Isoflavones and green kiwifruit:
A pilot study assessing the effects on bone turnover and lipid profile in healthy postmenopausal New Zealand women.

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Statement of contribution

Professor MC Kruger – study conception, design and funding.

Mrs CL Booth – Human studies coordinator – ethics application, recruitment, coordination and execution of the study.

Ms C Middlemiss – Support in sample collection and processing, assistance with the DPD and ucOC assays, dietary analyses, and independent data collection, statistical analyses and interpretation.

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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".

Signed ……..

Date ……..

Abstract

Background: The reduction in estrogen synthesis during menopause heightens the risk of development of osteoporosis and cardiovascular disease in postmenopausal women. Isoflavone (daidzein and genistein) interventions in postmenopausal women have reduced bone loss, and improved the serum lipid profile, which may reduce the risk of fracture and incidence of cardiovascular disease. However, skeletal benefits are inconsistent across interventions. This is partly a result of population heterogeneity in enteric bacterial daidzein metabolism – only ~30% of Caucasian women produce equol, a daidzein metabolite. Equol is more bioactive than its precursor and equol producers have more consistently shown skeletal benefits from isoflavone supplementation. Few interventions have accounted for equol production in postmenopausal study populations. Additionally, there is limited knowledge on how prebiotic foods, such as green kiwifruit, modulate daidzein-metabolising bacteria and equol production in humans. This pilot study aimed to assess bone turnover in response to isoflavone supplementation, and to determine the proportion of equol producers in postmenopausal women.

Objectives: The main objective was to measure the effect of short-term daily isoflavone supplementation alone or with the addition of green kiwifruit on biochemical markers of bone resorption, urinary deoxypyridinoline, plasma cross-linked C-terminal telopeptide of type I collagen, and plasma undercarboxylated osteocalcin in postmenopausal Caucasian women. A secondary objective was to measure the effect of isoflavones and kiwifruit on serum lipid profile. Additionally, equol production was determined in this population and assessed as a main effect.

Methods: This randomised crossover pilot study recruited 33 healthy postmenopausal Caucasian women, 1-10 years postmenopausal, and randomly allocated participants to treatment group A (n=16) or B (n=17) for a 16-week intervention. There were two consecutive 6-week treatment periods with a 2-week lead-in period at intervention commencement and a 2-week washout period between treatments. These treatments prescribed either: (1) daily isoflavone supplementation (50 mg/day aglycone daidzein

and genistein) alone or (2) with two green kiwifruit. Group A and B completed both treatments in opposite order. At treatment baseline and endpoints the following were measured (four time points): bone markers, serum lipid profile and both serum and urinary daidzein and equol. The hormones, serum follicle stimulating hormone, estradiol and thyroid-stimulating hormone, were also measured at baseline and endpoint to monitor potential adverse effects of isoflavones.

Results: Equol producers made up 30% of this study population (equol producers n=10; non-equol producers n=30). Serum equol rose significantly in equol producers. Plasma undercarboxylated osteocalcin decreased by 15.5% after the kiwifruit and isoflavone treatment and increased by 10.8% after the isoflavone only treatment. There were no changes in plasma C-terminal telopeptide of type I collagen or urinary deoxypyridinoline. In non-equol producers high-density lipoprotein cholesterol declined by an average of 4.9% with each treatment; there was no change in serum high-density lipoprotein cholesterol in equol producers following isoflavone treatment alone, and an 8.3% increase in serum high-density lipoprotein cholesterol following the combined kiwifruit and isoflavone combined. There were no other changes to the lipid parameters or hormones.

Conclusions: An aglycone isoflavone dose of 50 mg/day did not reduce bone resorption in the postmenopausal women in this study. Kiwifruit consumption decreased plasma undercarboxylated osteocalcin levels possibly due to the vitamin K content of green kiwifruit; however, alternative bioactive components in kiwifruit may have modulated this effect. The isoflavone treatment inhibited a decline in serum high-density lipoprotein cholesterol in equol producers and had synergistic effect with kiwifruit, which increasing this parameter. Equol and the carotenoid lutein from green kiwifruit may potentially modulate systemic inflammation. Kiwifruit may have a prebiotic effect in equol producers as shown by the increase in log ratio of daidzein to equol, but this requires further study. This equol producer subgroup was too small to detect a change the markers of bone resorption. Larger long-term studies are required to delineate the skeletal and cardiovascular effects of isoflavones and equol production in postmenopausal women.

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My family, Mum, Tim, Gen and Dad, I dedicate this work to you; your absolute pride and support for me in my academic endeavours has kept me going throughout the last few years. Mum, thank you for always being so interested in my work; I am truly lucky to have you care so much for my future.

“I’d rather have a mind opened by wonder than one closed by belief”

Albert Einstein

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Abbreviations

$1,25(OH)_2D$	Calcitriol
$24,25(OH)_2D$	Dihydroxycholecalciferol
25(OH)D	Calcidiol
AA	Arachidonic acid
AI	Adequate intake
B-ALP	Bone alkaline phosphatase
BMC	Bone mineral content
BMD	Bone mineral density
BMI	Body mass index
BMP	Bone morphogenetic protein
BRU	Bone resorptive unit
CaSR	Calcium sensing receptor
COX	Cyclooxygenase
CTx	Cross-linked C telopeptide of type 1 collagen
DBD	Vitamin D binding protein
DEXA	Dual-energy X-ray absorptiometry
DPD	Deoxypyridinoline
E2	Estradiol
ECM	Extracellular matrix
ERα/β	Estrogen receptor alpha/beta
FGF	Fibroblast growth factor
FOS	Fructooligosaccharide
FSD	Functional secretory domain
FSH	Follicle stimulating hormone
GC	Glucocorticoid
HDL-c	High-density lipoprotein cholesterol
HRT	Hormone replacement therapy
ID	Identification
IGF	Insulin-like growth factor
IL	Interleukin

LDL-c	Low-density lipoprotein cholesterol
LRP	Low density lipoprotein-related receptor
M-CSF	Monocyte colony stimulating factor
MCP	Monocyte chemoattractant protein
Mg	Magnesium
NHANES	National Health and Nutrition Examination Survey
NO	Nitric oxide
O-DMA	O-desmethylangolensin
OC	Osteocalcin
OPG	Osteoprotegerin
Ovx	Ovariectomised
PA	Physical activity
PBM	Peak bone mass
PGE	Prostaglandin
PPAR	Peroxisome proliferator-activated receptor
pQCT	Peripheral quantitative computed tomography
PTH	Parathyroid hormone
RANK	Receptor activator for nuclear factor κβ factor
RANKL	Receptor activator for nuclear factor κβ factor ligand
RB	Ruffled border
RCT	Randomised controlled trial
RDI	Recommended daily intake
SEM	Standard error of the mean
SD	Standard deviation
TAG	Triacylglycerol
TC	Total cholesterol
TC:HDL-c	Ratio of total cholesterol to high-density lipoprotein cholesterol
TGFβ	Transforming growth factor beta
TNF-α	Tumour necrosis factor alpha
TSH	Thyroid stimulating hormone
ucOC	Undercarboxylated osteocalcin
Wnt	Wingless type

Chapter 1

Introduction

1.1 Study background

Postmenopausal osteoporosis is a growing health burden in developed countries, due to the morbidity and mortality associated with increased prevalence of fracture and the increasing average lifespan (Brown, McNeill, Radwan, & Willingale, 2007). In 2013, the total healthcare costs associated with treatment of osteoporotic fractures in New Zealand was estimated at $1.38 billion dollars, and was projected to increase to $1.61 billion dollars in 2020 (Brown, et al., 2007). Proportionately more women contribute to total annual fracture incidence: 62.2% in women versus 38.8% in men (Brown et al., 2007). Hormone replacement therapy was once considered the gold standard treatment for postmenopausal osteoporosis but the use of HRT has diminished due to significant adverse health effects, such as increased risk of thromboembolic disease, cardiovascular disease (CVD), breast and endometrial cancer (Women's Health Initiative, 2002).

The isoflavones, daidzein and genistein, are phytoestrogens derived from soybean that have garnered research attention for their potential to reduce postmenopausal bone loss (Setchell & Lydeking-Olsen, 2003; Taku, Melby, Nishi, Omori & Kurzer, 2011). Asians consume soybean as part of their habitual diet, which exposes these populations to significant quantities of both daidzein and genistein (Uehara, 2013). Epidemiological studies have found that Asian postmenopausal women experience lower fracture rates and adverse CVD outcomes compared to Caucasian postmenopausal women (Poulsen & Kruger, 2008). Numerous observational studies have also reported a strong inverse association between isoflavone intakes and these co-morbidities in postmenopausal Asian women (Kim et al., 2002; Lauderale et al., 1996). Estrogen loss during menopause disrupts bone metabolism in postmenopausal women leading to a higher rate of bone resorption (Clarke & Khosla, 2010). Daidzein

and genistein are structurally similar to endogenous estradiol and activate the estrogen receptor beta (ER-β), expressed by osteogenic cells (Setchell & Lydeking-Olsen, 2008). Both cell and rodent studies have shown these isoflavones stimulate bone formation and inhibit bone resorption through ER-β stimulation (Morito et al., 2001; Poulsen & Kruger, 2008).

Randomised controlled trials (RCTs) of combined daidzein and genistein isoflavone interventions have been conducted in postmenopausal women of different ethnicities. Meta-analyses of some of these recent RCTs have found that bone mineral density (BMD) is improved at the lumbar spine compared to placebo when using isoflavone doses greater than 82 mg/day for at least 6-12 months (Taku et al., 2010b). Overall bone resorption was reduced by isoflavone doses greater than 52 mg/day used for at least 10 weeks (Taku et al., 2010a). Despite the plethora of positive findings from cell-based and rodent studies, isoflavone interventions have shown inconsistent skeletal benefits in postmenopausal women. There are numerous discrepancies between these RCTs, such as, isoflavone dose, isoflavone form (conjugate versus aglycone), trial duration, and years since onset of menopause (early versus late), which partially account for such inconsistent results. Another major contributing factor is the inter-individual heterogeneity in enteric daidzein metabolism to the more potent metabolite equol (Jackson et al., 2011). Only ~30% of Caucasian women can metabolise daidzein to equol compared to 50-70% in Asian women (Song et al., 2006).

Few human isoflavone interventions have accounted for the equol producer status of their postmenopausal participants. The isoflavone interventions that have prospectively screened for equol producer phenotype and randomly allocated equol producers to treatment, have reported significant benefits to bone density (Lydeking-Olsen, Beck-Jensen, Setchell, & Holm-Jensen, 2004, Wu et al., 2007) and lipid profile (Clerici et al., 2008) exclusively in equol producers. Such results suggest that equol production affects how postmenopausal women respond to isoflavone supplementation.

Animal and human observational studies suggest that soluble fibre intake is associated with the presence of an equol producer phenotype. This could be due to propagation of equol-producing intestinal bacteria (Tousen et al., 2013; Uehara, 2013). Combined isoflavone and soluble fibre supplementation has the potential to increase equol-production in equol-producers and could influence the establishment of an equol producer phenotype in non-equol producers. Green kiwifruit can act as a prebiotic, altering the composition of enteric microbiota (Lee et al., 2012), and can contribute to an increase in soluble fibre intake, providing 18% of daily dietary fibre requirements with a serving of two Zespri green kiwifruit (Zespri International, n.d). There have been no human studies regarding the effect of kiwifruit consumption on equol production.

1.2 Purpose of this study

There had been no previous isoflavone interventions in postmenopausal New Zealand (NZ) women in regards to bone health and CVD risk, in addition the prevalence of the equol producer phenotype had not been determined in NZ Caucasian women. This pilot study implemented a short-term isoflavone (mixed daidzein and genistein) and green kiwifruit intervention in NZ Caucasian postmenopausal women to evaluate the response of bone and cardiovascular health markers in this study population. The equol producer phenotype of participants was evaluated as a main effect. A combined kiwifruit and isoflavone intervention indirectly evaluated the prebiotic effect of kiwifruit on enteric equol-producing bacteria via serum equol measurements.

1.2.1 Study objectives

1.2.2.1 Primary objective

The primary objective of this pilot study was to evaluate the bone-sparing effects of a short-term isoflavone intervention in a post-menopausal Caucasian population by monitoring the change in bone turnover markers: urinary DPD, plasma CTx and ucOC. This study also evaluated the effect of equol producer status and kiwifruit consumption on these bone markers.

1.2.2.2 Secondary objective

The secondary objective was to measure the response of serum lipid parameters, total cholesterol (TC), low-density lipoprotein cholesterol (LDL-c), HDL-c and triacylglyerol (TAG), to isoflavone supplementation and measure the effect of the equol-producer phenotype.

1.2.2 Study hypotheses

It was predicted that postmenopausal women would experience a decrease in bone resorption (decreased CTx and DPD levels) compared to baseline levels following isoflavone supplementation. The equol producers were expected to have a greater reduction in bone resorption markers than non-equol producers. The addition of kiwifruit to isoflavone treatment was predicted to enhance the production of equol in equol-producers. It was also predicted that isoflavone supplementation would improve the serum lipid profile compared to baseline, i.e. reduce TC, LDL-c and TAG, and increase HDL-c, and that this effect would be greater in equol producers.

1.3 Layout of thesis

This thesis is presented in five chapters to describe the effect of isoflavone supplementation, alone and in combination with a green kiwifruit, on bone turnover and lipid parameters in equol producing and non-producing postmenopausal women. Chapter two is a review of the literature, which covers a background of bone metabolism, and subsequent evaluation of the role of isoflavones in bone in postmenopausal women. Chapter three describes the study design and methods. Chapter four presents the results of the study. Chapter five provides critical analysis and discussion of the results in context of relevant literature.

Chapter 2

Literature review

2.1 Bone composition

Bone is a dynamic organ with myriad roles in the human body; the skeleton envelops vital organs for protection, provides structural support and locomotion, and forms hematopoietic bone marrow (Clarke, 2008). Furthermore, bone acts as a mineral reserve, vital for mineral homeostasis.

The extracellular matrix (ECM) is comprised of organic and inorganic components. The ratio of organic matrix, mineral matrix and water is approximately 60:20:20, which creates an optimal balance between bone stiffness and flexibility (Crockett, Rogers, Coxon, Lynne, & Helfrich, 2011). The organic matrix is comprised primarily of collagenous protein fibrils (85-90%) arranged in lamellar orientation, providing a flexible structure for the mineral matrix to build upon. Non-collagenous proteins make up the remainder of this matrix and are thought to contribute to mineralisation and bone health (Clarke, 2008). The inorganic mineral is a crystalline structure of hydroxyapatite nano-particles, composed of calcium and phosphate ions with the molecular formula $Ca_{10}(PO_4)_6(OH)_2$ (Crockett et al., 2011).

There are 206 bones in the adult human body; each of these can be categorised based on their development, shape, anatomical location, and macroscopic structure (Clark, 2008). There are two macroscopic forms of bone, cortical and trabecular, which have distinct architecture and composition. Cortical bone lies superficially to trabecular bone and provides structural rigidity to the skeleton (Clarke, 2008) as it is highly calcified and arranged in dense cylindrical structures called osteons. Trabecular bone has a lattice-like arrangement of highly interconnected plates and rods and is less calcified than cortical bone; these features impart trabecular bone with a lightness and pliancy that balances the stiffness and weight of cortical bone (Vaughan, Mccarthy, & Mcnamara, 2012). The complex trabecular branching creates a greater surface area and metabolic activity than in cortical bone, which is suited to its role in mineral and

electrolyte homeostasis (Clarke, 2008). Bone exhibits site-specific ratios of trabecular and cortical compartments. This ratio ranges from 50:50 at the femoral head, to 75:25 at the vertebrae and 5:95 at the diaphysis of the radius (Clarke, 2008).

The periosteum, a fibrous connective tissue, sheaths the exterior cortical bone surface. This membrane houses arterial and venous networks, nerve endings, and is a reservoir of osteogenic cell precursors (Bayliss, Mahoney, & Monk, 2011). Capillary networks extend through cortical bone via ducts in cortical osteons (Brandi, 2009). Connective tissue lines the interior cortical surface and interface between cortical and trabecular bone to form the endosteum, which also provides osteogenic precursor cells and blood vessels (Brandi, 2009). Hematopoietic marrow occupies the central bone cavity and is interspersed throughout the trabecular voids (Clarke, 2008).

2.2 Bone metabolism

Bone modelling is the process that governs net bone formation during human development. Bone density increases 7-fold from birth to puberty and another 3-fold from puberty to adolescence (Peacock, 1991). Bone reaches its peak dimensions early in the second decade, whereas peak bone mass (PBM) is achieved early in the third decade (Bonjour, Chevalley, Ferrari, & Rizzoli, 2009). Following the attainment of PBM, bone density begins a gradual decline.

Throughout the lifecycle bone is continuously engaged in an independent remodelling cycle where fatigued bone is broken down and replaced by new bone (Crockett et al., 2011). This physiological process avoids the accumulation of skeletal micro-damage and bone fragility (Kular, Tickner, Chim, & Xu, 2012). Remodelling is also the basis of mineral homeostasis (Raggatt & Partridge, 2010). The osteogenic cells, osteoclasts, osteoblasts, and osteocytes, underpin bone remodelling. Osteoclasts degrade damaged bone, which osteoblasts subsequently replace, and osteocytes are involved in initiating the remodelling process (Crockett et al., 2011). Bone is remodelled in transient anatomical regions called bone-remodelling units (BRU) (Clarke, 2008).

An active BRU produces signal molecules to recruit a functional assemblage of osteogenic cells (Crockett et al., 2011). Bone lining cells envelop the BRU, which physically isolates the remodelling process and allows independent physiological regulation of the BRU. Bone turnover occurs in three main stages: initiation, reversal and termination (Kular et al, 2012). Initiation of remodelling involves the detachment of the bone lining cells, followed by recruitment, maturation and activation of osteoclasts and a subsequent period of bone resorption. The reversal phase entails osteoclast apoptosis coupled to osteoblast maturation and activation, followed by a period of bone formation. The reversal phase is the lengthiest in the remodelling cycle but is more rapid in cortical bone compared to trabecular bone (Kular et al., 2012). Termination of remodelling involves the controlled apoptosis of osteoblasts and reattachment of the bone lining cells; mature osteoblasts are also transformed to either osteocytes or bone lining cells (Raggatt & Partridge, 2010).

Myriad systemic and local activating and inhibiting factors regulate osteogenesis. In non-pathological conditions cross-talk between the osteogenic cells appropriately initiates a BRU and ensures the sequential activation and inhibition of osteoclasts and osteoblasts (Bayliss et al., 2011). This allows bone formation and resorption to occur in balance, so that bone density and strength are not compromised (Crockett et al., 2011; Matsuo & Irie, 2008).

2.3 Osteogenic cells

Osteoclastogenesis and osteoblastogenesis involve the sequential recruitment of osteoclast and osteoblast precursors and their lineage specification, cellular differentiation, maturation and activation (Crockett et al., 2011).

2.3.1 Osteoclastogenesis and osteoclast function

Osteoclasts resorb bone: these large multinucleated cells arise from the fusion of multiple monocytes of the monocyte-macrophage lineage. This lineage is derived from hematopoietic stem cells that alternatively give rise to macrophages or dendritic cells

depending on signal stimuli (Feng & Teitelbaum, 2013). Osteoclasts behave similarly to macrophages, exhibiting phagocytosis and activation in response to several macrophage-stimulating factors (including inflammatory cytokines) (Feng & Teitelbaum, 2013).

Monocyte precursors reside within the periosteum and endosteum. An active BRU recruits monocyte precursors via monocyte chemoattractant protein-1 (MCP-1), which is released into the local capillaries by bone lining cells at the remodelling site (Li et al., 2007). Monocyte-colony stimulating factor (M-CSF) produced by stromal cells and osteoblasts upregulates monocyte expression of receptor activator of nuclear κβ factor (RANK) to allow an appropriate response to RANK ligand (RANKL) (Burgess et al., 1999). Together RANKL and M-CSF coordinate specify osteoclast lineage and differentiation and direct the fusion of mononuclear cells to form the quiescent mature osteoclast (Suda, Nakamura, Jimi, & Takahashi, 1997).

RANKL has both soluble and membrane-bound forms and binds to its membrane-bound receptor RANK, on the osteoclast surface (Bayliss et al., 2011). RANKL is expressed by osteoclasts themselves, osteoblasts, and activated T cells. Binding of RANKL to RANK stimulates pre-osteoclast proliferation, differentiation into quiescent osteoclasts and maturation, in addition to the activity and survival of mature osteoclasts (Suda et al., 1999). The RANK/RANKL factors are key to the progression of bone resorption.

Osteoprotegerin (OPG) factor is a negative regulator of osteoclastogenesis secreted by osteoblasts and stromal cells (Crockett et al., 2011). OPG is secreted as a soluble decoy receptor for RANKL, causing its sequestration and reducing its availability for the RANK-bound stimulation of osteoclastogenesis (Crockett et al., 2011). Osteoclastogenesis is oppressed by an increasing ratio of OPG to RANKL, or by a decrease in osteoclastic RANK expression (Burgess et al., 1999). See figure 2.1 for a summary of osteoclastogenesis.

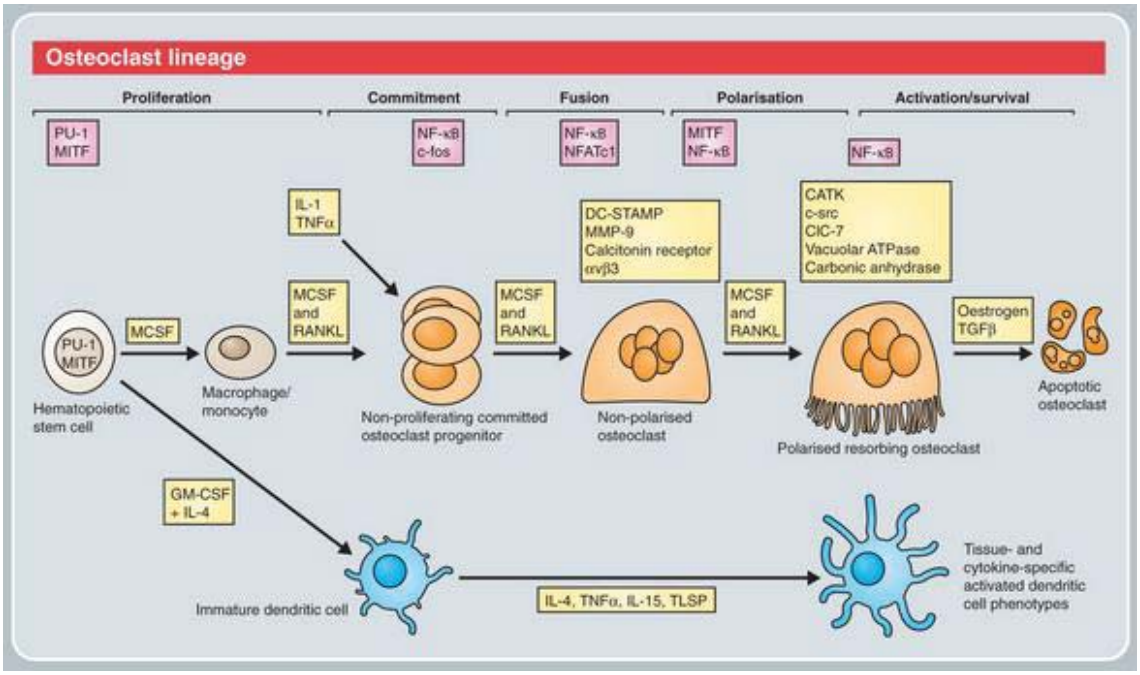

Figure 2.1 Illustration of osteoclast lineage specification, including the local and systemic factors involved and osteoclastic gene expression (shown in yellow boxes). Image reproduced with permission from Crockett et al. (2011).

