday					
Week 12	Friday					
Week 12	Saturday					
Week 12	Sunday					