An activated osteoclast becomes affixed to the bone surface by osteoclast-ECM interactions. Osteoclast integrin proteins bind to a specific amino acid sequence expressed by various proteins of the bone ECM (Duong, Lakkakorpi, Nakamura, & Rodan, 2000). Attachment of the osteoclast incites intracellular signalling and subsequent cytoskeletal changes that are vital to osteoclast function, particularly membrane polarization. Osteoclast microtubules arrange adhesion molecules, podosomes, into a dense ring formation in the membrane adjacent to bone. The transmembranous podosomes adhere specifically to the apatite component of hydroxyapatite (Luxenburg et al., 2007). This forms a sealed zone and generates osteoclast motility across the bone surface (Jurdic, Saltel, Chabadel, & Destaing, 2006). The sealed zone creates a microenvironment optimal for bone resorption and isolates the coupling factors that are released, which subsequently have a role in osteoblast coordination (Feng & Teitelbaum, 2013), moreover it prevents the inappropriate degradation of the surrounding bone (Jurdic et al., 2006). These cytoskeletal changes also induce osteoclast polarisation in which the osteoclast forms a ruffled border (RB) within the sealed zone, and the basolateral membrane, which acts as the functional

secretory domain (FSD) (Takahashi, Ejiri, Yanagisawa, & Ozawa, 2007). The convoluted membrane of the RB contains the appropriate transport proteins for proton secretion and secretory vesicle exocytosis (Takahashi et al., 2007). The adenosine triphosphate hydroxylase proton pump moves hydrogen ions across the RB (Vaananen et al., 1990). Proton secretion creates acidifies the resorption lacunae, dissolving the mineral and denaturing the proteins of the organic matrix. Subsequent dissolution of the mineral exposes collagen to the proteolytic action of low-pH-functioning enzymes (Takahashi et al., 2007). These enzymes are released from osteoclast secretory vesicles: tartrate resistant acid phosphatase, cathepsin K and metallomatrix proteinase-9. Collagenous and non-collagenous fragments, and free calcium and phosphate ions are transcytosed to the FSD for secretion (Mulari, Zhao, Lakkakorpi, & Väänänen, 2003).

2.3.2 Osteoblastogenesis and osteoblast function

Osteoblasts are responsible for the deposition of bone. Osteoblasts form the organic matrix and initiate and assist in mineralisation (Crockett et al., 2011). See figure 2.2 for a summary on osteoblastogenesis. Osteoblasts derive from multipotent stem cells present in the endosteum and periosteum. This stem cell line also differentiates into fibroblasts, chondrocytes and adipocytes (Crockett et al., 2011). Commitment to the osteoblast lineage relies on the canonical wingless type (Wnt)/β-catenin signalling pathway, which entails Wnt activation of Wnt co-receptors, low-density lipoprotein receptor-related proteins 5 and 6 (LRP5/6) and the frizzled receptor (Hill, Später, Taketo, Birchmeier, & Hartmann, 2005). The Wnt signalling pathway releases the transcription factors, Cfa-1 and Osx, which are indispensable for osteoblast gene expression. Bone morphogenetic proteins (BMPs) and transforming growth factor beta (TGFβ) are also essential for osteoblast lineage specification and differentiation; they upregulate the same transcription factors used in Wnt signalling by alternative intracellular pathways (Ahdjoudj, Lasmoles, Holy, Zerath, & Marie, 2002; Martinovic et al., 2006).

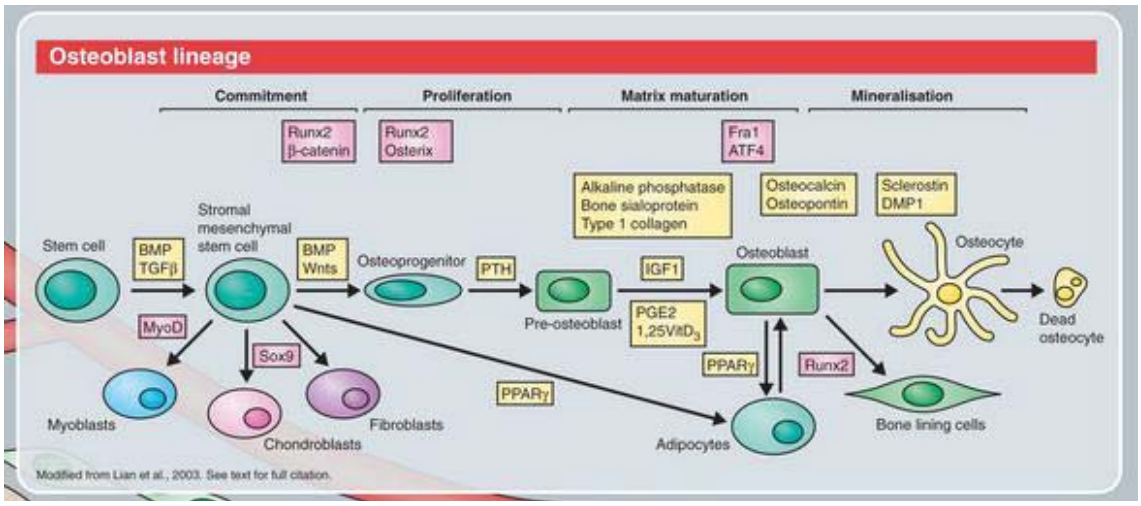

Figure 2.2 Illustration of osteoblast lineage specification including the local and systemic factors involved and osteoblastic gene expression (shown in yellow boxes. Image adapted and reproduced with permission from Crockett et al. (2011).

Osteoblasts attach to the bone surface through osteoblast-expressed integrins, which bind to exposed collagen (Crockett et al., 2011). After attachment osteoblasts secrete collagen fibrils, which are deposited in a directional manner: the fibrils within adjacent lamellae have alternate orientation. Prior to mineralisation this substance is referred to as osteoid (Clarke, 2008). Osteoblasts also secrete a range of non-collagenous proteins involved in the mineralisation process. Matrix vesicles containing bone alkaline phosphatase (B-ALP), inorganic phosphate and pyrophosphate, are secreted by active osteoblasts and initiate osteoid mineralisation (Orimo, 2010). Alkaline phosphatase cleaves inorganic phosphate from pyrophosphate; the enzymatic production of free phosphate ions regulates the rate of hydroxyapatite crystal formation (Crockett et al., 2011). Calcium ions are readily available in the extracellular solution if calcium intake is adequate. Hydroxyapatite crystals cultivate in clusters around the secreted matrix vesicles; these crystals enlarge and mineralise the surrounding osteoid (Orimo, 2010). Eventually osteoblasts become embedded in the bone they have secreted leading them to various fates, as osteocytes or bone lining cells. Bone lining cells cover both the periosteal and endosteal bone surfaces and produce signals initiating a BRU (Crockett et al., 2011).

Bone lining cells also envelop and canopy the BRU, creating physical isolation, and prepare bone for resorption by digesting the thin layer of osteoid for the formation of the osteoclastic sealed zone (Kular et al., 2012).

2.3.3 The signal triad regulating osteogenic cross-talk

The ratio of the triad of bone factors, RANK, RANKL, and OPG, is a primary regulator of osteoclastogenesis and due to the tight coupling of bone turnover will ultimately increase osteoblastogenesis (Bayliss et al., 2011). Factors that increase RANK and RANKL expression will upregulate osteoclastogenesis, whereas an increase in OPG will reduce the rate of osteoclastogenesis. Accordingly, the action of OPG is supported by a reduction in RANK or RANKL expression.

2.3.4 Osteocytes and mechanical loading

Osteocytes are formed by mature osteoblasts that have become embedded in the newly secreted matrix, and their cell bodies sit in lacunae (Neve, Corrado, & Cantatore, 2012). Osteocytes possess myriad dendritic projections that branch extensively through microscopic tunnels, canaliculi, in the bone. This ostoecytic network forms a common fluid space within mineralised bone, adept for the sensation of mechanical strain (Milovanovic et al., 2013). Osteocyte deformation occurs in response to a mechanical load and the resulting strain on bone (Klein-Nulend, Bakker, Bacabac, Vatsa, & Weinbaum, 2013). The mechanisms that underlie osteocyte mechano-sensation are poorly understood. It is known that strain signals are interpreted by osteocytes and this signal spreads via the ostoecytic network to the bone surface to it alter osteogenic cell activity (Milovanovic et al., 2013). Strain must surpass a programmed mechanical threshold to initiate remodelling; threshold stimulation increases osteocyte secretion of local regulators of bone cell activity. These local factors include: nitric oxide (NO), prostaglandin-E2 (PGE2), a PGE2 precursor arachidonic acid (AA) and cyclo-oxygenase 2 (COX2), and an enzyme converting AA to PGE2. These factors induce a rapid increase in osteoblast and osteoclast populations (Klein-Nulend et al., 2013).

Osteocyte signals mediate changes in the osteogenic cells. PGE2 increases osteoblast differentiation through an increase in Wnt signalling in pre-osteoblasts. Both NO and TGFβ downregulate the osteoblastic expression of RANKL (Neve et al., 2012; Raggatt & Partridge, 2010; Quinn et al., 2001) and upregulate OPG output (Neve et al., 2012), to suppress osteoclastogenesis. Conversely, PGE2 increases osteoclastogenesis indirectly by simultaneously increasing osteoblast expression of RANKL and reducing OPG (Neve et al., 2012). Strained osteocytes synthesise RANKL and M-CSF themselves, to directly upregulate osteoclastogenesis. Mechanical loading also decreases the osteocyte inhibition of osteoblastogenesis by repressing the constitutive secretion of sclerostin, a Wnt antagonist that blocks the Wnt co-receptor LRP-5 (Crockett et al. 2011).

The endocrine system influences the effect of mechanical loading on bone (Neve et al, 2012). The mechano-threshold changes with the relative levels of circulating E2, vitamin D and PTH; the receptors to these systemic hormones are displayed by osteocytes (Neve et al., 2012).

2.4 Factors affecting bone health

2.4.1 Physiological regulators

The calcium ion is vital to muscle and nerve function, and blood coagulation. Because these functions are vital to immediate survival there is a physiological demand to maintain serum calcium concentrations within a narrow range (Adams et al., 2013). If calcium balance cannot be maintained through intestinal absorption of dietary calcium, and renal calcium reabsorption, then skeletal calcium reserves are drawn upon to oppose hypocalcemia. Skeletal calcium is a vast and readily available store and osteolysis can contribute greatly to serum calcium levels, whereas, maximising intestinal absorption (which is limited by intake of calcium), and renal reabsorption, can contribute less to serum calcium levels (Adams et al., 2013). When dietary calcium falls below the required daily intake then skeletal calcium reserves are sacrificed to preserve normocalcemia based on the biological imperative of immediate survival.

Hormones can act directly on osteogenic cells to alter their rate or activity at different stages of their lifecycle. Hormones also modulate bone turnover indirectly by enhancing the transcription of local factors within the bone microenvironment that have paracrine effects on osteogenic cells. These localised factors include PGE, cytokines and growth factors (Crockett et al., 2011).

2.4.1.1 Vitamin D

Vitamin D is primarily of endogenous origin with small contribution from foods containing vitamin D (Anderson et al., 2013). The endogenous route begins with the vitamin D precursor, 7-prehydrovitamin D, which is synthesised and stored in the epidermis. The precursor undergoes photo-conversion to pre-vitamin D by exposure to the ultraviolet beta radiation from sunlight (Bickle, 2009). Subsequently, pre-vitamin D undergoes thermal isomerisation to vitamin D, and enters systemic circulation. Two consecutive hydroxylating steps are necessary to form biologically active vitamin D, calcitriol (St-Arnaud, 2010). Calcitriol binds to widespread target cells expressing the vitamin D receptor (VDR).

The primary hydroxylation produces calcidiol, 25-hydroxyvitamin D (25(OH)D), which occurs primarily in the liver via the enzyme 1α-hydroxylase. 25α-hydroxylase enzyme carries out the final hydroxylation to form bioactive calcitriol, 1,25-hydroxyvitamin D ($1,25(OH)_2D$) (St-Arnaud, 2010). Initially the expression of 25α-hydroxylase was considered to occur predominantly in the proximal renal tubules, however, extra-renal expression is evident in bone, thyroid, and myriad other tissues (Bickle, 2009). Calcidiol self-regulates its catabolism by inducing the expression of 24α-hydroxylase in target cells; 24α-hydroxylase transforms calcidiol to $24,25(OH)_2D$, and induces its breakdown (St-Arnaud, 2010).

Vitamin D synthesis is upregulated by parathyroid hormone (PTH) in the hypocalcemic state and suppressed by calcitonin in the hypercalcemic state. PTH and calcitonin modify the expression of renal 25α-hydroxylase. All three osteogenic cells express both 25α-hydroxylase and the VDR; this allows for bone-specific local activation and

paracrine action of vitamin D (St-Arnaud, 2010). However, the expression of 25α-hydroxylase is greater in mature osteoblasts and osteoclasts compared to their respective precursors. Thus the calcidiol response depends on osteogenic call maturity (Crockett et al., 2011). Calcidiol circulates in serum bound to vitamin-D-binding protein (DBP), which restricts cell permeability to target cells expressing the DBP-receptors cubulin and megalin. The osteogenic cells express both of these surface receptors (van Driel & van Leeuwen, 2014)

Vitamin D has an important role in the systemic regulation of serum calcium levels. 1,25$(OH)_2$D increases intestinal calcium absorption by ~85-90%, and increases renal calcium reabsorption and accelerates PTH-mediated increase in renal calcium uptake (Holick, 2009). In addition vitamin D has an autocrine role, acting directly on the osteogenic cells to induce osteolysis during hypocalcemia (Clarke, 2008).

Vitamin D status, as measured by 25(OH)D levels, is correlated to improved BMD and fracture incidence (Jackson et al., 2006; Lips et al., 2010). Serum calcitriol fails to show an association with bone health because the tissue-localised conversion of 25(OH)D to 1,25$(OH)_2$D cannot be accounted for by routine serum measures of 1,25$(OH)_2$D (Adams et al., 2013). The autocrine effects of vitamin D in bone are vital to bone health independent of calcium homeostasis. Calcium absorption and serum 1,25$(OH)_2$D are maximal when serum 25(OH)D reaches ~40 nmol/L (Anderson et al., 2013). However, it is apparent that greater serum 25(OH)D concentrations are required for its modulation of osteogenic cells, bone health and fracture risk: there is a reduction in fracture risk when comparing women with an average serum 25(OH)D level of 75 nmol/L versus 45 nmol/L (Anderson et al., 2013).

Vitamin D stimulates bone resorption indirectly via the upregulation of osteoblastic and osteocytic RANKL expression (Adams, 2013). Additionally, vitamin D stimulates osteocyte expression of fibroblast growth factor 23 (FGF23), which inhibits osteoblast activity. Active vitamin D acts on pre-osteoclasts to increase RANK expression and osteoclastic differentiation. Ultimately this increases the number of mature osteoclasts, which promotes bone resorption (Kogawa, Anderson, Findlay, Morris, & Atkins, 2010).

Vitamin D has a dual effect on osteoblast differentiation: osteoblast maturity determines the response to $1,25(OH)_2D$. Pre-osteoblast differentiation is inhibited by vitamin D, whereas the differentiation of more mature osteoblasts is enhanced by vitamin D (Baldock et al., 2006; Geng, Zhou, & Glowacki, 2011). Immature osteoblasts upregulate RANKL in response to vitamin D and mature osteoblasts increase OPG expression D (Baldock et al., 2006). Furthermore, vitamin D may increase the rate of bone mineralisation by increasing the formation of mature matrix vesicles in osteoblasts (Woeckel, van der Erden, Schreuders-Koedam, Eijken, & van Leeuwen, 2013).

Vitamin D appears to be catabolic to bone during hypocalcemia, but exerts anabolic effects during normocalcemia This biphasic response can be attributed to the differential effects of $1,25(OH)_2D$ on immature and mature osteoblasts and the relative levels of additional regulatory hormones and local factors in the bone microenvironment (Crockett et al., 2011).

2.4.1.2 Parathyroid hormone

PTH is synthesised by the parathyroid gland and its secretion is induced rapidly in response to declining extracellular calcium levels through the calcium-sensing receptor (CaSR). As serum calcium levels decline less calcium ions bind to the CaSR, which creates an intracellular signalling cascade upregulating PTH expression (Kular et al., 2012).

PTH is synthesised and secreted by parathyroid cells and is regulated in an intracrine manner by the intracellular formation of $1,25(OH)_2D$. Parathyroid cells express 25α-hydroxylase and VDR, which are both upregulated by serum 25(OH)D. Intra-parathyroid metabolism calcidiol to active vitamin D is a negative feedback inhibitor of PTH secretion. Serum 25(OH)D must be greater than 50 nmol/L to provide sufficient $1,25(OH)_2D$ to inhibit PTH secretion (Clarke, 2008).

PTH acts to restore normocalcemia in three ways: decreasing the renal loss of calcium (increasing renal calcium reabsorption), stimulating osteolysis, and increasing the renal output of $1,25(OH)_2D$ (Bayliss et al., 2011). A continuous increase in serum PTH induces osteolysis directly by binding to osteoblasts (Huang et al., 2004). Initially this reduces OPG synthesis, when bound to less mature osteoblasts, and subsequently increases RANKL output, when bound to more mature osteoblasts. Ultimately both actions enhance osteoclastogenesis by increasing RANK-RANKL binding (Huang et al., 2004). In bone lining cells PTH stimulates the expression of MCP-1, M-CSF, and RANKL, and oppresses OPG production; PTH also increases osteoblastic expression of MCP-1 (Crockett et al., 2011). These changes in local regulators of osteoclastogenesis increase pre-osteoclast recruitment, differentiation, activity and survival. PTH also stimulates osteocyte RANKL production (Saini et al., 2013).

However, PTH can favour bone formation. The anabolic effects of PTH require the presence of the local factor insulin-like growth factor-1 (IGF-1) and activation of Wnt signalling (PTH requires activation of LRP-5) to enhance osteoblastogenesis (Crockett et al., 2011). PTH suppresses sclerostin expression in osteocytes via the PTH-related peptide type 1 receptor to relieve its inhibition of Wnt signalling and thus increase osteoblastogenesis (Maeda, Takahashi, & Kobayashi, 2013; Saini et al., 2013).

2.4.1.3 Calcitonin

Calcitonin opposes PTH-induced osteoclast stimulation and reduces calcium release into circulation during hypercalcemia, but this role in calcium homeostasis is minor (Bayliss et al., 2011). Calcitonin is synthesised in the parafollicular C cells of the thyroid

gland and is expressed in response to an increasing number of calcium ions bound to the CaSR, which induces intracellular signalling that upregulates calcitonin expression (Crockett et al., 2011). Calcitonin binds directly to osteoclasts and suppresses their activity by inhibiting the formation of the cytoskeletal podosome ring and disrupting osteoclast adhesion (Suda et al., 1997), which is a reversible process (Crockett et al., 2011).

2.4.1.4 Estrogen

Estrogen is the female sex steroid hormone produced in majority by the follicular cells of the ovaries and in lesser amounts by the adrenal glands and adipose tissue (Pfeilschifter, Koditz, Pfohl, & Schatz, 2002). In women, ovarian E2 production begins at first menses and rapidly declines with menopause when the ovaries go through natural degeneration (Riggs, 2002). Estrogen has a protective effect on bone mass (Almeida et al., 2010), and bone health is impaired in postmenopausal women. Estrogen maintains a slow rate of bone turnover by reducing both osteoblast and osteoclast maturation via the induction of pre-osteoclast apoptosis. Estrogen promotes apoptosis via upregulation of local apoptotic factors in pre-osteoblasts and mature osteoclasts (Almeida et al., 2010). Estrogen also favours bone formation by promoting mature osteoblast activity and survival and osteocyte survival (Almeida et al., 2010). Promotion of osteocyte survival acts to maintain the sensitivity of the ostoecytic network and responsiveness of bone to mechanical stimuli (Klein-Nulend et al. 2013).

2.4.1.5 Local factors

PGE2 is part of a larger family of eicosanoids, derived from polyunsaturated fatty acids, which are important in bone biology. PGE2 formation is regulated by osteocytes, osteoblasts, and osteoblastic precursors (Arikawa, Omura, & Morita, 2004). These cells express PGE2, AA, and COX-2 the rate-limiting enzyme for AA conversion to PGE2.

At physiological concentrations PTH induces COX-2 expression in osteoblasts (Coetzee, Haag, Claassen, & Kruger, 2007), whereas, in osteocytes mechanical stimulation is required for COX-2 expression (Crockett et al., 2011).

PGE2 increases bone turnover by increasing both the rates of resorption and formation. PGE2 binds to osteoclasts to increase their expression of RANK, and also binds to osteoblasts to simultaneously increase RANKL expression and reduce OPG output. These actions combined promote osteoclastogenesis (Liu, Kirschenbaum, Yao, & Levine, 1991). Vitamin D and PTH act directly on pathways similar to those activated by PGE2 and their effects are enhanced by the presence of PGE2. Mice, with COX-2 deletion in the osteocyte, are PGE2 deficient, and exhibit a lower rate of resorption in response to both PTH and vitamin D (Liu et al., 1991). PGE2 stimulates osteoblast maturation through a direct signalling pathway that upregulates expression of crucial osteoblastic genes (Liu et al., 1991). PGE2 also acts indirectly on osteoblasts by upregulating osteoblastic expression of BMP-2, which positively regulates osteoblastogenesis (Arikawa et al., 2004; Xinping Zhang et al., 2002). Overall, PGE2 in low concentrations coupled to a mechanical stimulus has an anabolic effect on bone. Conversely, higher PGE2 concentrations act in a catabolic manner on bone (Blackwell, Raisz, & Pilbeam, 2010).

The cytokines, interleukin-1 and -6 (IL-1/6) and tumour necrosis factor-alpha (TNF-α), are produced by resident macrophages within the bone microenvironment (Hofbauer, Brueck, Shanahan, Schoppet, & Dobnig, 2007; Lee, Kalinowski, Jastrzebski & Lorenzo, 2002). These factors feed forward to stimulate their expression from osteoblasts and osteoclasts. The effects of Il-1, IL-6 and TNF-α synergise to enhance osteoclastogenesis: all three factors upregulate RANK expression in osteoclasts and IL-1 and TNF-α strongly stimulate RANKL expression by osteoblasts and stromal cells (Hofbauer et al., 1999; Lee et al., 2002; Nakashima et al., 2000). IL-1 modulates the pro-osteoclastogenic effects of vitamin D and both IL-1 and TNF-α induce further osteoblastic expression of IL-1 (Lee et al., 2002). Conversely, OPG expression, which is upregulated by bone anabolic factors, inhibits IL-1 mediation of osteoclastogenesis (Lee et al., 2002).

IGF-1 is synthesised by active osteoblasts during bone formation and is incorporated into the bone matrix (Imai et al., 2009). IGF-1 may assist in coupling bone formation to resorption as it is released during bone degradation. IGF-1 stimulates osteoblast differentiation and maturation and downregulates osteoclastic RANK expression but also upregulates stromal cell synthesis of RANKL (Rosen, 2004). Local factors. IL-1, PGE2, BMPs, and TGF-β, and hormones, PTH, and E2, all stimulate IGF-1 production in osteoblasts and stromal cells (Rosen, 2004).

The complexity of intracrine signalling in the regulation of bone turnover explains why hormones have biphasic effects on bone. Effects of local factors depend on the maturity of osteogenic cells and the relative levels of local osteogenic factors and their inhibitory and activating cross-talk (Huang, Ren, Ma, Smith, & Goodman, 2010; Okada et al., 2002).

2.4.2 Lifestyle factors

2.4.2.1 Physical activity

Premature osteocyte apoptosis can occur due to bone damage, sex steroid deficiency, and senescence, and is thought to reduce the mechano-sensitivity of bone to loading (Crockett et al., 2011). However, physical activity (PA) is a major determinant of bone mass during adolescence and adulthood (Harvey et al., 2012). Regular PA during former years maintains greater bone mass and is protective against fracture, especially in senescence when inevitable bone loss occurs (Troy, Edwards, Bhatia, & Bariether, 2013). Mechanical stimulation of bone is created by muscle tension during physical activity: osteocytes and osteoblasts respond by initiating bone remodelling in mature bone and favouring bone formation. Disuse of bone results in loss of mass (Troy et al., 2013).

2.4.2.2 Nutrition

Different dietary components can have positive or negative effects on bone health (Cashman, 2007). There are several micronutrients that are essential to bone health throughout the human lifecycle (Cashman, 2007).

2.4.2.1.1 Calcium

Calcium is the primary component of bone mineral. Inadequate daily intake or low bioavailability of dietary calcium leads to hypercalcemia and stimulates secretion of the calcitropic hormones PTH and vitamin D to maintain serum calcium (Lips et al., 2010). The recommended daily intake (RDI) of calcium in NZ women (aged 51-70) is 1300 mg/day (NHMRC & MoH, 2006). Lower calcium intakes are associated with increased bone resorption, lower bone mass and greater fracture susceptibility (Feskanich, Willett, & Colditz, 1997; Kalkwarf, Khoury, & Lanphear, 2003), particularly in postmenopausal women due to diminished E2 levels. Myriad randomised controlled trials show that long-term calcium supplementation can reduce bone loss in postmenopausal women with low calcium intake (Chee, Suriah, Chan, Zaitun, & Chan, 2003). Calcium supplementation reduces bone loss in postmenopausal women and may translate to a decrease in fracture incidence, however, only a few trials have measured fracture incidence as a primary outcome (Feskanich, Willett, & Colditz, 2003). The proportion of women with vitamin D insufficiency increases in the aging population, which negatively impacts calcium balance. Vitamin D supplementation increases serum 25(OH)D and reduces the rate of bone loss in postmenopausal women (von Hurst, Stonehouse, Kruger, & Coad, 2010) and vitamin D and calcium supplements are synergistic in the prevention of bone loss (Di Daniele et al., 2004; Lips et al., 2010). Calcium is most abundant and bioavailable in dairy products with smaller amounts provided from bony fish, legumes, nuts and leafy green vegetables (Nieves, 2005).

2.4.2.2.2 Phosphate

Dietary phosphorus provides phosphate, which is another vital component of the hydroxyapatite mineral. Prolonged hypophosphatemia reduces bone matrix mineralisation (Clarke, 2008); despite this, bone mass is not negatively impacted by low phosphorus intake. More importantly, high phosphorus intake in combination with low calcium intake is damaging to bone health due to the prolonged elevation of PTH (Bergwitz & Harald, 2010); serum PTH opposes hyperphosphatemia, secondary to hypocalcemia. A rise in PTH increases serum calcitriol and FGF-23, which act on the renal tubules to increase phosphate excretion (Bertzwig & Harald, 2008). Vitamin D also reduces intestinal absorption of phosphorus (Bertzwig & Harald, 2008).

2.4.2.2.3 Vitamin K

Vitamin K is an essential cofactor in the enzymatic carboxylation of the metal-chelating glutamyl side chains present on osteocalcin (OC) (Koitaya, Sekiguchi, Tousen, Nishide, & Morita, 2014). OC is embedded in bone mineral following the carboxylation of its side chains**.** Low vitamin K intake results in an increase in serum undercarboxylated OC (ucOC), which cannot bind to the hydroxyapatite matrix. Higher levels of plasma ucOC are associated with greater fracture risk (Booth et al., 2000; Koitaya, et al., 2014). Vitamin K supplementation in postmenopausal women is effective in reducing plasma ucOC by increasing OC carboxylation levels (Binkley, Krueger, Engelke, Foley, & Suttie, 2000; Binkley et al., 2002; Binkley et al., 2009). Vitamin K has two forms: phylloquinone (vitamin K1), which is abundant in green leafy vegetables, and the menaquinones (vitamin K2), which are produced by bacteria. Only one subtype of menaquinones, menaquinone-4 (MK-4), is found in some foods such as fermented soy, cheese, eggs, fish, meat, and liver (Nieves, 2005). Intestinal microbiota also produce menaquinone-7 (MK-7), however the literature suggests it has minimal impact on serum vitamin K levels, as vitamin K is not absorbed in the colon (Tsugawa et al., 2006).

2.4.2.2.4 Magnesium

Magnesium (Mg) forms a complex with hydroxyapatite mineral in bone and regulates mineralisation, particularly crystal formation and size (Nieves, 2005). In addition, Mg is a cofactor for many enzymatic processes that occur in bone metabolism. Mg deficiency in mice is detrimental to bone health in several ways (Cashman, 2007; Rude, Gruber, Wei, Frausto, & Mills, 2003). In Mg-deficient mice bone exhibited a reduced population of mature osteoblasts due to Mg-dependent osteoblast proliferation (Rude et al., 2003). In addition Mg-deficient mice exhibited increased levels of circulating pro-inflammatory cytokines (TNF-α and IL-1) (Rude et al., 2003) and a subsequent increase in osteoclastic activity. Moreover, PTH and vitamin D secretion and activity are impaired in Mg-deficient mice (Rude et al., 2003). Mg-salts may play a role in the systemic regulation of acid-base homeostasis; Mg-salts are alkaline and can counteract diet-induced acidosis, typical of western diets and in the elderly (Castiglioni, Cazzaniga, Albisetti, & Maier, 2013). Unopposed acidosis induces osteolysis to release alkaline ion buffers regardless of bone health (Tucker et al., 1999). Dietary sources of magnesium include a wide variety of vegetables and fruits (Nieves, 2005).

2.4.2.2.5 Vitamin C

Vitamin C is an essential cofactor of the osteoblastic enzyme that hydroxylates the collagen amino acid side-chains, lysine and proline (Nieves, 2005). Collagen hydroxylation is essential to the establishment of stabilising collagen cross-links in the bone extracellular matrix (Nieves, 2005). A novel role of vitamin C has been shown in an animal study wherein dietary vitamin C increased dietary calcium bioavailability by increasing intestinal calcium uptake and retention (Morel & Wolber, unpublished data, as cited in Wolber et al., p249, 2013). Although human studies are required to confirm this action, vitamin C may indirectly reduce bone resorption by increasing fractional calcium absorption, which is important in individuals with low calcium intake. Fruit and vegetables are good sources vitamin C, particularly kiwifruit and citrus fruits (Cashman, 2007).

2.5 Osteoporosis

2.5.1 Definition and causes of osteoporosis

Osteoporosis is a pathological state of skeletal dysfunction in which bone remodelling becomes unsynchronised: bone resorption outweighs bone formation. Osteoporotic bone is characterised by structural deterioration and a large reduction in BMD (Civitelli, Armamento-Villareal, & Napoli, 2009). The World Health Organization diagnoses osteoporosis as BMD of the total hip and spine that is less than -2.5 standard deviations (SD) from a healthy reference population (National Institute of Health, 2000). Osteopenia is low BMD, defined as BMD between -1 and -2.5 SD from the healthy reference population (Barrett-Connor et al., 2005). Aging, glucocorticoid excess and sex steroid deficiency are the major factors underlying the pathophysiology of osteoporosis (Marie & Kassem, 2011). As such, osteoporosis is most prevalent in the elderly and in postmenopausal women and as life span increases globally the economic burden is projected to rise substantially (Brown, McNeill, Leung, Radwan & Willingale, 2011).

2.5.1.1 Glucocorticoid excess

Glucocorticoid (GC) excess can occur by pharmacological use of glucocorticoids in disease or by endogenous means (a pathophysiological deregulation of the adrenal gland or hypothalamus stimulating GC release). GCs slow bone formation by inhibiting osteoblastogenesis and osteoblast activity, directly binding to pre-osteoblasts and mature osteoblasts (O'Brien et al., 2004). Intestinal calcium absorption and renal reabsorption are inhibited leading to calcium deficit and hyperparathyroidism (Reid, 2000). Moreover osteocytes are stimulated to resorb bone subsequently enlarging their lacunae (O'Brien et al., 2004).

2.5.1.2 Aging

Senescence causes considerable lifetime bone loss in both genders. Peak BMD is attained early in the third decade of life and is followed by an inevitable decline in density with age (Guglielmi & de Terlizzi, 2009). This bone loss is correlated to a decreased systemic secretion of growth hormone (GH) with age, and a subsequent decline in bone derived IGF-1 and TGFβ, factors that are vital to bone formation (Nicolas et al., 1994; Ohlsson, Bengtsson, Isaksson, Andreassen, & Slootweg, 1998). With aging, a greater proportion of stromal cells commit to the adipocyte lineage, which limits the available osteoblast population (Moerman, Teng, Lipschitz, & Lecka-czernik, 2004).

2.5.1.3 Sex steroid deficiency

The sex steroids protect bone mass in both genders. In males, androgen deficiency occurs gradually with age due to a slow decline in testicular activity. In females the onset of E2 deficiency occurs rapidly over the menopausal period and causes a sharp increase in the rate of bone loss, prevalence of osteoporosis, and fracture rate in postmenopausal women (Moerman et al., 2004). Henceforth osteoporosis that results from sex steroid deficiency and aging will be referred to as postmenopausal osteoporosis, as it predominantly afflicts postmenopausal women.

2.5.2 Postmenopausal osteoporosis

Menopause is defined as the terminal cessation of the menstrual period, due to a loss of ovarian follicular function that occurs naturally with reproductive aging in women, at around 51.4 years (Gold et al., 2001). Premature menopause occurs in some women and is defined as menopause occurring before 40 years of age (Luborsky, Meyer, Sowers, Gold, & Santoro, 2003). Premature menopause and surgical menopause also reduce E2 production and contribute to significant bone loss but these types of menopause will not be considered further.

Natural menopause causes a hormonal transition in which E2 levels rapidly decline (Pfeilshifter et al., 2002) and is defined by serum follicle stimulating hormone (FSH) levels >20 IU/L (Brink et al., 2008).

Bone loss occurs in both men and women at an average rate of 3% for cortical bone and 7-11% for trabecular bone per decade after PBM (Riggs, 2002). In women, the onset of menopause accelerates bone loss to 9-12% and 13% per decade for cortical and trabecular bone respectively (Riggs, 2002). Compared to premenopausal women, postmenopausal women experience approximately double the rate of bone resorption, meanwhile, their rate of bone formation rate is nearly halved (Riggs, 2002). Bone loss occurs at this rate over the first 1-5 years post-menopause, then slows over the subsequent 5-10 years to a similar rate experienced by men of the same age (Clarke & Khosla, 2010; Riggs, 2002). Rapid bone loss in postmenopausal women increases the proportion of women with low bone densities, impaired bone structure, and increased fracture risk. In the NZ population over 60 years of age, more than 50% of women have diagnosed osteoporosis compared to only one third of males, and fracture incidence is considerably higher in women at 56% compared to 29% in men (Osteoporosis New Zealand, n.d.).

2.5.2.1 Structural changes to bone in postmenopausal osteoporosis

In postmenopausal women there is a rapid loss of bone density and microarchitecture in both the cortical and trabecular bone compartments (Akhter, Lappe, Davies, & Recker, 2007; Burghardt, Kazakia, Ramachandran, Link, & Majumdar, 2010). Detrimental microarchitectural changes occurs: the trabecular network becomes increasingly perforated and fragmented, and cortical porosity increases (Akhter, et al., 2007; Burghardt, et al., 2010). The total resorptive area in postmenopausal women is much greater than age-matched men (Burghardt et al., 2010). Periosteal expansion occurs in both aging men and women as a physiological compensation to weakening bone structure, which partially offsets torsion and bending of the axial skeleton (Clarke & Khosla, 2010).

Bone loss occurs more rapidly in the trabecular compartment due to its greater metabolic activity: 20-30% of lifetime trabecular bone loss occurs in the 5-10 years after menopause compared to a 5-10% loss of cortical bone (Riggs, 2002). The most common fracture sites in postmenopausal women are the distal radius, vertebrae and femoral neck, as they are predominantly composed of trabecular bone (Barratt-Connor et al., 2005).

2.5.1.2 Changes to bone metabolism in postmenopausal osteoporosis

Estrogen has a critical role in bone health, positively regulating bone mass in both direct and indirect manners. The estrogen receptors (ER-α and ER-β) are expressed in bone, intestinal, and myriad other tissues (Braidman et al., 2001). Estrogen withdrawal during menopause induces metabolic changes that increase bone resorption and decrease bone formation (Riggs, 2002; Wehrli, Rajapakse, Magland, & Snyder, 2010).

Estrogen directly and indirectly inhibits osteoclastogenesis; this inhibition is lost in E2 deficiency (Bayliss et al., 2011). Intestinal calcium absorption declines as fewer vitamin D receptors are expressed in the jejunum, which reduces enterocyte responsiveness to vitamin D (Rizzoli et al., 2008). Intestinal calcium absorption declines and subsequently PTH levels rise (Burghardt et al., 2010). Estrogen inhibits the catabolic effects of PTH but is permissive of anabolic PTH effects. In menopause the catabolic effects of PTH are unopposed (Chen, Noland, & Kalu, 1997).

In E2 deficiency stromal and mononuclear cells increase their expression of pro-osteoclastogenic cytokines: IL-1, IL-6 and TNF-α (Crockett et al., 2011). Furthermore, E2 has mild antioxidant activity, and this loss exacerbates oxidative stress in the bone microenvironment (Pfeilschifter et al., 2002). Free radical activity enhances cytokine production from macrophages, monocytes and stromal cells (Pfeilschifter et al., 2002). Osteoclastogenesis is also enhanced by an increase in stromal cell and osteoblastic expression of RANKL, combined with a suppression of OPG expression by osteoblasts. Estrogen deficiency also increases monocyte-macrophage lineage specification resulting in larger population of immature osteoclasts (Pfeilshifter et al., 2002).

Estrogen deficiency increases the lifespan of mononuclear cells and osteoclasts have a longer lifespan due to loss of E2-induced apoptosis in these cells (Taxel, Kaneko, Lee, & Aguila, 2008).

In E2 deficiency there is a loss of direct and indirect stimulatory effects on osteoblastogenesis. Estrogen loss causes osteocyte apoptosis: this reduces osteocyte network connectivity and increases the mechanical strain required to stimulate of bone formation (Tomkinson, Gevers, Wit, Reeve, & Noble, 1998). The ER-α is required for the response to mechanical loading, however, this is a ligand-independent effect (Tomkinson et al., 1998). Skeletal expression of pro-osteoblastic IGF-1 is reduced in menopause with the loss of E2-osteocyte stimulation (Almeida et al., 2010; Chen et al., 2005). Moreover osteoblast differentiation is reduced and osteoblast activity and lifespan are reduced (Windahl et al., 2013).

2.6 Assessing bone health

2.6.1 Bone mineral density and bone turnover

BMD is the measure of bone density in each gram per square metre of bone (g/m^2). BMD is the current gold standard for estimating bone health, fracture risk and clinical response to anti-resorptive treatments (Pfeilschifter et al., 1998). As bone strength cannot be directly measured, BMD acts as a surrogate measure of bone strength: BMD is inversely correlated to fracture risk. Single-nucleotide polymorphisms that regulate BMD are strong correlates of fracture incidence (Ralston et al., 2005). However, there are myriad additional aspects of skeletal infrastructure that affect fracture risk independently of BMD (Brandi, 2009). Many fractures occur in women that have a normal BMD (greater than -1 SD from the healthy reference population) (Brandi, 2009). Additionally, a substantial change in BMD must occur before it can be detected; it takes ≥6 months, the completion of approximately three bone turnover cycles, before BMD changes significantly (Bouxsein, 2008). Biochemical markers of bone turnover are used to assess rates of bone formation and resorption as they correspond to the metabolic state of bone, on a short-term basis (Bouxsein, 2008).

2.6.2 Biochemical markers of bone resorption: CTx and DPD

Collagen fragments are released during resorption of bone (Seibel, 2005). These peptide fragments, C- and N-terminal cross-linked telopeptides of type I collagen (CTx and NTx respectively), can be detected in both serum and urine. Collagen matures within the organic bone matrices changing the aspartyl side chain from α to β conformation (Seibel, 2005). Serum βCTx is selectively measured for bone resorption markers because αCTx is associated with various other bone pathologies (Vasikaran, 2008).

Deoxypyridinoline (DPD) forms as a covalent cross-link in mature collagen, and is also released into circulation during the proteolysis of resorbed collagen peptides (Seibel, 2005). DPD represents the breakdown of mature bone and is be detectable in urine as these cross-links are not metabolised (Civitelli et al., 2009). DPD is found in both bone and dentine but DPD levels primarily originate from bone due to its more rapid turnover in comparison to dentine.

Bone metabolism has a circadian rhythm, potentially due to the cyclical release of PTH, (Shlemmer & Hassager, 1999). Humans exhibit diurnal variation in CTx and DPD with highest levels early morning (7 a.m.) declining from 10 a.m. to its lowest point in the afternoon (Ju et al., 1997). When measuring urinary DPD the use of either a 24-hour urine collection or using the second void within a specific time frame (i.e. before 10 a.m.) reduces this variation. Fasting reduces diurnal variation in plasma CTx due to a dietary influence on CTx levels, and measuring within a specific time frame (Schlemmer & Hasseger, 1999).

2.6.3 Biochemical marker of bone health: ucOC

OC is a marker of osteoblast activity and bone formation. OC embeds in the organic matrix bound to hydroxyapatite; the adsorption of OC to hydroxyapatite relies on the carboxylation of OCs three glutamyl side chains (Sokoll et al., 1997; Tsugawa et al., 2006). 10-40% of the OC produced by osteoblasts is imperfectly carboxylated (one or

more of the side chains are not carboxylated), which is reflected by serum OC measures. Serum vitamin K has a strong inverse correlation with plasma ucOC because vitamin K acts as an essential cofactor for the OC carboxylase enzyme (Binkley et al., 2000). There is increased risk of hip fracture in postmenopausal women with high levels of ucOC, as ucOC is non-functional in bone (Vergnaud et al., 1997; Booth et al., 2000). Vitamin K supplementation lowers plasma ucOC (Sokoll et al., 1997), which may improve fracture risk, although this requires confirmation from RCT measuring fracture incidence as an outcome. Vitamin K supplementation alone does not positively affect bone mass, however, vitamin K supplemented in conjunction with vitamin D and calcium enhanced the reduction of bone loss at the femoral neck in healthy postmenopausal women (Braam et al., 2003). Serum ucOC measures can be used to indicate vitamin K insufficiency and a potential increase in fracture risk in postmenopausal women (Binkley et al., 2009).

2.7 Treatment for postmenopausal osteoporosis

Hormone replacement therapy (HRT) is a prescribed combination of E2 and progesterone that decreases the risk of hip fracture in postmenopausal women, and additionally reduces the risk of colorectal cancer. However, use of HRT has diminished following extensive risk-benefit analyses by the Women's Health Initiative (WHI): HRT increases the incidence of breast cancer, blood clots, stroke and heart attack, which outweigh its benefits to bone health (Women's Health Initiative, 2002). Postmenopausal women are increasingly looking to alternative non-hormonal treatments that do have adverse health effects (Vasikaran, 2008). There is ongoing research into the relevance of bioactive dietary compounds for bone health.

2.7.1 Phytoestrogens: specific effects on bone health and general effects on cardiovascular health

2.7.1.1 Phytoestrogens: bone turnover and bone density

Phytoestrogens are plant-derived compounds consumed in the human diet that have generated research potential due to their structural and functional similarity to endogenous E2 (Women's Health Initiative, 2002). Diet in many Asian countries is habitually high in soy, which contains a subgroup of phytoestrogens called isoflavones (Vatanparast & Chilibeck, 2007). Epidemiological studies present a well-founded association between soy consumption and bone health in populations of postmenopausal Asian women that is independent of soy protein and alternative soy food constituents (Morito et al., 2001). Women in the highest quartile of soy consumption, which corresponds with the highest quartile of isoflavone intake (determined by serum or urinary isoflavones levels) have significantly greater BMD, lower levels of bone resorption markers and lower hip fracture incidence compared to Western women consuming low levels of soy (Lau et al., 1994; Lauderale et al., 1996; Ling et al., 1996; Zhang et al., 2005). Early postmenopausal Asian women (within 10 years of menopause) in the highest quintile of soy consumption were half as likely to experience a fracture compared to women in the lowest quintile of soy consumption (relative risk ratio 0.51) (Zhang et al., 2005).

2.7.1.2 Phytoestrogens: lipid metabolism and cardiovascular disease, the evidence from human studies

Heart disease also becomes more prevalent following menopause (Women's Health Initiative, 2002). Soy consumption is correlated to lower levels of heart disease in both Asian and Caucasian postmenopausal populations (Potter et al., 1998). Some studies have shown an improvement in lipid profiles in postmenopausal women consuming larger quantities of soy (Goodman-Gruen & Kritz-Silverstein, 2001; Nagata, Takatsuka, Kurisu, & Shimizu, 1998; Vatanparast & Chilibeck, 2007).

Epidemiological studies show that Asian postmenopausal women have a lower CVD burden that is strongly correlated to isoflavone intake (Goodman-Gruen & Kritz-Silverstein, 2001; Nagata, Takatsuka, Kurisu, & Shimizu, 1998; Vatanparast & Chilibeck, 2007. The physiological mechanisms that may mediate the favourable effect of isoflavone on CVD risk are diverse and include modulation of lipid metabolism pathways, which may be modulated by estrogenic activity (Chen, Yang, Zhang, & Yang, 2014). Benefits to the serum lipid profile are described as a decrease in TC, LDL-C, TAG and an increase in HDL-C (Chen et al., 2014).

Despite the cardiovascular benefits found in epidemiology and meta-analysis presenting the lipid-lowering effects of soy protein containing isoflavones (Anderson, Johnstone, & Cook-Newell, 1995; Zhan, & Zo, 2005), it has been difficult to establish an effect of independent isoflavone supplementation in postmenopausal women. Few human intervention studies have directly assessed the effect of isoflavone supplementation on lipid parameters. Moreover, there are further caveats to these studies: small study groups, which limit statistical power, variable isoflavone doses and bioavailability, variable intervention duration and the participant characteristics, including age, gender, health status, and stage of menopause (Taku, Umegaki, Sato, Taki, Endoh, & Watanabe, 2007).

Some meta-analyses of the available RCT have reported positive changes to lipid metabolism (Taku et al., 2007) and other show no change whatsoever (Hooper et al., 2008; Taku, Umegaki, Ishimi, & Watanabe, 2008; Yeung, & Yu, 2003). Nonetheless, Hooper and colleagues failed to state the average amount of isoflavones that was considered in their meta-analysis. Meta-analysis by Yeung & Yu (2003) averaged 28.5-150 mg/d isoflavone doses across observed studies, but it was not disclosed what proportion of these were genistein and daidzein; this meta-analysis was further hindered by its considerable study heterogeneity.

The first meta-analysis by Taku and colleagues (2007) found a significant decrease in serum LDL-C and TC across 11 studies, with greater effect in hypercholesterolemic women. Isoflavone intake in this study averaged at 102 mg/d (Taku et al., 2007). but

future meta-analysis by Taku and colleagues (2008) contradicted this finding, reporting no effect of isoflavones at 70 mg/d in normocholesterolemic women. Meta-analyses that examined the effect of soy protein (Taku et al., 2007; Zhan & Ho, 2005) also derived a positive correlation between isoflavone concentration within the soy protein, and a positive effect on lipid profile in both hyperlipidemic and normolipidemic postmenopausal women. Another study found that isoflavones increased HDL-C in postmenopausal women but only when taken in combination with prescribed exercise (Wu et al., 2006).

Several individual studies conclude that isoflavones exert no effect on lipid metabolism (Dewell, Hollenbeck, & Bruce, 2002). An 80 mg/d isoflavone supplement did not alter serum lipid concentrations in healthy postmenopausal women (Simons, von Konigsmark, Simons, & Celermajer, 2000). This is in agreement with another study in normolipidemic postmenopausal women that found no effect of 40 mg/d or 80 mg/d isoflavone supplements on lipid metabolism (Nestel et al., 1998). However, in the previous study genistein and daidzein were only present in small quantities in the isoflavone supplement (3.5 and 4.5 mg respectively). Some studies describe a hypolipidemic response to isoflavone supplementation in hypercholesterolemic postmenopausal women, with either a lesser response (Taku et al., 2007) or no effect at all in normolipidemic participants (Greany, Nettleton, Wangen, Thomas, & Kurzer, 2004).

The scientific literature concerning the underlying physiological interaction between isoflavones and lipid metabolism is sparse and results of the studies so far warrant further investigation.

2.7.2 Soy isoflavones and their metabolism

Genistin and daidzin are the predominant phytoestrogens found in soy foods and belong to the phytoestrogen subclass, isoflavones (Chen, Ho, Lam, Ho, & Woo, 2003a). Daidzin and genistin cannot be absorbed *in vivo* due to their glycosidic side chains; primary intestinal metabolism of isoflavones cleaves glycosides, via *β-glucosidase*, a

bacterial enzyme produced by resident microbiota (Potter et al., 1998; Wu et al., 2006). The resulting aglycone isoflavones, daidzein and genistein, are bioavailable and readily undergo trans- and paracellular intestinal absorption (Setchell et al., 2002). The conjugate and aglycone isoflavone structures are shown in figure 2.3. A second phase of enteric metabolism also occurs: daidzein gives rise to the metabolites equol and O-desmethylangolensen (O-DMA), and genistein produces the metabolite 5-hydroxyequol or is alternatively degraded to hydroxyphenylpropionic acid (Sanchez-Calvo, Rodriguez-Iglesias, Molinillo & Macias, 2013). Genistein is mostly degraded to hydroxyphenylpropionic acid (Chen et al., 2003a).

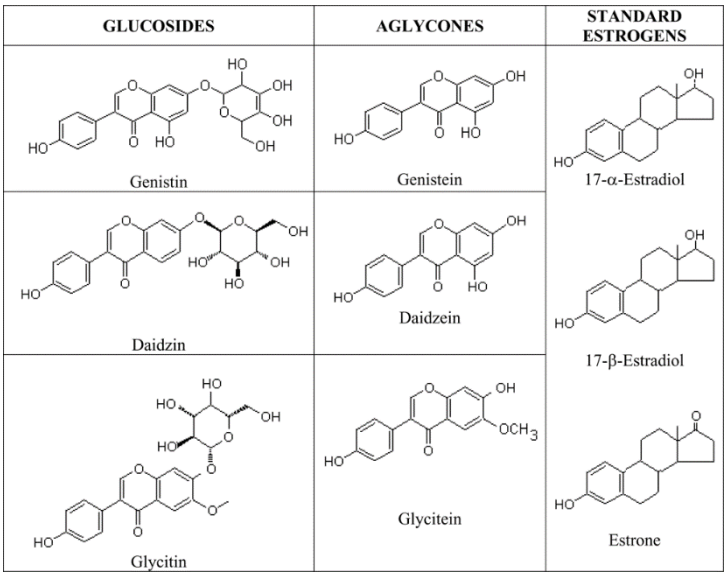

Figure 2.3 The molecular structures of soy isoflavones in their natural conjugate and aglycone forms following intestinal *β-glucosidase* cleavage, and the comparative structure of standard endogenous estrogens. Image reproduced with permission from (Lye, Kuan, Ewe, Fung, & Liong, 2009).

Daidzein metabolism exhibits large inter-individual variation: 80-90% of humans produce O-DMA, whereas only 30-50% of humans are capable of equol production (Jackson et al., 2011). The daidzein-metabolising phenotype is reliant on specific intestinal bacteria responsible for daidzein metabolism (Matthies, Loh, Blaut, & Braune, 2012). The presence of daidzein-metabolising bacteria is influenced by both hereditary and dietary factors (Setchell et al., 2005).

2.7.3 Daidzein and genistein: mechanisms of bone health modulation

2.7.3.1 *In vitro* studies

2.7.3.1.1 Estrogenic activity

Daidzein and genistein are selective ER modulators: both preferentially bind to ER-β with a relative binding affinity of 1% and 13% compared to endogenous E2 (Raimondi et al., 2009; Setchell et al., 2002). Thus daidzein has relatively weak binding affinity compared to genistein. ER-β is expressed in bone, by the osteogenic cells, and is more prevalent than ER-α (Matthies et al., 2012). Findings *in vitro* show promising bone protective effects of both daidzein and genistein by the modulation of osteogenic activity, osteoblastogenesis and osteoclastogenesis in an ER-dependent manner (Schmitt, Dekant, & Stopper, 2001).

Daidzein and genistein can increase the differentiation of osteoblast precursors and augment osteoblast activity: B-ALP activity and the mineralisation rate are increased in isoflavone-treated osteoblasts (Braidman et al., 2001; Morito et al., 2001). VDR expression increased in daidzein-treated osteoblast precursors, which could mediate increased osteoblast responsiveness to vitamin D (de Wilde et al., 2004).

Isoflavones can indirectly inhibit osteoclastogenesis. Treatment of osteoblasts with isoflavones, 0.0001 mg/day and 0.1 mg/day from a soybean extract, stimulated an increase in osteoblast output of OPG and a concomitant reduction in RANKL expression (Park et al., 2014). This resulted in a reduced maturation of pre-osteoclasts (Park et al., 2014). The isoflavone composition of the soybean extracts was not identified, which makes it difficult to draw comparisons with similar experimental work. These findings are reiterated in previous studies showing that daidzein and genistein concomitantly upregulate OPG and downregulate RANKL expression in osteoblast cell lines (Castelo-Branco & Hidalgo, 2011). Osteoblasts treated with daidzein alone had significantly increased OPG output and reduced RANKL expression (de Wilde et al., 2004). Daidzein was equally as effective as E2 used at the same dose,

which contradicts the low relative binding affinity of daidzein. This may be explained by a specific daidzein-ER-β binding conformation that enhances transcription factor affinity. The increased ratio of OPG to RANKL favoured the inhibition of osteoclast proliferation and survival in a dose-dependent manner (de Wilde et al., 2004). Both daidzein and genistein inhibited pro-osteoclastic cytokine synthesis from osteoblasts: IL-6 (Chen, Garner, Quarles, & Anderson, 2003b) and PGE-2 (via blocking TNF-α stimulation) (Suh et al., 2003).

Moreover, genistein and daidzein can directly inhibit the differentiation and activity of osteoclast-like cell precursors (Rassi, Lieberherr, Chaumaz, Pointillart, & Cournot, 2002; Tadaishi, Nishide, Tousen, Kruger, & Ishimi, 2014; Yamagishi, Otsuka, & Hagiwara, 2001). Daidzein treatment decreased the survival of pre-osteoclast monocytes and decreased the formation of active multinucleated osteoclasts, ultimately reducing bone resorptive area (Rassi et al., 2002). In addition, both daidzein and genistein inhibited the osteoclastic effects of TNF-α in an ER-dependent manner (Karieb, & Fox, 2011).

2.7.3.1.2 Other mechanisms

Additional to estrogenic activity, isoflavones modulate peroxisome proliferator-activated receptor (PPAR) activity and lineage specification of osteoblast precursors. A balance between PPAR and ER activation determines lineage specification of mesenchymal stem cell as either osteoblasts or adipocytes. Genistein and daidzein modulate the PPAR differently depending on their interaction with the PPAR types: PPARγ, PPARδ and PPARα (Dang, Audinot, Papapoulos, Boutin & Löwik, 2003).

Daidzein has a reciprocal relationship with PPARδ: at low doses (≤ 20 μM) daidzein binds to PPARδ to stimulate osteoblastogenesis but this effect is lost at higher concentrations (Dang & Lowick, 2004). At higher doses daidzein activates PPARγ, favouring adipogenesis (Dang & Löwik, 2004; Bao, Zou & Zhang, 2011). Genistein inhibits PPARγ at low doses (>1 nM for genistein) possibly through ER-mediated

synthesis of a specific inhibitory protein, which promotes osteoblastogenesis. At higher doses genistein acts as a PPARγ-ligand and upregulates PPARγ activity, increasing adipogenesis and consequently reducing osteoblastogenesis (Dang & Löwik, 2004).

Independently of its action on the ER, genistein acts as a tyrosine-kinase inhibitor. In bone this affects the tyrosine kinase receptor for M-CSF, a factor crucial to osteoclastogenesis (Gao & Yamaguchi, 2000). Genistein inhibits activation of multinucleated osteoclasts; this disrupts cytoskeletal rearrangement and the inward K^+ channel which results in reduced secretory activity, attachment to bone and survival of mature osteoclasts (Gao & Yamaguchi, 2000; Comalada et al., 2006). The inhibition of osteoclast proliferation was dose specific, occurring only at daidzein doses ≥50 μM (Comalada et al., 2006).

Daidzein and genistein both possess mild antioxidant activity (Hung et al., 2005). Genistein protected osteoblast-like cells from oxidative damage (Lee, Chen, & Anderson, 2001). Inflammatory cytokines cause oxidative damage *in vivo*, and contribute to postmenopausal bone loss due to decreased osteoblast survival (Pfeilshifter et al., 2002).

2.7.3.2 Animal studies

Ovariectomy induces bone loss in murine species in a similar manner to postmenopausal bone loss. Ovariectomised (Ovx) murine species are used to assess the effects of anabolic bone treatments and their potential application to human bone health. Importantly, isoflavones reach plasma levels equivalent to those attained in humans (Taku, Melby, Nishi, Omori, & Kurzer, 2011).

Isoflavones provided in the diet of skeletally mature Ovx rats suppressed bone loss and preserved microarchitectural elements at four skeletal sites (Picherit et al., 2001b). Protection against bone loss was similar for the higher doses of 40 and 80 μg/g body weight/day (Picherit, et al., 2001b). Moreover, failure load of the femur decreased in Ovx mice but this mechanical impairment was completely counteracted by isoflavone

supplementation. In addition, Isoflavone treatment both reduced urinary DPD and maintained BMD in Ovx rats, whereas BMD was significantly reduced in control rats (Picherit et al., 2001b).

In adult Ovx rats with established osteopenia isoflavones suppressed bone resorption but did not reverse structural damage (Bitto et al., 2008; Picherit et al., 2001a). This suggests that isoflavone treatment in postmenopausal women may be more effective in early menopause, when the rate of bone loss peaks, compared to later in menopause when bone quality has already deteriorated significantly. Conversely, another study found that a large isoflavone dose mildly improved Ovx-induced microarchitectural damage (Devareddy et al., 2006): trabecular separation was reduced and there was a trend toward increasing trabecular number, however, BMD loss was unaffected. Furthermore, Devareddy et al. (2006) compared group soy protein containing isoflavones and isoflavone-devoid soy protein. Rats consuming soy without isoflavones did not have an improvement in trabecular structure, which would suggest that the isoflavones mediated the change in bone structure regardless of the additional soy protein constituent. The amount of isoflavones received by these study animals is inconclusive because the isoflavone dose was based on the weight of the diet consumed rather than animal weight and data on consumption was not provided.

Another study shows isoflavone supplementation and calcium supplementation has a positive effect on BMD in Ovx rats, however isoflavone supplementation alone had no benefit to BMD (Breitman, Fonseca, Cheung, & Ward, 2003). The isoflavone dose in this study relative to the weight of diet consumed, without any indication of consumption, moreover, the specific isoflavone composition was not provided, nor was a measure of isoflavone bioavailability; this hampers the extrapolation of such results to human isoflavone studies.

When administered as separate isoflavones, daidzein was more effective than genistein at preventing bone loss in Ovx rats (Picherit et al., 2000). BMD at the total body, femur and lumbar spine was maintained at a density equivalent to that in sham-operated rats; genistein did not prevent Ovx-induced bone loss at these sites (Picherit

et al., 2000). Daidzein treatment (10 mg/g/day) prevented the deterioration of trabecular microarchitecture in adult Ovx mice (Tyagi et al., 2011). Daidzein eliminated detrimental immune changes that are associated with ovariectomy: an expansion of high TNF-α producing T-cell population and an increase in RANKL expression from B-cells. As a result, osteoclastogenesis was reduced with daidzein supplementation (Tyagi et al., 2011).

Daidzein increased trabecular thickness and restored trabecular morphology in the tibia of young Ovx rats (Somjen, Katzburg, Kohen, Gayer, & Livne, 2008). In addition, there was a significant reduction in the adipocyte population in the bone marrow, and thus a larger population of pre-osteoblasts. Genistein-treated rats did not experience these bone-protective effects (Somjen et al., 2008). However, because the skeleton is developing in immature Ovx rats these results are not as translatable to postmenopausal bone health.

2.7.3.3 Human studies

Western populations typically consume negligible levels of soy and thus are exposed to very low levels of isoflavones (Setchell et al., 2013; Taku et al., 2010a). Numerous RCTs have examined the effects of isoflavone supplementation on parameters of bone health in these populations, examining BMD and biochemical markers of bone turnover; no trials have examined fracture incidence. Table 2.1 summarises some of the recent RCTs examining isoflavone supplementation and BMD in postmenopausal women. Despite epidemiological evidence and the multitude of positive findings in animal and *in vitro* studies, the outcomes in human trials have been inconsistent. Some RCTs report a moderate protection against bone loss and bone resorption with isoflavone supplementation, while other studies found no difference between isoflavone and placebo treatment groups (Alekel et al., 2010; Levis, Strickman-Stein, Ganjei-Azar, & Xu, 2011).

Women on a one-year genistein intervention, 54 mg/day, exhibited a significant reduction in markers of bone resorption, urinary DPD and pyridinoline (PYD), compared to the placebo group. Genistein also increased markers of bone formation, serum OC and urinary B-ALP. Furthermore, BMD was increased by ~3% at the femoral neck and lumbar spine in the genistein group (Morabito et al., 2002). Similarly, three-year genistein supplementation, 54 mg/day, modestly increased BMD compared to the placebo group who lost bone mass over the trial period (Marini et al., 2007). Potter et al., (1998) found that in a postmenopausal study group 90 mg/day soy protein containing isoflavones significantly increased BMD at the lumbar spine by 2.2% compared to a 6.4% decrease in the control group. The control group received isoflavone-devoid soy protein, which suggests the bone-sparing effects were correlated with the isoflavones independent from the soy protein.

Regardless of these heterogeneous results, two meta-analyses found robust inhibition of bone loss at the lumbar spine (Ma, Qin, Wang, & Katoh, 2008a; Taku et al., 2010a). Taku et al. (2010a) found that isoflavone supplementation, ≥82 mg/day for ≥1 year, increased lumbar spine BMD by 2.4% or 20.3 mg/m^2. These results are in conjunction with an earlier meta-analysis: isoflavone supplementation, ≥90 mg/day for >6 months, increased spinal BMD by 20.6 mg/m^2 (Ma et al., 2008a). Only three RCTs examined hip BMD, which is insufficient for meta-analysis. Sub-group analysis within these studies restricts the significant bone sparing effects to Caucasian women (Taku et al., 2011). A separate meta-analysis found no benefit of isoflavone supplementation to BMD (Liu et al., 2009). Liu et al. (2008) excluded all trials less than one-year in duration concluding studies of this duration only represent transient effects limiting their sample size and statistical power. In addition this meta-analysis calculated the net change in BMD per year as opposed to overall change in BMD, which could reduce the apparent change in BMD (Taku et al., 2011). BMD at the trochanter and total hip were unaffected by isoflavone treatment (Taku et al., 2011), however, there was a limited number of trials (n=5) measuring BMD at these sites. When separately analysing trials with longer durations (>2-years) there was a trend of increasing femoral neck BMD 2.5% (P=0.08) with isoflavone supplementation (Taku et al., 2011).

Meta-analyses have also shown a consistent decrease in urinary DPD in response to isoflavone supplementation (Ma, Qin, Wang, & Katoh, 2008b; Taku et al., 2010b). Urinary DPD decreased significantly with isoflavone supplementation, 50-90 mg/day >10 weeks (Ma et al., 2008b; Taku et al., 2010b). Only a limited number of RCTs (n=2) assessed the resorption marker CTx; one trial found that genistein supplementation, 54 mg/day, significantly reduced plasma CTx over the second to third of supplementation compared to baseline (Marini et al., 2008). More trials are required to see if this effect is consistent in postmenopausal women. No human isoflavone trials have measured plasma ucOC as an outcome (Taku et al., 2010b).

2.7.3.4 Discrepancies in human studies

Several factors may contribute to the discrepancies between RCTs examining postmenopausal bone health in response to isoflavones.

In meta-analyses, osteopenic postmenopausal women experienced greater increases bone mass compared to postmenopausal women with normal BMD (Marini et al., 2007). Also, some study groups had a considerably lower yearly rate of bone loss than the general postmenopausal population (Levis et al., 2011). Menopausal status of the study population was important; a greater effect was seen in early postmenopausal women, within 10 years of the onset of menopause, compared to women in late menopause (Marini et al., 2007). A large epidemiological study corroborates this finding: comparing women within the highest quintile of isoflavone consumption women within 10 year of menopause had a greater reduction in fracture risk (Zhang et al., 2005). Alternatively, Ma et al. (2008a), found bone loss was significantly inhibited only in women in the first five years of menopause experience. Meta-analysis of bone turnover in response to isoflavone supplementation determined at least 6-months intervention duration is needed to elicit an effect on BMD through changes to the bone remodelling cycle (Taku et al., 2010; Weaver & Cheong, 2005). Each remodelling cycle takes at least 120 days to complete and it is thought that it takes 1-3 cycles to detect significant change in BMD (Weaver & Cheong, 2005).

Meta-analysis found isoflavone supplementation had greater bone protective effects in Westerners. This may be due to high dietary isoflavone consumption in Asians, ~90 mg of soy isoflavones daily; benefits to bone health may be limited above this dose. Alternatively, detecting any additional benefits in Asian populations may require a larger sample size to detect an effect (Ma et al., 2008a).

Isoflavone composition varies widely across RCTs (Ma et al., 2008a): many studies used isoflavones in their glycosidic form without measuring isoflavone bioavailability. In these trials the exact dose is unknown because glycosidic isoflavones do not provide the same amount of aglycone isoflavones as a pure aglycone supplement of an equal dose. Delineating an optimal isoflavone dose is difficult (Kenny et al., 2009). Moreover, animal studies show that daidzein and genistein have different effects on bone metabolism at different doses. Supplements of mixed daidzein and genistein ratios may act synergistically or antagonistically.

Many trials did not duly account for crucial confounding factors, such as calcium intake, serum vitamin D, and habitual soy intake (Poulsen & Kruger, 2008). These factors may change significantly over the course of an intervention, which will may confound results and mask the bone-sparing effects of isoflavones.

Research attention is now focussed on the large inter-individual heterogeneity in daidzein metabolism: equol is more bioactive than its precursor, and some studies have shown equol-producers are more responsive to isoflavone supplementation than non-equol producers (Uehara, 2013). In this case, the skeletal benefits of isoflavone supplementation will depend on the number of equol-producers within the study group (Jackson et al., 2006; Setchell & Clerici, 2010; Vatanparast & Chilibeck, 2007).

Table 2.1 A summary of some recent isoflavone interventions in postmenopausal women and the effect on BMD

Study	Sample size (n) and average number of years post-menopause	Duration	Isoflavone type, preparation and form (glycoside or aglycone)	BMD measures: site/s and method	Significant BMD outcomes (P value ≤ 0.05)
Alekel et al., 2010 Randomised double-blinded placebo-controlled RCT	n=224 1-5 years postmenopausal 80 mg/d protected against bone loss at the lumbar spine	3 years	120 mg/d or 80 mg/d of soy protein isolate containing genistein, daidzein and glycitein in the ratio 1.3:1:0.3 DEXA	BMD of the femoral neck, proximal femur, lumbar spine and whole body	80 mg/d protected against bone loss at the lumbar spine
Brink et al., 2008 Randomised double-blinded placebo-controlled RCT	n=37 2.8 years postmenopausal	1 year	110 mg/d aglycone isoflavones 60-75% genistein, 25-35% daidzein, 1-5% glycitein incorporated into biscuits	DEXA BMD of the whole body and lumbar spine	No protective effect of isoflavones on BMD loss
Huang et al., 2006 Randomised non-blinded placebo-controlled RCT	n=43 ≤6 years postmenopausal	1 year	100 mg/d (71 mg genistein; 29 mg daidzein) or 200 mg/d aglycone isoflavones (142 mg genistein; 58 mg daidzein)	DEXA BMD of the lumbar spine, femoral neck, Ward's triangle and trochanter	Isoflavones had no protective effect on bone loss No report of treatment compliance
Kreijkamp-Kaspers et al., 2004 Randomised double-blinded placebo-controlled RCT	n=175 17 years postmenopausal	1 year	99 mg/day aglycone isoflavones: 52 mg/d genistein, 41 mg/day daidzein, 6 mg/day glycitein	DEXA BMD of proximal femur and lumbar spine (L1-4)	No protective effect of isoflavones on BMD loss

Levis et al., 2011 Randomised double-blinded placebo-controlled RCT	n=248 ≤5 years postmenopausal	2 years Sub-group of equol producers identified	99 mg/day aglycone isoflavones: 52 mg/d genistein, 41 mg/day daidzein, 6 mg/day glycitein	DEXA BMD of the femoral neck, total hip and lumbar spine	No effect on BMD Non-significant trend in equol producers for inhibition of bone loss
Shedd-Wise et al., 2011 Randomised double-blinded placebo-controlled RCT	n=216 1-8 years postmenopausal	2 years	120 mg/d or 80 mg/d of isoflavone capsules containing aglycone genistein, daidzein and glycitein (ratio 1.3:1:0.3)	pQCT Trabecular and cortical Volumetric BMD of the distal tibia and midshaft femur	120 mg/day had a modest protection of volumetric cortical BMD of mid-shaft femur with increasing time since onset of menopause
Tai et al., 2012 Randomised double-blinded placebo-controlled RCT	n=431 <10 years postmenopausal	2 years	300 mg/day aglycone isoflavones: 172.5 mg genistein; 127.5 mg daidzein.	DEXA BMD of the lumbar spine and femoral neck	Isoflavones had no protective effect on bone loss
Wong et al., 2009 Randomised double-blinded placebo-controlled RCT	n=403 ≤14 years postmenopausal	2 years	80 mg/d or 120 mg/d of aglycone isoflavone capsules containing:	DEXA BMD of the femoral neck, lumbar spine, total hip, trochanter, and whole body	120 mg/d dose significantly decreased whole body bone loss

2.8 The importance of equol

Despite the well-established population heterogeneity of the equol-producer phenotype and evidence that equol possesses significant bioactivity, very few human trials have accounted for equol production (Jackson et al., 2011; Uehara, 2013).

2.8.1 Equol benefits to bone

2.8.1.1 *In vitro* studies

Equol is a chiral molecule that exists as two enantiomers, S-equol and R-equol (Muthyala et al., 2004). In both humans and many other animal species the bacterial metabolism of daidzein invariably produces the S-equol conformation. The S-equol enantiomer has strong selective affinity for ER-β whereas R-equol has moderate affinity for ER-α. S-equol has 100-fold greater affinity to ER-β compared to its parent molecule, daidzein (Kimira et al., 2012). Equol bioactivity is further increased owing to the greater proportion of free serum equol, thus equol has greater opportunity to bind the ER-β (Kimira et al., 2012). In comparison, serum-binding proteins sequester a large proportion of daidzein, genistein, and endogenous E2. Furthermore, equol is a more potent antioxidant than either daidzein or genistein (Sanchez-Calvo et al., 2013). Equol was shown to repress adipogenesis, stimulate osteoblastogenesis, and inhibit osteoclastogenesis in bone marrow MSC via anti-inflammatory mechanisms (Nishide et al., 2013).

2.8.1.2 Animal studies

Complete daidzein metabolism occurs in murine species without inter-individual variation. Daidzein supplementation in Ovx mice and rats has produced more consistent benefits to bone health compared to human trials. The bone-sparing effects of daidzein in Ovx mice and rats may be accounted for by equol production (Ishimi, 2010).

Equol (0.5 mg/day) treatment reduced bone loss in the whole body, femur, and lumbar spine in Ovx mice (Fujioka et al., 2004) to an similar levels as E2 treatment. Plasma equol levels reached 1550.6 nmol/L. Another study found that equol administration in Ovx mice prevented loss of trabecular structure and density at the proximal tibia and lumbar spine (Rachoń, Seidlová-Wuttke, Vortherms, & Wuttke, 2007). Equol intake was estimated at 6.87 mg/day based on consumption of a 400 mg equol/kg diet, but it was not stated whether pure S-equol or a racemic supplement was used and serum equol was not measured to confirm this (Rachoń et al., 2007). A racemic equol supplement in Ovx mice increased femoral calcium content but had no effect on femoral BMD at a high dose of 200 mg/kg diet. This racemic mix provided S-equol at 100 mg/kg diet consumed (Legette et al., 2009) and total serum equol was between 30-40 μmol/L (30,000-40,000 nmol/L), which is considerably higher than serum equol levels obtained by Fujioka et al. (2004). However, R-equol and S-equol have different affinities for the ER subtypes and it is unknown whether R-equol can interfere with the ER-β bioactivity of S-equol (Kimira et al., 2012). Further studies are required to address this issue.

Equol (4.65 mg/day) supplementation in Ovx rats maintained femoral microarchitecture and mechanical integrity (Tezval et al., 2010), whereas genistein failed to exhibit an effect despite using doses similar to previous studies (Ishimi et al., 2000). Equol treatment increased the force was required to induce fracture and there was a significant increase in trabecular area and width (Tezval et al., 2010), however, plasma equol was not measured in this study. Trabecular density and cortical area in Ovx rats were increased with equol treatment, estimated 12 mg/day equol from 4 mg/g feed consumed, but equol did not inhibit detrimental changes to biomechanical properties or total BMD (Sehmisch et al., 2010). The equol supplement used by Sehmisch et al. (2010) was racemic and provided 6 mg/day of bioactive S-equol, but this dose of equol had previously elicited a positive response in Ovx rats (Tezval et al., 2010). Nonetheless, without measuring serum equol the extent of skeletal exposure to equol is unknown.

One study investigated how inflammatory mechanisms may potentiate the bone protective effects of equol. Expression of the pro-inflammatory cytokines, TNF-α and IL-I, are downregulated in the bone marrow Ovx mice following equol treatment (Nishide et al., 2013). In E2 deficiency these cytokines increase T-cell senescence and B-cell maturity, which subsequently induces RANKL expression. The RANKL/OPG ratio was lowered in equol supplemented Ovx rats and this downregulated osteoclastogenesis and reduced bone resorption (Nishide et al., 2013). Ultimately bone loss was completely inhibited at the distal femoral metaphysis (Nishide et al., 2013). Once again the exact dose of equol was unknown as equol was provided as 0.064 g/100g feed consumed and not measured in the serum.

2.8.1.3 Human studies

In humans, the equol producer status is defined by a fasting serum concentration of >20 nmol/L or >5 μg/L (Setchell et al., 2005). Alternatively a 24-hour urine collection concentration of >82 nmol/L or >20 μg/L is indicative of equol-production (Setchell et al., 2005). A urinary ratio of equol to daidzein, greater than -1.75, is a more accurate measure of equol status than an absolute value as it accounts for proportionate metabolism of a known dose of daidzein (Setchell et al., 2005).

Three isoflavone trials that have accounted for equol production found bone-sparing effects were exclusive to equol producers (Jackson et al., 2011). The equol producer groups in these trials had serum equol concentrations ranging from 44-150 ng/mL from dose of daidzin/daidzein ranging from 38-39 mg/day.

One study in postmenopausal Caucasian women (average of 52.8 years) found that aglycone isoflavones, 76 mg/day in soymilk, protection from bone loss was much higher in the subgroup of equol producers (Lydeking-Olsen et al., 2004). Equol producers experienced a 2.4% increase in lumbar spine BMD while the control group lost 4% of their BMD at this site (consuming isoflavone-devoid soymilk). Comparatively, the non-equol producers had only 0.6% increase in bone mass.

However, equol status was not prospectively screened and the resulting equol producer group was too small to detect significance (n=10) (Lydeking-Olsen et al., 2004).

Isoflavone supplementation over 24 weeks in Japanese postmenopausal women significantly attenuated bone loss in the equol producer subgroup compared to non-equol producers and placebo group (Wu et al., 2006). Bone mineral density decreased by 0.53% and increased by 0.13% at the whole body and total hip respectively in equol producers. In non-equol producers bone loss was equivalent to the control group, with a 1.35% and 1.77% decline in BMD at the whole body and total hip respectively (Wu et al., 2006). All subjects consumed their regular diet of 40-45 mg soy isoflavones but the equol producers within the control group had much lower serum equol levels of 14 ng/mL than the isoflavone-treated equol producers.

One-year supplementation of isoflavone conjugates, 76 mg/day, inhibited bone loss in postmenopausal Japanese women, who were within 5 years of menopause (Wu et al., 2007). BMD decreased at the total hip and inter-trochanteric region by 2.38% and 2.61% in the non-equol producers, whereas equol producers experienced a significantly smaller decline of 0.46% and 0.04% at the same sites. Bone loss in non-equol producers was equivalent to the control group.

Another intervention in Caucasian postmenopausal women had contradictory results, reporting no effect of isoflavone supplementation even when stratifying groups in to equol producers and non-producers (Kenny et al., 2009). In this trial, equol producers were delineated appropriately by a serum equol >5 ng/mL and the average serum equol of >35 ng/mL in the equol producers is similar to the previous trials measuring equol production. However, the study population was on average 22-25 years postmenopausal and evidence suggests that isoflavones are only effective in early menopause, within the first 10 years after cessation of menses (Ma et al., 2008a).

Levis and colleagues (2011) trial report that isoflavone had no effect on bone loss in postmenopausal Caucasian women bone regardless of equol production and the early menopausal status of the group (within 5 years). However, equol-producer status was defined as the presence of any amount of equol in urine; this criteria would have falsely identified participants as equol producers because serum equol levels <5 ng/mL are classed as non-equol producers and this would mask the effects of equol production. The findings of Brink et al. (2008) in postmenopausal Caucasian women within three years since menopause, also suggest that equol producers do not experience reduced bone loss compared to non-equol producers. In this study a serum equol level of >1000 nmol/L was used to determine equol producers, however, equol screening was retrospective. Equol producers were not identified and assigned randomly to groups prior to the study, which could bias results. Several other isoflavone RCTs measured equol levels that did not prospectively screen for this phenotype (Alekel et al., 2010; Liu et al., 2009; Wong et al., 2009).

Bone resorption was significantly decrease in non-equol producing early postmenopausal Japanese women receiving a pure S-equol supplement for 12 months (Tousen et al., 2011). Equol treatment reduced DPD by 24% compared to 3% in the control group (Tousen et al., 2011). Only the highest equol dosage was effective, 10 mg/day, which produced serum equol concentrations ranging from 26.3-85.1 nmol/L. Wu et al. (2006) measured serum equol at 503.3-515.8 nmol/L in their postmenopausal study group, a level considerably higher than Tousen et al., (2010), which may account for their lack of change in BMD. Equol administered as a pure supplement reaches peak serum concentration one hour after ingestion, whereas equol produced from daidzein metabolism slows the rate of absorption and could account for the difference in serum equol levels (Tousen et al., 2011).

More human trials are required to elucidate the physiological effects of equol on bone health and the effective dose requirements for skeletal health (Jackson et al., 2011). Of particular interest is the potential to modulate equol producer status in the postmenopausal population.

2.8.2 Factors influencing the equol producer phenotype in humans

Equol production is completely reliant on the composition of the enteric microbiome, and several candidate bacteria have been identified as equol-producing (Bolca et al., 2007). Several bacterial species isolated from human equol producers are capable of daidzein metabolism *in vitro,* e.g. *Adlercreutzia equolifaciens, Slackia isoflavoniconvertens, Slackia equolifaciens,* and *Lactococcus garvieae* (Sanchez-Calvo et al., 2013). It is apparent that daidzein conversion to equol relies on myriad species and explains the inter-individual herterogeneity (Sanchez-Calvo et al., 2013). The equol producer phenotype is stable over time and has significant hereditary regulation, but environmental factors also contribute the development of this phenotype (Jackson et al., 2011). Antibiotic treatment in equol-producers temporarily abolishes their ability to metabolise daidzein (Atkinson, Frankenfeld, & Lampe, 2005; Sutherland et al., 2012). There is also inter-individual variation in response to antibiotic due to antibiotic resistance of particular equol-producing bacteria (Setchell et al., 2013).

Differences in diet and body composition (which is related to diet) may modulate equol-production. Overweight and obesity have been inversely associated with equol production in an Asian population (Wu et al., 2012). Asian populations and vegetarians have a greater proportion of equol producers compared to Westerners/omnivores and this has been correlated to the different dietary patterns (Setchell et al., 2013; Uehara, 2013). Numerous observational studies report that in adult Asian populations and vegetarians ~60% are equol producers compared to 25-30% in non-vegetarian Western populations (Setchell et al., 2005; Setchell et al., 2013).

2.8.2.1 Dietary factors

The impact of dietary composition on the daidzein-metabolising phenotype has produced conflicting results. Older Asian populations consume greater quantities of isoflavones and this correlates to a greater proportion of equol producers compared to younger generations: 45% in teenagers and 40% in 20-30 year olds compared to 65% and 80% in 40 and 50 year olds, respectively (Fujimoto, 2008). Daidzein and genistein are not exclusively found in soy, but this is the predominant source of these isoflavones (Chen et al., 2003a) and it is likely that the greater quantity of soybean protein consumed in this population would account for the higher daily isoflavone intake. Other studies suggest that isoflavone intake does not influence equol-producer phenotype but that it is simply a prerequisite for equol-production, as well as with the appropriate microbiome (Setchell et al., 2013).

More recent extensive dietary analysis of Caucasian equol producers reports a correlation between equol production and the intake of polyunsaturated fatty acids, calcium, vitamin A and E, and maltose (Setchell et al., 2013). It is possible that these dietary components are required as cofactors for growth and activity of daidzein-metabolising bacteria (Setchell et al., 2013). The same study found no effect of macro- or micronutrients on the epidemiology of the equol-producer phenotype. Different studies have found positive associations between the equol producer phenotype and high-carbohydrate/low-fat diets (Rowland, Wiseman, Sanders, Adlercreutz & Bowey, 2000), and the number of vegetable servings per day (Atkinson et al., 2005). However these findings are inconsistent and alternative studies contradict these findings (Gardana, Canzi, & Simonetti, 2009). The association between vegetable intake and an equol-producer phenotype may be related to soluble fibre: there is significant evidence that soluble fibre modulates the gut microenvironment, and this could influence daidzein-metabolising bacteria presence and activity (Tousen et al., 2013).

Fructooligosaccharides (FOS), polydextrose and raffinose are specific type of soluble dietary fibre with prebiotic effects, stimulating the growth and activity of some species of symbiotic enteric bacteria (Bouhnik, Raskine, Simoneau, Paineau, & Bornet, 2006). The daidzein-metabolising microbiome may be promoted by FOS consumption. An animal trial found the bone sparing effects of daidzein were greater with the supplementation of FOS even at the lowest isoflavone dose which had previously lacked significance (Mathey et al., 2007). Ohta et al. (2002) reported a similar result in Ovx mice: the combination of isoflavone and FOS supplementation increased the metabolism of daidzein to equol, and restored femoral bone density to a greater extent than isoflavones alone. Similarly, a combination of polydextrose and raffinose increased serum equol in Ovx rats and further inhibited femoral bone loss compared to equol alone (Tousen, et al., 2013).

In human trials, the association between equol production and combined isoflavone/FOS supplementation is less clear (Tousen et al., 2013). In non equol-producers supplementation of dietary fibre did not alter daidzein metabolism (Lampe et al., 2001; Larkin, Price, & Astheimer, 2007). Nonetheless research is currently limited to these trials, both of which had limited sample sizes. Short-term FOS intake in a postmenopausal Japanese population did not affect serum equol levels in either equol producers or non-producers (Tousen et al., 2013). However, this trial was a pilot study with a small sample size and short duration. Larger trials are warranted to more accurately assess the interaction between soluble fibre intake and daidzein metabolism in postmenopausal women.

2.8.2.2 Green kiwifruit, equol production and bone health

Green kiwifruit (*Actinidia deliciosa*) contains both soluble and insoluble fibre and has been shown to function as a whole food prebiotic (Lee, Low, Siah, Drummond, & Gwee, 2012; Rosendale et al., 2012).

A few studies have attempted to evaluate whether green kiwifruit consumption can modulate the equol-producing phenotype *in vivo* (Tousen et al., 2013). Daidzein and kiwifruit supplementation significantly increased the inhibition of bone loss in Ovx rats compared to daidzein alone. However, equol productions were not affected by kiwifruit consumption (Tousen et al., 2013).

Green kiwifruit contains the micronutrients: vitamins A, B6, C, E, and K, and minerals, iron, calcium, magnesium, copper, folate, and potassium (Agricultural Research Service, 2011). Green kiwifruit is a superior source of vitamin K in comparison to gold kiwifruit (table 2.2). Table 2.2 displays the micronutrient composition of green kiwifruit and the relative contribution to national RDI guidelines (NHMRC & MoH, 2006).

Table 2.2 Green kiwifruit and gold kiwifruit: selected micronutrient content and percentage contribution to RDI in NZ women (51-70 years) (Agricultural Research Service, 2011; NHMRC & MoH, 2006)

Micronutrient	**Content in green kiwifruit (mg/100g)**	**Content in gold kiwifruit (mg/100g)**	**RDI or AI (mg/day women aged 51-70)**	**RDI/100g green kiwifruit (%)**	**RDI/100g gold kiwifruit (%)**
Vitamin K	0.04	0.006	0.06	66.7	10
Vitamin C	93	105	45	206.7	233.3
Calcium	34	20	1300	2.6	1.5
Magnesium	17	14	320	5.3	4.4
Phosphorus	34	29	1000	3.4	2.9

Vitamin A and E have been shown to increase the frequency of the equol-producer phenotype, thus an increased intake of these micronutrients could increase propagate equol-producing bacteria (Setchell et al., 2013). To date, there has been no research regarding the effect of vitamin A or E on the equol-producer phenotype in humans.

Consumption of kiwifruit may impact bone health independently of effects on daidzein metabolising bacteria. Vitamin K and C, calcium, magnesium and phosphorus are particularly important micronutrients in bone health. Green kiwifruit is also a good source of carotenoids, in particular lutein (Sommerburg, Kuenen, Bird, & van Kujik, 1998), which has the potential to modulate bone health (Tousen et al., 2013). Lutein has been shown to enhance the action of equol in reducing osteoclastogenesis (Tadaishi et al., 2011).

Vitamin K is abundant in green kiwifruit and promotes bone health by maximising OC carboxylation. Calcium intake is often insufficient in postmenopausal women (Cashman, 2007) and vitamin C has been shown to increase the bioavailability of calcium in an animal study (Morel & Wolber, unpublished data as cited in Wolber et al., p249, 2013. Calcium uptake is facilitated and retention is increased *in vivo* in piglets fed green kiwifruit, which was due to the vitamin C content. The vitamin C content of kiwifruit could affect calcium absorption and calcium balance in calcium deficient populations and thus the rate bone resorption. Magnesium deficiency is also a risk factor for postmenopausal osteoporosis due to its structural role in bone and modulation of bone metabolism, however, kiwifruit only contribute a small amount of the Mg RDI for 51-70 year old women (Orchard et al., 2014).

The soluble fibre in kiwifruit could also increase dietary calcium and magnesium absorption, based on studies of mineral absorption and FOS supplementation in mice (Ohta et al., 1995; Ohta et al., 1997).

2.9 Summary

Menopausal reduction in endogenous E2 levels significantly increases the incidence of osteoporosis and CVD in the postmenopausal population (Jackson et al. 2011; Zhang et al., 2005). The estrogenic soy isoflavones, daidzein and genistein, act as ER-β agonists in bone and other tissues (Taku et al., 2011). In Asian populations high soy isoflavone consumption is associated with a reduced fracture risk, increased BMD and reduced risk factors for CVD (Lau et al., 1994; Zhang et al., 2005). Osteogenic cell culture studies and trials in Ovx rodents support the use of soy-derived isoflavones, genistein and daidzein, for the reduction of bone loss (Taku et al., 2011) and improvement of the lipid profile (Taku et al., 2007). The literature on human isoflavone interventions has shown inconsistent benefits to bone and cardiovascular outcomes. A major factor underlying these discrepancies may be participant heterogeneity in intestinal daidzein metabolism to the potent metabolite equol. Rodent and cell studies have shown equol to exhibit greater bioactivity in bone metabolism, and in the few human trials that have measured the equol-producer phenotype, equol positively impacted bone loss and serum lipid parameters to a greater extent than non-equol producers. However, research in this area is lacking and future daidzein interventions are required to clarify the effects of equol on postmenopausal bone and cardiovascular health. Furthermore, green kiwifruit represent a suitable prebiotic food to investigate the effect of soluble fibre on daidzein metabolism in human isoflavone interventions. The additional micronutrients and phytochemicals present in kiwifruit, such as vitamin K, lutein, and vitamin C may modulate bone metabolism independently of prebiotic effects.

Chapter 3

Methods

3.1 Intervention overview

This study was a 16-week randomised crossover pilot study in postmenopausal women carried out at Massey University, Palmerston North during August-December 2013. This study implemented a green kiwifruit and soy isoflavone intervention. Participants made a total of four trips to the Human Nutrition Research Unit at Massey University at weeks 2, 8, 10 and 16. During these visits blood and urine samples were collected.

3.2 Intervention design

Healthy, postmenopausal Caucasian women (1-10 years postmenopause, >50 years of age) were the target population for this study. Participants were taken through a health-screening questionnaire prior to the study commencing to assess inclusion and exclusion criteria (appendix 3).

Participants were randomly allocated to either treatment group A or group B. Group A received daily isoflavones in the first intervention period followed by daily kiwifruit and isoflavones in the second intervention period and vice versa for group B. Each intervention period was 6-weeks in duration preceded by a 2-week lead-in period and followed by a 2-week washout period, shown in figure 3.1. There was no placebo group in this experimental design. The two-week lead-in and washout periods allowed for participant adjustment to the dietary restrictions and reduced confounding during the intervention periods (appendix 1).

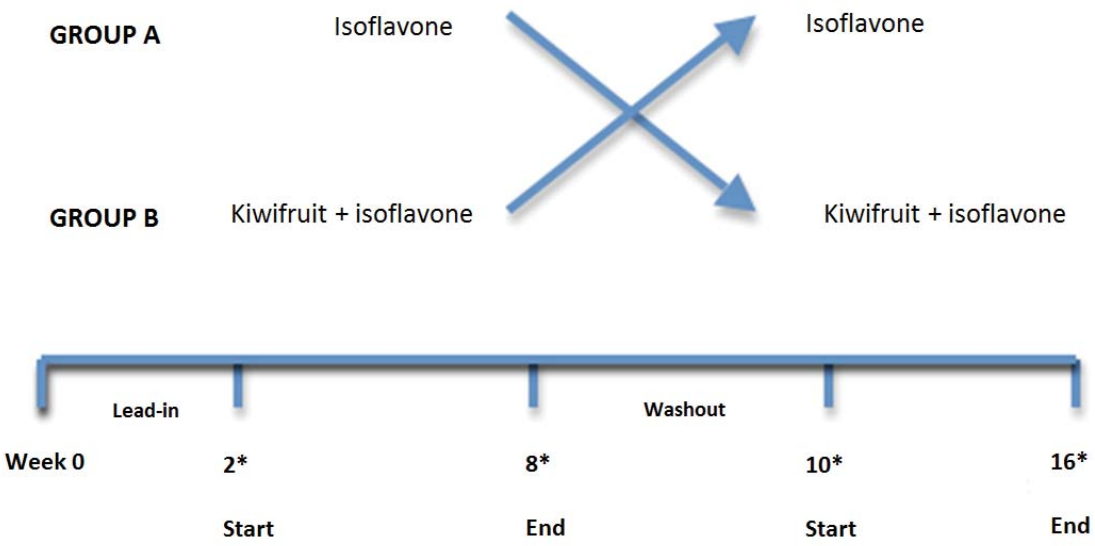

Figure 3.1 An illustration of this 16-week crossover intervention. At week 0 participants were allocated to group A or B. The lead-in period occurred over weeks 0-2. The treatments proceeded during weeks 2-8 and weeks 10-16 with a washout period in weeks 8-10. An * indicates the weeks in which blood and urine samples were collected.

The isoflavone supplement was in oral capsule form; participants were instructed to take two per day in the morning. During the kiwifruit and isoflavone intervention participants were directed to eat two kiwifruit per day with the isoflavone capsules. Compliance to the intervention regime was monitored through a compliance chart completed by the participants (appendix 6).

3.2.1 Soy isoflavone supplement

The soy isoflavone supplement 'Nature Made Soy Isoflavone™' consisted of aglycone daidzein and genistein, and was manufactured by Otsuka Pharmaceutical Company Ltd., Tokyo, Japan. In total, participants received 50 mg of isoflavones daily during each 6-week treatment of which 47.2 mg were aglycone and 2.8 mg were glycoside-conjugates. Participants were provided with a 16-week supply of isoflavone supplement at the initiation of treatment (week 2).

3.2.2 Green kiwifruit

The green kiwifruit used in this study were provided by Zespri® International, Mt Maunganui, NZ, and were delivered to Plant and Food Research, Palmerston North. Participants received an initial tray of kiwifruit from the Human Nutrition Research Unit at the beginning of their kiwifruit/isoflavone treatment, during week two and week 10 for groups B and A respectively. Additional trays of kiwifruit were either picked up from the Nutrition Unit or delivered to participants upon request.

3.2.3 Dietary restrictions

Dietary and supplement restrictions were implemented throughout the study period to reduce confounding factors (this included the lead-in and washout periods). Participants were asked to remove the following items from their diet: probiotics, i.e. yoghurt (uncooked), fermented food products or probiotic supplements, and fibre supplements, due to their potential influence on intestinal microbiota; soy foods, i.e. soy-based bread, tofu, soy-milk and other soy-based desserts, due to the confounding of isoflavone intake. Participants were instructed to avoid the consumption of kiwifruit, except for those that were provided for the 6-week kiwifruit/isoflavone treatment to accurately assess the effect of kiwifruit on equol production. Participants were also asked to abstain from taking omega-3, calcium or vitamin D supplements for the duration of the study due to their modulatory effects on bone metabolism.

3.3 Ethics approval and considerations

This study was reviewed and granted ethics approval by the Massey University Human Ethics committee: Southern A, Application 13/15. All participants gave written informed consent prior to the study (appendix 1). Participants were advised of the study procedures, requirements and associated risks or benefits; this information was also provided in a detailed information sheet (appendix 2).

Participants were made aware of their right to withdraw from the study at any time and participant confidentiality was assured by the assignment of individual identification (ID) codes at the beginning of the study. All paperwork and electronic data is safely stored for 10 years post-study completion and will be destroyed after this period of time.

3.4 Participants

3.4.1 Eligibility criteria

Prospective participants were contacted via phone call or email and taken through a screening questionnaire (appendix 3). Upon meeting the inclusion/exclusion criteria these individuals underwent a blood-screening test at Medlab™ Central Palmerston North, an accredited diagnostic laboratory employing standardised methodology. Kidney and liver function, and haematology were tested to ensure participants met the exclusion criteria. BMD was screened to prospectively exclude osteoporotic individuals.

3.4.1.1 Inclusion criteria

This study recruited women who were 1-10 years postmenopausal and had undergone a natural menopause. Menopause was defined prospectively as ≥12 consecutive months of amenorrhea. An ideal study population would consist of women in their first five years of menopause, as skeletal responsiveness to isoflavone intervention may be greater in women at this stage (Ma et al., 2008). However, the sample size would have been too small with such a limited postmenopausal interval alongside the stringent exclusion criteria of this study. The postmenopausal status of participants was confirmed retrospectively by serum FSH levels >20 IU/L.

3.4.1.2 Exclusion criteria

Participants had to be willing to comply with the dietary and supplement restrictions for the duration of the intervention. These restrictions included fibre products for bowel movements so individuals with frequent constipation were excluded. Participants were required to be non-smokers and consume no more than two standard units of alcohol per day (on average). Women were excluded if they had experienced fracture in the previous six months, had a T score less than -2.5 S.D at the hip or spine, or were diagnosed with any disease of the bone or systemic disease affecting bone density. These pathologies include Paget's disease and cancer metastatic to bone. Participants were excluded if they had gastrointestinal disease (excluding appendicitis and irritable bowel syndrome) or had experienced any gastrointestinal infections in the previous month. Women were omitted from the study if they had cholesterol levels ≥6 mmol/L, current kidney and/or liver impairment or history of these impairments, or any endocrine disorders, i.e. diabetes mellitus or hyperthyroidism, hyperparathyroidism, hypercorticolism or hypogonadism. Some medications were incompatible with the study due to their effects on bone and mineral metabolism and lipid metabolism, such as corticosteroids and heparin. Participants with long-term or recent antibiotic use were excluded due to the disruption of intestinal microbiota, which lasts for 10-14 days after cessation of antibiotic treatment.

3.4.2 Recruitment

Prospective participants were recruited via an advertisement in the local newspaper (The Manawatu Standard), posters placed around Massey University, and an email sent to Massey staff and word of mouth. The recruitment period was carried out over two weeks from the middle to end of August.

3.4.3 Participants of this study

36 healthy postmenopausal women were recruited for this study. 34 of these participants were living in Palmerston North, while the other two participants (affiliated with Massey University) were living in the Hawke's Bay and arranged to travel to Palmerston North for the blood and urine samples.

3.4.4 Sample size

Sample size calculations were based on detecting a significant change in the bone markers, DPD, CTx and ucOC, with a statistical power of 80% and significance of 5%. A significant change was defined as a difference that was at least two-fold greater than the inter-assay coefficient of variation (CV) for each respective bone marker. For DPD, a significant difference occurs with a change of ≥1.79 nmol/mmol creatinine; 27 participants were required to detect a change of this size. For ucOC, a significant difference occurs with a change of ≥1.35 ng/ml; 25 participants were required to detect a change of this size. For CTx, a significant difference occurs with a change of ≥0.09 μmol/L; 20 participants were required to detect a change of this size.

3.4.5 Randomisation

A random number generator, sourced from Randomization.org, produced numbers to randomly allocate participants to group A or B. These numbers also acted as anonymous ID for each participant. Due to the trial's crossover design participants and research personnel were not blinded to the treatment groups.

3.5 Data collection

3.5.1 Anthropometric measurements

Body height and weight were measured at baseline for each participant, without their shoes and outer layers of clothing. A stadiometer (Holtain Ltd., England) was implemented to measure height (m) to the nearest 0.1 of a centimetre; participants were directed to assume an upright posture with their heels directly against the heel board before measurement. Weight (kg) was measured on a manual scale (Seca, Model 762, Vogel & Halke, Germany) to the nearest 0.1 of a kilogram. BMI was calculated from height and weight, by the formula:

BMI = weight (kg)/ height $(m)^2$.

3.5.2 Measurement of bone mineral density

For each participant, BMD of the lumbar spine (L1-4), neck of hip and total hip, were measured using DEXA (model: Hologic QDR, Discovery A, Bedford, MA, USA) at the Human Nutrition Research Unit, Massey University. Three different qualified operators carried out the measurements using a standardised methodology. A control scan was carried out on each day of measurements to ensure that the machine was appropriately calibrated (coefficient of variation <0.5%). Participants with T scores in the osteopenic range were notified and prompted to inform their general practitioner and arrange a diagnostic DEXA scan.

3.5.3 Dietary assessment

Participants completed a 3-day food diary including two week days and one weekend day. Food diaries (appendix 4) were given to participants at week two, alternatively participants could opt for an electronic copy of the food diary. Research personnel informed participants of the method used to fill out a dietary record; written instructions were also included in the first page of the food diary. Participants were asked to complete diaries during the first intervention period. Once the diaries were

completed and returned they were analysed for the daily intake of energy, calcium, magnesium, phosphorus, and vitamin C, using Foodworks® Version 7 (Xyris Software, Australia). Participants were sent an electronic record of their dietary analysis for personal interest.

3.5.4 Blood and urine samples and biochemical analyses

3.5.4.1 Sample collection requirements

Participants were asked to visit the Human Nutrition Research Unit, Massey University between the hours of 7.30 and 10.30 a.m. following an overnight fast to give blood and spot urine samples. Participants were asked to refrain from exercise on the morning of their sample collection. Participants completed a 24-hour urine collection the day prior to their sample collection. Samples were collected between Monday to Friday during weeks 2, 8, 10, and 16 and each sample collection was carried out on the same day, where possible, to ensure a complete lead-in, washout, and treatment period. All blood and urine samples (1ml aliquots) were stored at -80˚C until the completion of the intervention.

3.5.4.2 Blood sampling

The bone markers, CTx and ucOC, were measured in plasma. The remaining parameters, lipids, TC, LDL-c, HDL-c and TAG, hormones, E2, FSH, and thyroid stimulating hormone (TSH), and isoflavones (daidzein and equol) were measured in serum. A qualified phlebotomist took all blood samples. Blood (6ml) was collected by venipuncture into vacutainers containing either EDTA or serum separation gel and clotting activator. The EDTA tube was refrigerated immediately while the serum separator tube was clotted for 30 minutes at room temperature. All tubes were centrifuged for 15 minutes at 4˚C on 2100 G.

3.5.4.3 Urine sampling

During their visit to the Nutrition Research Unit, Massey University, participants produced a spot sample of urine (2 ml). Urinary DPD and creatinine were measured from the spot urine sample. Creatinine measures were used to correct DPD levels according to urinary concentration

Participants were asked to carry out their 24-hour urine collection the day prior to their scheduled blood and spot urine samples, from which urinary daidzein and equol were measured. For the 24-hour collection participants used a 500 mL jug to collect and measure the volume of each urinary void. Participants recorded the volume of each void on a chart provided with the information sheet on 24-hour urine collection (appendix 5). Urine from the jug was then emptied into a discrete 24-hour urine collection device, the sumius U-Container™ (appendix 5), which subsamples and stores 1/50th of each urinary void. The remainder of each void was discarded. To estimate total 24-hour urinary volume the sum of the subsample volumes were multiplied 50-fold, which was verified against the total urinary void volumes.

3.5.5 Biochemical analyses

3.5.5.1 Bone markers

CTx was measured by commercial enzyme-linked immunoassay (Canterbury Health Laboratories Ltd., Canterbury) with an inter-assay CV of 6% and intra-assay CV of 5.2%. DPD and ucOC were analysed at the Institute of Human Health and Nutrition (IFNHH) laboratories, Massey University. DPD was measured by a competitive enzyme-linked electrochemiluminescence immunoassay kit (MicroVue, California, U.S) with an inter-assay CV of 4.8% and intra-assay CV of 8.4%. Creatinine was measured in urine by the Jaaffe colorimetric method. ucOC was also measured by a competitive enzyme-linked electrochemiluminescence immunoassay kit (Takara, Tokyo, Japan) with an inter-assay CV of 5.7% and intra-assay CV of 4.6%.

3.5.5.2 Lipids and hormones

All hormones (E2, FSH and TSH) were measured by commercial enzyme-linked immunoassay (Medlab Central Ltd., Palmerston North). Lipids (TC, LDL-c, HDL-c and TAG) were measured by standard enzymatic colorimetric assay (Medlab Central Ltd., Palmerston North).

3.5.5.3 Isoflavones and vitamin D

Daidzein and equol were measured by time-resolved fluoroimmunoassay and vitamin D was measured by isotope-dilution liquid chromatography-tandem mass spectrometry (Canterbury Health Laboratories Ltd., Canterbury). Equol producing participants were determined by a log base 10 ratio of urinary daidzein to equol ≥ -1.70 in at least two of the three measured time points: the spot urine sample at week 8, and the 24-hour urine collections at week 8 and 16.

3.6 Compliance

Participants were asked to record their compliance to the treatments and dietary/medication restrictions on the compliance charts provided (appendix 6). Participants also returned their residual isoflavone supplements at the completion of the intervention. Residual isoflavones were counted and the compliance charts were tallied to calculate compliance to the isoflavone and kiwifruit/isoflavone treatments. Adherence <80% to either treatment would result in exclusion from data analysis.

3.7 Statistical analysis

Normality of the data was tested via the Anderson-Darling normality test in Minitab® version 16.1.1. (2010), where P-values >0.05 indicated a normal distribution. If possible non-normally distributed data was transformed by log base 10 to a normal distribution. If a small number of participants had outlier values that skewed the data from normal distribution they were removed from data analysis. Data was analysed

using SAS version 9.4 (SAS Institute Inc., Cary, NC, USA). Mean values and standard errors were calculated. Multivariate analysis was used to assess the main effects of treatment, intervention period, time (start or end), order of interventions, and the interaction of treatment over time. Further multivariate analysis accounted for equol producer status as a main effect and the additional 3-way interaction between equol producer status, treatment and time. Baseline characteristics were analysed for group differences using one-way ANOVA in Minitab®; differences were considered significant it the P value was <0.05.

Chapter 4

Results

36 postmenopausal women (≤10 years since the onset of menopause) were recruited for this study and randomly allocated to treatment group A (n=18) or B (n=18). However, three participants withdrew during the first intervention period due to unexpected surgery, sickness and loss of interest. 33 participants completed the intervention (group A n=16; group B n=17) and all were compliant with the intervention (>80% adherence to both treatments). Results are presented as mean ± standard error of the mean (SEM).

4.1 Baseline characteristics

Three participants were not available for DEXA measurements during the intervention period, thus 30 participants were included in analysis of BMD and BMC (group A n=14; group B n=16). One participant chose not to have height or weight measured for personal reasons, so 32 participants were included in the analysis of nutrient intake, vitamin D status, serum estradiol and anthropometric measurements (group A n=16; group B n=16).

The baseline characteristics are presented in table 4.1. Anthropometric measurements were not significantly different between group A and B. Participants had a mean age of 57.2 ± 0.8 years, height of 164.5 ± 1.4 cm, weight of 67.8 ± 2.6 kg, and BMI of 25.1 ± 1.1. Groups A and B had similar BMD, BMC, and T and Z scores for each skeletal site ($P>0.05$). Mean T scores were greater than -1 SD from the reference BMD distribution. The exception was for group B: the T scores at the neck of hip (-1.12 ± 0.215) and lumbar spine (-0.850 ± 256) were slightly osteopenic. The total energy intake, 7734.0 ± 446.0 kJ, and micronutrient intakes were not different between groups ($P>0.05$). Mean calcium intake was 931.0 ± 93.8 mg/day; vitamin C intake averaged at 142.2 ± 19.5 mg/day; magnesium intake was 336.4 ± 22.6 mg/day on average; mean phosphorus

intake was 1414.0 ± 84.6 mg/day. Serum hormone levels, vitamin D, E2, and FSH, were also comparable between groups (P>0.05), with means of 70.2 ± 5.7 nmol/L, 41.0 ± 3.2 pmol/L, and 68.1 ± 5.0 IU/L respectively. Minimum serum FSH concentrations were 28.6 IU/L and 25.9 IU/L for group A and B respectively. All participants had serum FSH >20 IU/L, the threshold value used to confirm postmenopausal status.

Table 4.1 Baseline characteristics of groups A and B (mean ± SEM); P values <0.05 represent significantly different means.

Baseline characteristics	**Group A**	**Group B**	**P value**
Age (years)	56.8 ± 0.9	57.6 ± 0.7	0.912
Height (cm)	164.6 ± 1.4	164.3 ± 1.4	0.877
Weight (kg)	69.2 ± 2.9	66.3 ± 2.2	0.427
BMI (kg/m^2)	25.6 ± 1.1	24.6 ± 1.0	0.525
BMD (g/cm^2)			
Lumbar spine	1.04 ± 0.047	0.995 ± 0.028	0.126
Neck of the hip	0.806 ± 0.034	0.724 ± 0.024	0.066
Total hip	0.918 ± 0.030	0.852 ± 0.021	0.087
BMD T score			
Lumbar spine	-0.012 ± 0.428	-0.850 ± 0.256	0.116
Neck of the hip	-0.394 ± 0.310	-1.12 ± 0.215	0.071
Total hip	-0.187 ± 0.247	-0.743 ± 0.168	0.082
BMD Z score			
Lumbar spine	1.16 ± 0.427	0.471 ± 0.242	0.190
Neck of the hip	0.725 ± 0.303	0.471 ± 0.242	0.086
Total hip	0.569 ± 0.241	0.114 ± 0.241	0.130
BMC (g)			
Lumbar spine	61.3 ± 4.13	53.2 ± 2.42	0.114
Neck of the hip	4.10 ± 0.191	3.59 ± 0.176	0.086
Total hip	31.9 ± 1.17	29.2 ± 0.826	0.077
Vitamin D (nmol/L)	70.3 ± 6.90	70.1 ± 4.50	0.987
Baseline FSH (IU/L)	69.1 ± 4.91	67.1 ± 5.09	0.788
Baseline E2 (pmol/L)	41.3 ± 4.25	40.6 ± 2.09	0.888
Dietary intake			
Total energy (kJ/day)	7517.0 ± 407.0	7951.0 ± 485.0	0.501
Calcium (mg/day)	948.5 ± 94.2	913.4 ± 93.3	0.793
Magnesium (mg/day)	334.0 ± 18.9	338.8 ± 26.3	0.884
Phosphorus (mg/day)	1440.0 ± 85.2	1387.9 ± 83.9	0.664
Vitamin C (mg/day)	131.6 ± 16.3	152.7 ± 22.6	0.458

4.2 Biochemical analyses

Multivariate analysis was used to assess the significance of the main effects: treatment, the period (1 or 2 - equivalent to weeks 2-8 and 10-16 respectively) and order of treatment (1 or 2 - equivalent to group B and A respectively), time of treatment (start or end) and equol producer status (equol producer or non-equol producer) (table 4.2). The effect of interaction between time and treatment and a three-way interaction between time, treatment, and equol producer status were also assessed. The P values for the main effects and interactions are shown in table 4.2. The mean values ± standard error of the mean (SEM) at baseline and end of treatment for each parameter are displayed in table 4.3. The mean percentage change for the bone markers (table 4.4) and HDL-c (table 4.5) were calculated in addition to the mean absolute change to display the treatment outcomes in a more effective manner. Percentage change was not analysed for serum equol and the log ratio of daidzein to equol analysed for due to the non-parametric distribution of these parameters as percentage change.

A few participants had outlier values for CTx (participants 9 and 28 from group B) and DPD (participant 18 from group B), which caused these data sets to skew from a normal distribution. Removal of these participants from their respective data sets created a normal distribution. However, when comparing the original and data sets there was no difference in the outcomes of multivariate analysis, thus the final results use the original data set for these parameters.

Table 4.2 The P values for the main effects and interaction effects of the bone markers, lipid parameters, hormones and isoflavones. * highlights P values <0.05, which indicate statistically significant effects.

Effects	**ucOC (ng/mL)**	**CTx (µmol/L)**	**DPD (nmol/mmol Cr)**	**TC (mmol/L)**	**HDL-c (mmol/L)**	**LDL-c (mmol/L)**
Order	0.8643	0.7639	0.23	0.0804	0.1684	0.2709
Equol	0.605	0.0463 *	0.3941	0.5601	0.1513	0.8137
Period	0.0679	0.3192	0.614	0.6413	0.3691	0.5868
Treat	0.2696	0.7067	0.7734	0.8179	0.563	0.2597
Time	0.2706	0.3827	0.928	0.0136 *	0.4477	0.0007 *
Treat*Time	<0.0001 *	0.0789	0.2485	0.4053	0.0516	0.8737
Treat*Time*Equol	0.2391	0.5452	0.751	0.334	0.0059 *	0.192
Effects	**TC:HDL-c**	**TAG (mmol/L)**	**FSH (IU/L)**	**TSH (mIU/L)**	**Log daidzein:equol ratio**	**Serum equol (nmol/L)**
Order	0.9071	0.1041	0.7589	0.2766	0.1501	0.7963
Equol	0.2122	0.2709	0.6441	0.1208	0.0235 *	0.3451
Period	0.1576	0.6698	0.9506	0.5878	0.0418 *	0.0043 *
Treat	0.1853	0.1424	0.2382	0.5657	0.3666	0.2615
Time	0.1278	0.3011	0.6272	0.2059	0.0212 *	<0.0001 *
Treat*Time	0.0696	0.8908	0.4798	0.7079	0.7178	0.3448
Treat*Time*Equol	0.0935	0.0334 *	0.5973	0.7475	0.0047 *	0.0005 *

Table 4.3 The mean values of bone markers, lipid parameters, hormones and isoflavones over time for the different treatments.

Treatment	Time	ucOC (ng/mL)	CTx (µg/L)	DPD (nmol/mmol Cr)	TC (mmol/L)	HDL-c (mmol/L)	LDL-c (mmol/L)
Isoflavone	Start	$5.76^{a,x}$	0.502	6.61	5.79	1.67	3.53
	End	$6.37^{b,y}$	0.491	7.65	5.49	1.62	3.36
	Difference	0.61	-0.011	1.04	-0.30	-0.05	-0.17
	SEM	0.226	0.0176	1.164	0.165	0.066	0.136
Kiwi / Iso	Start	$6.37^{b,y}$	0.476	7.82	5.65	1.62	3.58
	End	$5.40^{a,x}$	0.509	6.93	5.55	1.64	3.42
	Difference	-0.97	0.033	-0.89	-0.10	0.02	-0.16
	SEM	0.226	0.0176	1.164	0.165	0.066	0.136
Treatment	**Time**	**TC:HDL-c**	**TAG (mmol/L)**	**FSH (IU/L)**	**TSH (mIU/L)**	**Log ratio daidzein:equol**	**Serum equol (nmol/L)**
Isoflavone	Start	3.44	0.989	68.7	1.38	-0.65	3.0
	End	3.45	1.032	68.4	1.29	-0.86	9.9
	Difference	0.01	0.0430	-0.3	-0.09	-0.21	6.9
	SEM	0.0165	1.075	4.14	1.11	0.113	1.74
Kiwi / Iso	Start	3.55	0.943	69.2	1.48	-0.51	3.2
	End	3.43	0.974	70.6	1.31	-0.80	12.4
	Difference	-0.12	0.0302	1.4	-0.17	-0.29	9.7
	SEM	0.0165	1.075	4.14	1.11	0.113	1.74

Superscripts with different letters indicate significantly different means: $^{a/b}$ compares means within treatment; $^{x/y}$ compares means between treatments at the same time point (SEM = standard error of the mean).

4.3 Bone markers

Table 4.4 Average percentage change in the bone markers from baseline to end of treatment (mean ± SEM). P values <0.05 indicate statistically significant changes.

	Percentage change (%) ucOC		Percentage change (%) DPD		Percentage change (%) CTx	
Treatment	**Mean**	**P value**	**Mean**	**P value**	**Mean**	**P value**
Iso	10.8 ± 4.4	0.0205	28.2 ± 20.4	0.1769	0.8 ± 3.5	0.8247
Kiwi / Iso	-15.5 ± 4.4	0.0015	8.2 ± 21.1	0.7	7.0 ± 3.7	0.0672

4.3.1 Serum ucOC

There was a significant change in plasma ucOC according to treatment over time (P<0.0001) (table 4.2 and 4.3). Overall participants experienced a 10.8% increase in plasma ucOC with the isoflavone treatment, and a 15.5% decrease with kiwifruit/isoflavone treatment (figure 4.1 and table 4.4). No other main effects or interactions were significant (table 4.2).

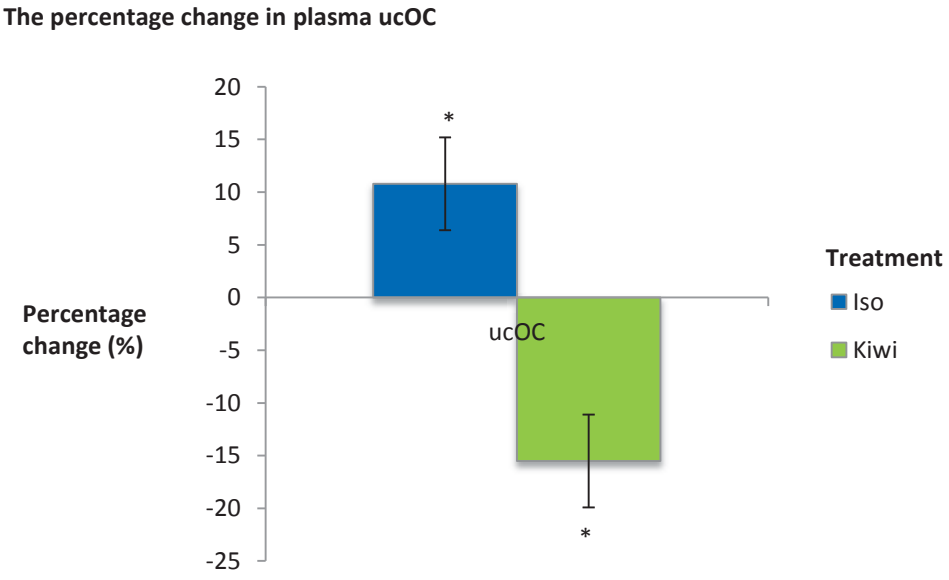

Figure 4.1 Bar graph showing the mean percentage change in plasma ucOC over the treatment periods. Treatment: Iso = isoflavone; Kiwi = Kiwifruit/isoflavone. Error bars represent the SEM. * represents a significant change (P<0.05).

4.3.2 Plasma CTx and urinary DPD

No change in plasma CTx or urinary DPD occurred with either treatment (table 4.2; figures 4.2 and 4.3 respectively). There was a significant main effect of equol producer status on plasma CTx levels: equol producers had lower plasma CTx than non-producers overall (P=0.0463) (table 4.8). No other effects were significant for these bone markers.

The percentage change in plasma CTx

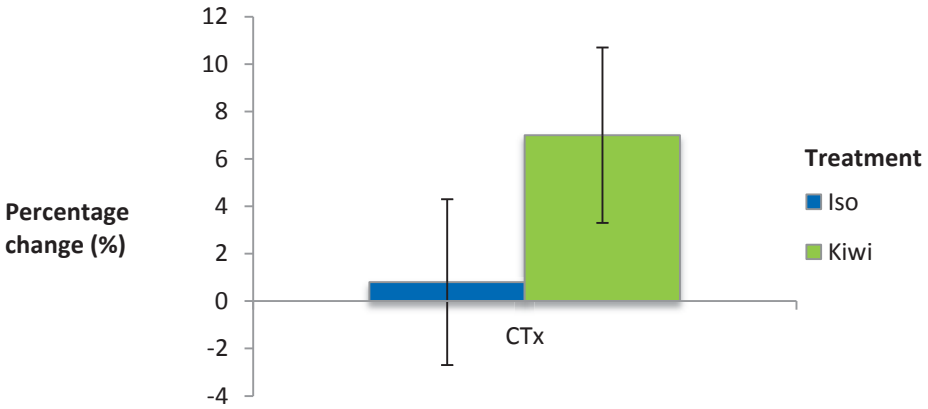

Figure 4.2 Bar graph showing the mean percentage change in CTX over the treatment periods. Treatment: Iso = isoflavone; Kiwi = kiwifruit/isoflavone. Error bars represent the SEM. * represents a significant change (P<0.05).

The percentage change in urinary DPD

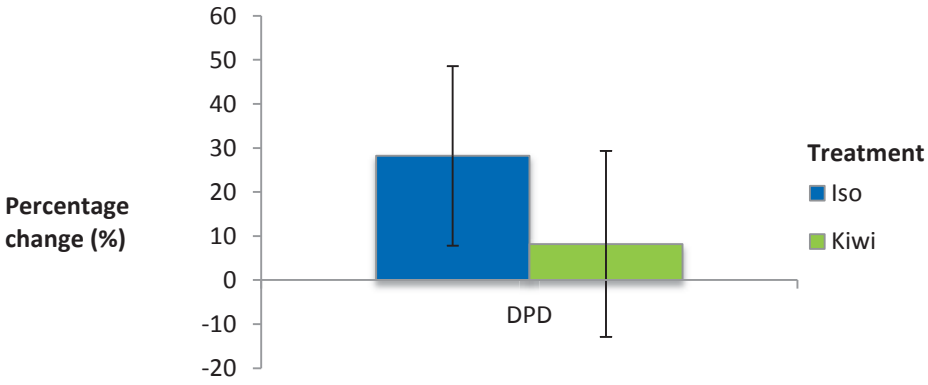

Figure 4.3 Bar graph showing the mean percentage change in urinary DPD over the treatment periods. Treatment: Iso = isoflavone; Kiwi = kiwifruit/isoflavone. Error bars represent the SEM. * represents a significant change (P<0.05).

4.4 Serum lipid profile

4.4.1 Serum HDL-c

Non-equol producers experienced a significant decrease in serum HDL-c with both treatments: 5.0% over the duration of the isoflavone treatment (P=0.0089) and 4.7% over the duration of the kiwifruit/isoflavone treatment (P=0.0130) (tables 4.3 and 4.5; figure 4.4). Serum HDL-c did not change in equol producers during the isoflavone treatment (P>0.05). Equol producers on the kiwifruit/isoflavone treatment experienced a significant, 8.3%, increase in serum HDL-c (P=0.0044). Overall, the non-equol producers had significantly higher serum HDL-c at the beginning of treatment compared to equol producers (P=0.0227).

Table 4.5 The mean percentage change in serum HDL-c according to treatment and equol producer status (mean ± SEM). A P value >0.05 indicates a statistically significant change.

		Percentage change (%) HDL-c	
Treatment	**Equol producer**	**Mean**	**P value**
Iso	Yes	-1.2 ± 2.7	0.6528
	No	-5.0 ± 1.8	0.0089
Kiwi / Iso	Yes	8.3 ± 2.7	0.0044
	No	-4.7 ± 1.8	0.0130

The percentage change in HDL-c from baseline in equol producers (+) and non-producers (-)

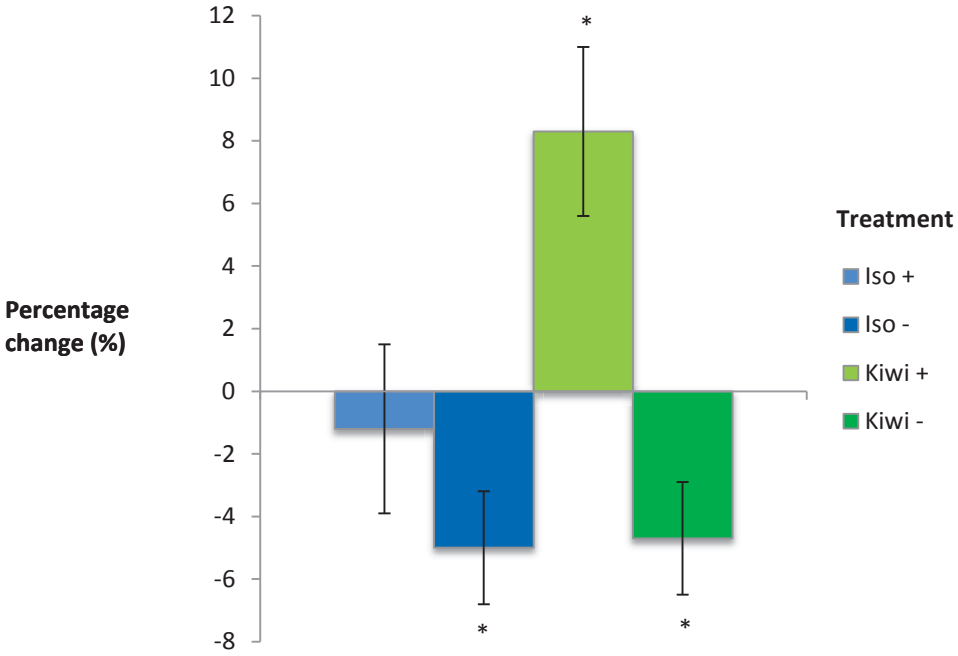

Figure 4.4 Bar graph showing the mean percentage change in serum HDL-c, over the treatment periods, in equol producers (+) and non-equol producers (-). Treatment: Iso = isoflavone; Kiwi = kiwifruit/isoflavone. Error bars represent the SEM. * represents a significant change (P<0.05).

4.4.2 Serum TC, TAG, LDL-c, and the ratio of TC to HDL-c

The data for TAG and the ratio of TC to HDL-c was log transformed to a normal distribution for multivariate analyses. There was no significant change in any of the serum lipid parameters, TAG, LDL-c, TC or the ratio of TC to HDL-c, with either treatment (tables 4.2 and 4.3). Time was a significant main effect for both serum LDL-c and the ratio of TC:HDL-c: on average participants experienced a significant decrease in these parameters over the intervention period regardless of treatment (P=0.0136 and P=0.0007 respectively) (table 4.8). Equol producer status had a significant effect on serum LDL-c, which overall was significantly higher in non equol-producers compared to equol producers (P=0.0235) (table 4.8).

4.5 Equol production

4.5.1 Log ratio of urinary daidzein to equol

Equol non-producers experienced a significant decrease in the log ratio of urinary daidzein to equol over the treatments, isoflavone (P=0.016) and kiwifruit/isoflavone (P<0.0001) (figure 4.5 and table 4.6). The log ratio of urinary daidzein to equol did not change in equol producers over either treatment (P>0.05).

Log ratio of urinary daidzein to equol over the treatment periods according to equol producer status

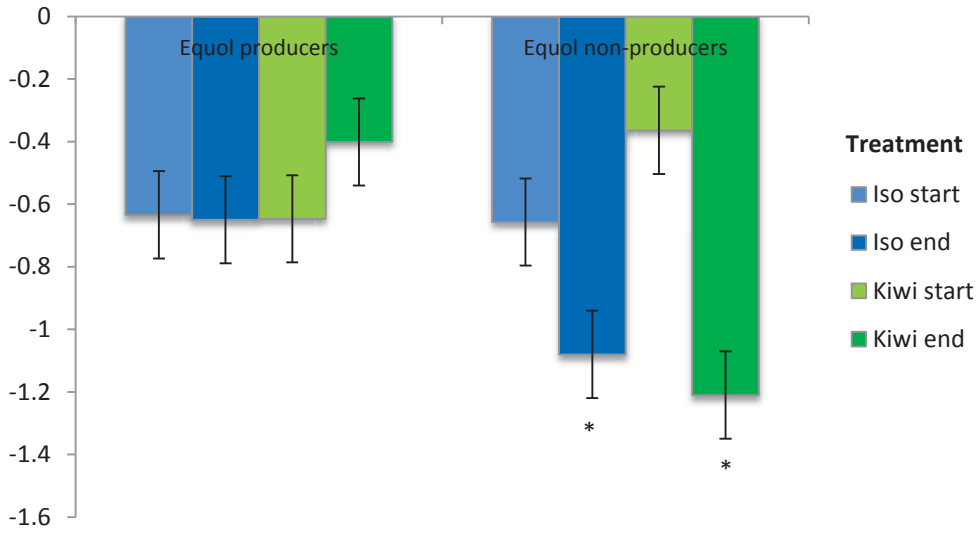

Figure 4.5 Bar graph showing the mean change in log ratio of urinary daidzein to equol over the treatment periods, stratified into equol producers and non-equol producers. Treatment: Iso = isoflavone; Kiwi = kiwifruit/isoflavone. Error bars represent the SEM. * represents a significant effect (P<0.05).

4.5.2 Serum equol

Serum equol did not change in non-equol producers with either treatment. In equol producers serum equol increased significantly over both the isoflavone (P=0.0008) and kiwifruit/isoflavone treatment (P<0.0001) (figure 4.6 and table 4.6). Serum equol was similar in equol producers at the end both treatments, however there was a trend (P=0.0629) for higher serum equol in participants following the kiwifruit/isoflavone treatment. As a main effect, serum equol increased over time with significantly higher serum equol in at the end of treatments (table 4.8).

Serum equol at treatment baseline and endpoint according to equol producer subgroups

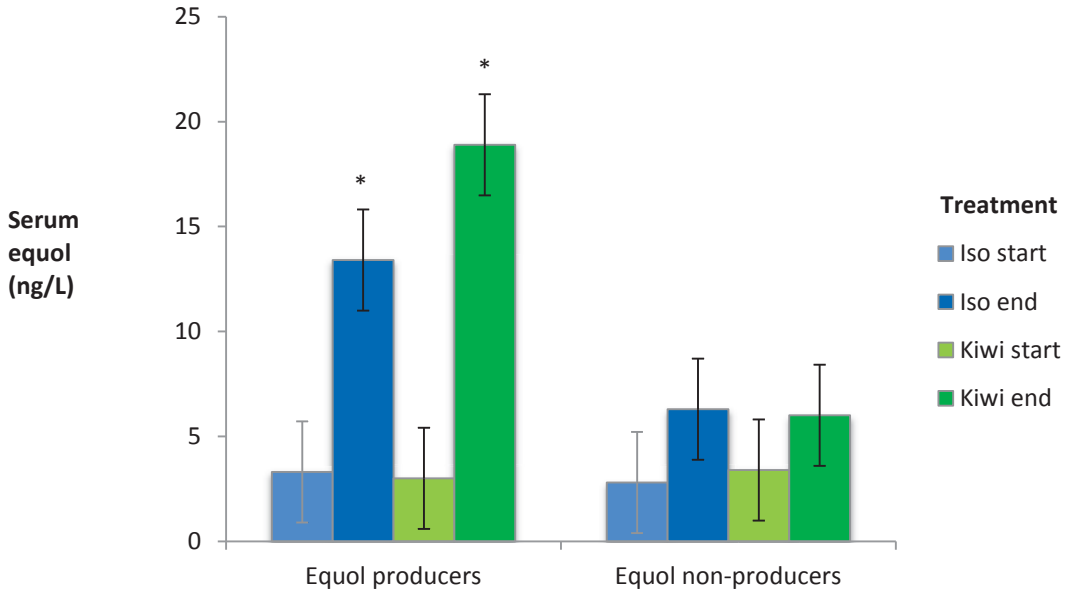

Figure 4.6 Bar graph showing the mean change in serum equol over the treatment periods, stratified into equol producers and non equol-producers. Treatment: Iso = isoflavone; Kiwi = kiwifruit/isoflavone. The error bars represent the SEM. * indicates a significant change (P<0.05).

Table 4.6 The biochemical parameters exhibiting a significant three-way interaction between treatment, time and equol producer status (mean values and SEM).

		HDL-c (mmol/L)				Log ratio of daidzein:equol				Serum equol (nmol/L)			
Treatment	**Equol producer**	**Start**	**End**	**SEM**	**P value**	**Start**	**End**	**SEM**	**P value**	**Start**	**End**	**SEM**	**P value**
Iso	Yes	$1.56^{a,xy}$	$1.53^{a,x}$	0.0505	0.5538	$-0.634^{a,x}$	$-0.650^{a,yz}$	0.2607	0.9518	$3.267^{a,x}$	$13.39^{b,y}$	2.910	0.0008
	No	$1.79^{b,y}$	$1.70^{a,x}$	0.0333	0.0105	$-0.657^{b,x}$	$-1.08^{b,xy}$	0.1719	0.016	$2.776^{a,x}$	$6.337^{a,x}$	1.919	0.0666
Kiwi / Iso	Yes	$1.48^{a,x}$	$1.62^{b,x}$	0.0505	0.0116	$-0.647^{a,x}$	$-0.401^{a,z}$	0.2607	0.349	$3.000^{a,x}$	$18.89^{b,y}$	2.910	<0.0001
	No	$1.75^{b,y}$	$1.67^{a,x}$	0.0333	0.0209	$-0.364^{b,x}$	$-1.21^{a,x}$	0.1719	<0.0001	$3.449^{a,x}$	$5.969^{a,x}$	1.919	0.1993

Superscripts with different values represent significantly different means (P value <0.05): $^{a/b}$ compares means within a treatment; $^{x/y}$ compares means between treatments at the same time point. The SEM is for the difference between start and end of treatment. Treatments: Iso = isoflavone; Kiwi / Iso is kiwifruit/isoflavone

4.6 Hormones: serum E2, FSH and TSH

The data for TSH was log transformed to a normal distribution. Neither serum TSH or FSH changed significantly from baseline over the treatment periods (tables 4.2 and 4.3). The data for serum E2 did not fit a normal distribution and could not be transformed. At baseline the majority of participants had serum E2 <37 pmol/L, however, some participants exhibited E2 levels >37 pmol/L, ranging from 38-272 pmol/L**.** Pearson Chi-square analysis was carried out to confirm that there no change in the proportion of participants with serum E2 <37 or >37 pmol/L due to a treatment effect (P=0.0854), and that there was no difference between treatments in the proportion of participants with serum E2 <37 and >37 pmol/L (P=0.0854) (table 4.7).

Table 4.7 Pearson Chi-Square table showing distribution of participants with E2 levels <37 and >37 pmol/L.

Treatment	Time	Participants with E2 <37	Participants with E2 >37 pmol/L	All
Iso	Start	30	4	34
		88.2	11.8	100
		26.6	21.1	25.8
	End	29	5	34
		85.3	14.7	100
		25.7	26.3	25.8
Kiwi / Iso	Start	28	4	32
		87.5	12.5	100
		24.8	21.1	24.2
	End	26	6	32
		81.3	18.8	100
		23	31.6	24.2
All		113	19	132
		85.6	14.4	100
		100	100	100

N.B. Cell contents from top to bottom: participant count, % of row, % of column. Pearson Chi-Square = 0.779; P value = 0.0854.

Table 4.8 Mean values for the biochemical parameters that have significant overall main effects (mean ± SEM).

Parameters	Main effects	Equol producer status	Mean	P value
CTx (µmol/L)	Equol	Yes	0.426 ± 0.0579	0.0463
		No	0.563 ± 0.0374	
TC (mmol/L)	Time	Start	5.67 ± 0.1595	0.0136
		End	5.52 ± 0.1595	
LDL-c (mmol/L)	Time	Start	3.56 ± 0.1317	0.0007
		End	3.39 ± 0.1317	
	Equol	Yes	3.27 ± 0.2259	0.0235
		No	3.45 ± 0.1614	
Log ratio daidzein:equol	Period	1	-0.586 ± 0.0793	0.0418
		2	-0.832 ± 0.0793	
	Time	Start	-0.575 ± 0.0818	0.0212
		End	-0.834 ± 0.0818	
	Equol	Yes	-0.5828 ± 0.1181	0.0418
		No	-0.8265 ± 0.1181	
Serum equol (ng/ml)	Time	Start	3.02 ± 1.07	<0.0001
		End	11.1 ± 1.07	
	Period	1	6.60 ± 1.146	
		2	7.68 ± 1.146	

Chapter 5

Discussion

5.1 Overall outcomes of the study

The aim of this pilot study was to determine the effect of short-term supplementation of aglycone isoflavones, daidzein and genistein, alone or in combination with green kiwifruit, on biochemical markers of bone turnover and lipid profile in healthy non-vegetarian postmenopausal women. The study group was also assessed for equol-producer status and this was included as a main outcome effect. No prior studies have evaluated equol producer prevalence in NZ Caucasian women.

Bone resorption was not reduced by isoflavone supplementation; urinary DPD and plasma CTx levels were unaffected by both treatments. Treatment effect remained insignificant when differentiating between equol producers (n=10) and non-equol producers (n=20) (table 4.2). The combined supplementation of kiwifruit and isoflavones significantly decreased plasma ucOC, which indicates a beneficial increase in the proportion of bone-functional carboxylated OC (tables 4.3 & 4.4). However, the isoflavone supplement alone caused a significant elevation in plasma ucOC, which may have a negative impact on bone health. These changes were similarly significant in both equol producers and non-equol producers (table 4.2).

Isoflavones had no effect on the serum lipid profile (table 4.2) with the exception of a significant association between equol production and serum HDL-c (tables 4.5 & 4.6). Serum HDL-c declined significantly in non-equol producers over both treatments. In equol producers serum HDL-c did not change over the isoflavone treatment, but increased significantly with the kiwifruit and isoflavone treatment.

Serum equol rose significantly in equol producers over both treatments, however there was no additional effect of kiwifruit consumption on equol production (table 4.6). Serum equol was unchanged in non-equol producers.

5.2 Study group characteristics

At baseline the two study groups were not significantly different in their age, anthropometric measures (height, weight, and BMI) or in BMD and BMC at the skeletal sites measured. The average age and BMI of the study group were 57.2 $\pm$ 0.8 years and 25.1 $\pm$ 1.1 kg/m^2 respectively. This BMI is bordering normal (<25 kg/m^2) and overweight (>25 kg/m^2) according to the BMI index for women 19+ years of age (Papakitsou et al., 2004). Women in this study have a BMI very similar to that found in healthy postmenopausal Dutch Caucasian women, 25.6 kg/m^2 (55.3 years) (Braam et al., 2003); this BMI is lower than the national median BMI (27.6 kg/m^2) in NZ women (19+ years) according to the National Nutrition Survey (NNS08/09) (Ministry of Health, 2012). BMD in this study group ranged from normal to slightly osteopenic. At each skeletal site measured, the mean T scores were greater than -1 SD from the healthy reference BMD, indicating normal bone density (T scores >-1 SD) (National Institute of Health, 2000). The exceptions were the T scores for group B at the neck of hip (-1.12 $\pm$ 0.215) and the lumbar spine (-0.850 $\pm$ 256), which were in the osteopenic range (-1 to -2.5 SD). Although group B had slightly osteopenic BMD at the lumbar spine and neck of hip, these T scores were not significantly different between group A and B.

Baseline serum FSH levels were 68.1 $\pm$ 5.0 IU/mL. Menopausal status is verified by serum FSH >20 IU/ml (Brink et al., 2008), therefore all women in this study were postmenopausal. The baseline serum estradiol level was 41.0 $\pm$ 3.2 pmol/L and there was no change in serum E2 over the treatment period (table 4.7). Serum E2 was monitored to confirm that isoflavone supplementation had no deleterious effects on endogenous E2. The current study group recruited women who were 1-10 years postmenopausal, however, the average number of years since onset of menopause was not reported. The average serum E2 levels in this study are comparable to values reported by a large prospective study of postmenopausal women that were on

average 8-10 years postmenopausal (~12 pg/ml which is equivalent to 44.1 pmol/L) (Sowers et al., 2008), whereas women who were less than 5 years postmenopausal had average serum E2 that was >80.8 pmol/L (converted from 22 pg/ml). Thus it could be estimated that the women in this study were closer to 8-10 years postmenopausal. Participants had an average serum 25(OH)D level of 70.2 $\pm$ 5.7 nmol/L. According the Institute of Medicine ≥50 nmol/L serum 25(OH)D is considered to be a sufficient concentration for the maintenance of calcium homeostasis (Institute of Medicine, 2010). Recent literature, albeit controversial, suggests that this level of 25(OH)D is insufficient for the maintenance of bone health and reduction in fracture risk (Anderson et al., 2013; Holick, 2009). Anderson and colleagues (2013) define serum 25(OH)D between 51-75 nmol/L as insufficient. Nonetheless, the mean 25(OH)D in this study lies at the top end of this range and exceeds the national median serum 25(OH)D (63.1 nmol/L) for NZ women aged 55-64, as reported in the NNS08/09 (Ministry of Health, 2012). Serum vitamin D has significant seasonal variation, according to the fluctuation in UV light levels; minimum serum 25(OH)D levels occur over winter (Feskanich et al., 2003). In this study, the blood samples for vitamin D were taken in the second week of the intervention, during August, which is the final month of NZ winter. Thus the vitamin D average in this group of NZ women would be at its lowest.

Thyroid function, measured by serum TSH, ranged from 1.43-1.30 mIU/L over the duration of the study and did not change significantly with either treatment. The TSH levels seen in this group were within a healthy range: high and low levels of TSH are defined as serum TSH concentrations >4.5 mIU/L and <0.1 mIU/L respectively (Hollowell et al., 2002). Serum TSH was measured as a precaution, to monitor potential adverse effects of isoflavone supplementation (Setchell, Zhao, Shoaf, & Ragland, 2009; Tousen et al., 2011), although no adverse events were expected.

Baseline dietary assessment of the groups showed no significant difference in total energy intake or in the intakes of the calcium, phosphorus, magnesium and vitamin C. The average total energy intake was 7734.0 kJ/day, which is higher than the median value (6927 kJ/day) reported for 51+-year-old NZ women (University of Otago and Ministry of Health, 2011). The BMI of this study group was lower than the median for

similarly aged NZ women; it can be speculated that the higher energy intake in this group, compared to the national median, may be reflective of higher activity level. However, this cannot be verified, as physical activity was not measured in this study. Compared to reports from the National Nutrition Surveys (NNS97 and NNS08/09), nutrient intakes of women in the current study were higher than the median national intakes of 51-70 year old NZ women, which are: calcium, 737 mg/day, and vitamin C, 97 mg/day (University of Otago and Ministry of Health, 2011), magnesium, 265 mg/day, and phosphorus, 1297 mg/day (Russell et al., 1999) (NB. The NNS97 was consulted for magnesium and phosphorus, as these micronutrients are not reported in the NNS08/09). The micronutrient intakes reported by this study also exceed the NZ RDI except for calcium (table 5.1). The NNE08/09 did not report an average intake for vitamin K but a recent study in premenopausal Caucasian NZ women indicated an average vitamin K intake of 237 $\pm$ 45.2 μg/day (mean $\pm$ SEM) at baseline (Kruger et al., 2006), according to a food-frequency questionnaire. Nonetheless, these participants were younger (26.9 $\pm$ 0.91 years) than the current study group and likely have different dietary habits and micronutrient intakes.

Table 5.1 The average daily micronutrient intakes in this study and the relative contribution to RDI (for NZ women aged 51-74) (NHMRC & MoH, 2006).

Micronutrient	**NZ RDI (mg/day)**	**Mean daily intake (mg/day)**	**Mean daily Intake relative to RDI (%)**
Calcium	1300	931.0	72%
Vitamin C	45	142.2	316%
Magnesium	320	333.3	104%
Phosphorus	1000	1490.5	149%

5.3 Bone markers

5.3.1 Decrease in plasma ucOC with kiwifruit and isoflavone treatment

Serum ucOC, a marker of vitamin K status, decreased significantly with the kiwifruit and isoflavone treatment, from 6.37 ng/L to 5.40 ng/mL (figure 4.1). There was, however, a significant increase in ucOC during the isoflavone only treatment. The average plasma ucOC level at baseline was 6.07 ± 0.226 ng/ml, which is similar to values reported by an isoflavone intervention in postmenopausal Irish women, 6.2 ± 1.3 ng/ml (50-64 years) (Collins, Cashman, & Kiely, 2005), and postmenopausal Japanese women, 6.50 ± 4.9 ng/ml (mean age 58.9 years) (Yasui et al., 2006). No other human isoflavone trials have measured the response of plasma ucOC to an isoflavone and kiwifruit intervention. From this study, it is apparent that some bioactive component/s of green kiwifruit affected plasma ucOC independently of the isoflavone treatment and kiwifruit consumption may modify fracture risk in postmenopausal women through OC carboxylation.

5.3.1.1 Kiwifruit consumption, vitamin K, and a decrease in plasma ucOC

ucOC is a marker of vitamin K status: plasma ucOC levels are inversely correlated to serum vitamin K levels (Binkley et al., 2000). Vitamin K acts as an essential cofactor for the enzymatic carboxylation of OCs glutamyl side chains. Carboxylation is required for OC to bind to skeletal hydroxyapatite and undercarboxylation negatively impacts bone health. Increased plasma ucOC levels are correlated to increased fracture risk and reduced BMD at the femoral neck (Szulc, Chapu, Meunier, & Delmas, 2003; Vergnaud et al., 1997).

In a large prospective study in women (age-adjusted for the age range of 39-63 years), vitamin K intake lower than 109 μg/day was associated with 30% increase in fracture incidence compared to vitamin K intakes in the top four quintiles (Feskanich et al., 1999). Another prospective study found significantly lower fracture incidence in elderly women with higher vitamin K intake: fracture incidence was 3.1% in the highest

quartile of vitamin K intake, 254 μg/day, compared to 7.1% in lowest quartile, 56 μg/day (Booth et al., 2000). However participants were older (mean 79.6 years) than this study group and would have greater fracture rates. Tsugawa and colleagues (2006) report a strong negative correlation between vitamin K intake and urinary DPD levels, which suggests that vitamin K could reduce bone resorption.

By NZ and Australian standards the adequate intake (AI) of vitamin K is 60 μg/day for women of 51-70 years, which is at considerably less than the lowest quintile of vitamin K intake in the study by Feskanich et al. (1999) that showed heightened fracture risk. Vitamin K supplementation has been shown to decrease plasma ucOC over both short and long term interventions. A vitamin K fortified diet containing 420 μg/day decreased plasma ucOC by 41% over five days (Sokoll et al., 1997), and supplementing vitamin K AT 1000 μg/day decreased plasma ucOC by 48% over 12 months (Binkley et al., 2002). Moreover, Tsugawa et al. (2006) found older participants require a higher serum vitamin K concentration to maximise OC carboxylation than younger participants: in 70+ year olds 2.5 ng/ml vitamin K was required for maximal OC carboxylation compared to 50-69 year olds, who required only 1.4 ng/ml. Binkley et al. (2002) suggest that an vitamin K intake of 1000 μg/day induces maximal OC carboxylation. This vitamin K dose reduced plasma ucOC by 74%, which is significantly higher than the alternative doses of 250, 376, and 500 μg/day.

The two green kiwifruit (~74g each) consumed daily in the kiwifruit/isoflavone treatment was approximately a 148 g serving (Zespri® International, n.d.). According to the vitamin K content of green kiwifruit this serving would provide ~68.4 μg daily, which is 114% of the vitamin K AI (60 μg/day) this age group. The vitamin K intake during the kiwifruit/isoflavone treatment is additional to normal dietary vitamin K; this may have significantly increased serum vitamin K, which could have subsequently increased OC carboxylation. In this study vitamin K intake was not estimated due to lack of vitamin K content in the Foodworks® database, moreover, serum vitamin K was not measured. A previous study has defined cut-off values for plasma ucOC that indicate vitamin K deficiency (≥ 4.5 ng/ml) and increased fracture risk (≥ 5.5 ng/ml)

(Nishizawa et al., 2013). The current study reports a decrease in plasma ucOC from 6.37 to 5.40 ng/ml with kiwifruit/isoflavone treatment. According to Nishizawa et al. (2013) this current study group are deficient in vitamin K, and the kiwifruit/isoflavone treatment has reduced plasma ucOC to a level associated with lower fracture risk. Daily consumption of ≥2 green kiwifruit may reduce fracture risk in postmenopausal women by improving vitamin K status and increasing OC functionality in bone.

5.3.1.2 Increase in plasma ucOC with the isoflavone treatment

It is unlikely that the increase in ucOC during the isoflavone treatment was a direct effect of the isoflavone treatment itself. One previous crossover trial measuring vitamin K supplementation (420 μg/day) and plasma ucOC in a healthy population reported a similar increase in ucOC in the control group, however, participants were young males and females (mean age 28.7 ± 4.6 years), which does not match the current postmenopausal group. Participants consuming ~100 ug/day vitamin K experienced a 28% increase in ucOC over the 15-day intervention period (Sokoll et al., 1997). However, a small sample size (n=10) may have biased the results of this study (Sokoll et al., 1997). A long-term RCT found that plasma ucOC increased significantly in the placebo group at 12 and 24 months compared to the vitamin K supplemented groups (Bolton-Smith et al., 2007). It is possible that vitamin K intake declined during the isoflavone treatment. Alternatively, if bone formation increased during isoflavone treatment, this may have increased total serum OC and therefore increased ucOC proportionately. However, previous studies of isoflavone supplementation report decrease bone resorption, which in turn reduces bone formation due to the tight coupling of these processes. Moreover, meta-analyses of isoflavone intervention and bone turnover markers did not find a significant effect on serum OC levels (Taku et al., 2010b).

Acute and long-term physical activity can affect the concentration of bone turnover markers, including plasma ucOC (Seibel, 2005; Wu et al., 2006). In this study participants were asked to refrain from heavy exercise in the 24 hours prior to phlebotomy to reduce acute exercise-induced fluctuation in serum bone markers.

However, long-term physical activity was not evaluated in this study. It is possible that physical activity decreased during the isoflavone treatment and led to increased bone turnover, however, CTx and DPD levels were unchanged. Ultimately, it is ambiguous what underlying factor mediated the increase in plasma ucOC in this study.

5.3.2 No change in the rate of bone resorption: DPD and CTx did not respond isoflavone treatment

This study reports no significant changes in bone resorption markers, urinary DPD and plasma CTx (figures 4.2 and 4.3). The current study group had similar mean values of urinary DPD and plasma CTx compared to postmenopausal women of the same age. Baseline plasma CTx in this study was 0.476-0.502 $\pm$ 0.018 ng/ml. A previous study reported plasma CTx of 0.43 $\pm$ 0.15 ng/ml in healthy postmenopausal women (57.5 $\pm$ 4.7 years, n=27) (Valderas et al., 2009). This study reported average baseline urinary DPD of 6.61-7.82 $\pm$ 1.16 mmol/mmol creatinine. Previously, healthy postmenopausal women (62.1 $\pm$ 2.4 years, n=42) had an average of 6.99 $\pm$ 0.72 mmol/mmol creatinine (Arjmandi et al., 2003).

The results regarding DPD are in contrast to a recent meta-analysis (Taku et al., 2010b): 50-90 mg/day of aglycone isoflavone for 10 weeks to 12 months, decreased urinary DPD by 18.5% in postmenopausal women. The current study was a six-week intervention and may have been be too short to assess a change in DPD. Two previous short-term isoflavone interventions, each of four-week duration, found a significant decrease in DPD in women receiving aglycone isoflavone supplementation, 14 mg/day (Mori et al., 2004), and 38.4 mg/day Uesugi et al., 2002), which are lower doses than the dose used in the current study. However, the perimenopausal group in the trial by Uesugi and colleagues (2002) may have been more responsive to isoflavone treatment (Ma et al., 2011) compared to the 1-10 year postmenopausal women in this study. Furthermore, the trial by Mori et al. (2004) was considered low quality (Taku et al., 2010a). In regards to equol production and bone resorption markers, Wu et al. (2006) measured urinary DPD in response to 75 mg/day isoflavone conjugates, and found no change in DPD in either equol producers or non-producers. Equol levels reached 81

ng/mL in the equol producers (mean age 55 years) (Wu et al., 2006). Interestingly, BMD loss was inhibited at the subtotal body and hip exclusively in the equol producers (Wu et al., 2006). Urinary DPD decreased by 24% in non-equol producers after treatment with a pure S-equol supplement, 10 mg/day for 12 months (Tousen et al., 2011). Serum equol reached 85.1 nmol/L (20.7 ng/ml) during supplementation (Tousen et al., 2011).

This study adds to the limited literature regarding the effect of isoflavone supplementation on plasma CTx in postmenopausal women. There have been no meta-analyses in this area as there are only two relevant studies that have measured this parameter (Albertazzi, Steel & Botazzi, 2005; Marini et al., 2008). In these studies, plasma CTx did not respond to isoflavone supplementation. Pure genistein supplements were used in the studies by Albertazzi et al. (2005) and Marini and colleagues (2008) whereas this study used a mixed supplement. Marini and colleagues (2008) found that when analysing each year separately that there was a significant decrease in plasma CTx over years 2-3, but there was high participant dropout for the third year that would bias the results. More long-term RCT are needed to elucidate the relationship between isoflavone and plasma CTx in postmenopausal women. It is important that these future studies measure equol production.

One potential effect on bone turnover not estimated in this study was the effect of the vitamin C content of kiwifruit on calcium absorption from the diet. Vitamin C has been shown to facilitate calcium absorption and retention in an animal study (Morel & Wolber, unpublished data, as cited in Wolber et al., 2013, P249). Given that consumption of two green kiwifruit daily provided an additional 412-464% of the RDI for vitamin C, the kiwifruit and isoflavone treatment had the potential to increase the fractional absorption of intestinal calcium. However, serum calcium and PTH were not measured, so the effect of kiwifruit on calcium retention is unknown. Nonetheless, this effect seems unlikely as neither of the bone resorption markers decreased in response to the kiwifruit and isoflavone treatment.

There are several potential reasons for the lack of association between isoflavone supplementation and bone resorption observed in this study. Firstly, the intervention duration was too short: meta-analysis by Taku et al., (2011) found significant change in urinary DPD following isoflavone interventions ≥10 weeks. In addition, the equol producer subgroup (n=10, 30% of participants) was underpowered to detect a change in bone turnover markers (discussed further in the limitations section). At least 27 participants were required to detect a significant change in urinary DPD (≥1.79 nmol/mmol creatinine) and at least 20 participants were required to detect a significant change in CTx (≥0.09 nmol/L).

Tousen and colleagues (2011) report a significant decrease in urinary DPD in response to equol supplementation in non-equol producing postmenopausal Japanese women. Serum equol levels in the equol producers of this study were comparatively low and may explain the absence of an effect on bone turnover. The average serum equol obtained in the current study was 16.6 nmol/L, which is equivalent to 3.93 ng/ml, whereas in the study by Tousen et al. (2011) serum equol reached 20.7 ng/ml in equol producers. Moreover, Tousen et al. (2011) conducted a longer intervention with a larger sample size (12 months; n=23).

Finally, the women in this study ranged from 1-10 years postmenopause, and based on serum E2 levels it is estimated that participants were on average 8-10 years postmenopausal (Sowers et al., 2008). Bone turnover and bone loss is most rapid in the in early menopause (1-5 years since the onset of menopause) and isoflavone intervention has been shown to be more effective at preserving bone health in early postmenopausal women (Ma et al., 2008). The current study group may have been less responsive to an isoflavone intervention.

5.4 Lipid parameters

The baseline levels of serum lipid parameters are similar to those obtained in other groups of healthy postmenopausal women (Kreijkamp-Kaspers, et al., 2004; Welty, Lee, Lew, & Zhou, 2007). In the current study serum lipids values for TC, LDL-c, HDL-c and TAG are as follows: 5.68 $\pm$ 0.165 mmol/L, 3.57 $\pm$ 0.136 mmol/L, 1.65 $\pm$ 0.066 mmol/L, and 0.966 $\pm$ 1.08 mmol/L. These values are similar to reports by a comparable to another study of healthy postmenopausal women for TC, LDL-c, HDL-c and TAG: 5.91 $\pm$ 1.01 mmol/L, 3.70 $\pm$ 0.83 mmol/L, 1.50 $\pm$ 0.39 mmol/L and 3.32 $\pm$ 2.51 mmol/L (Welty et al., 2007). TAG and higher HDL-C were slightly lower in this study group compared to these previous studies, and may indicate a comparatively 'healthy' lipid profile in terms of CVD risk (Wu et al., 2006).

In the current study isoflavones did not have a hypo-cholesterolemic effect. There were no changes in TC, LDL-c or TAG. This contrasts with results from Clerici et al. (2007) that isoflavone-enriched pasta reduced both serum TC and LDL-c by 7.3% and 8.6% respectively over a 4-week intervention. Equol producers (n=20) in the group exhibited a similar decrease in serum TC but a larger decrease in serum LDL-c than non-equol producers (Clerici et al., 2007); serum equol was 25.5 $\pm$ 4 ng/ml in equol producers, which is considerably higher than the equol producers of the current study. The hyper-cholesterolemic sample population recruited by Clerici et al. (2007) may exhibit greater response to isoflavone supplementation (and serum equol) than normolipidemic populations.

In agreement with the results of this study, lipid parameters were not improved after one year of isoflavone supplementation (99 mg/day aglycone) in healthy postmenopausal women (Kreijkamp-Kaspers et al., 2004), nor in the equol producer subgroup (n=60). However, Kreijkamp-Kaspers et al. (2004) did not measure the final serum equol levels and cannot adequately account for the effect of equol production. In another study of postmenopausal women lipid parameters did not respond to isoflavones even when analysing the equol producers (n=117) separately (Hall et al., 2006).

The current study reported a synergistic effect between kiwifruit consumption and isoflavone supplementation exclusively in equol producers; serum HDL-c increased by 9.5% following the kiwifruit/isoflavone treatment. Equol producers receiving isoflavone treatment alone had no change in serum HDL-c, whereas non-equol producers experienced a decline in serum HDL-c across both treatments (-5.0% in the isoflavone and -4.6% in the kiwifruit/isoflavone treatment). No other interventions have reported similar findings relative to equol production. Although serum HDL-c decreased in non-equol producers, HDL-c was significantly higher at baseline and decreased to a level equivalent to the equol producers after each treatment.

Potter et al. (1998) reported an increase in serum HDL-c in postmenopausal women receiving 56 mg isoflavone per day for 24 months; it is not known whether equol production modulated this effect, as serum equol was not measured. A study by Wu et al. (2006) found a significant increase in serum HDL-c following 6-month isoflavone supplementation in conjunction with walking, but this effect was significant for walking independently of isoflavone intake.

The findings of this study suggest that some bioactive component/s of green kiwifruit may act in synergy with equol, due to the fact serum HDL-c dropped in non-equol producers consuming kiwifruit. Serum HDL-c is inversely associated with risk of CVD development; this relationship has been partially attributed to the action of sequestering detrimental oxidised-LDL-c (Navab, Reddy, Van Lenten, & Fogelman, 2011). Regular long-term supplementation of isoflavones and kiwifruit in postmenopausal equol producing women may reduce the risk of CVD development. The physiological mechanism underlying this benefit in equol producers is unclear but there are several possibilities that require further research. It is not currently known how HDL-c synthesis is affected by ER-β agonist activity. Equol's antioxidant qualities may reduce systemic inflammation: an inflammatory environment is detrimental to the anti-atherogenic role of HDL-c (Navab et al., 2011) and a study by Nishide et al. (2013) found a reduction in inflammatory cytokine expression in the bone marrow cells of Ovx mice treated with equol. Moreover, equol and the carotenoid lutein, which is present in kiwifruit, have been shown to act synergistically in their inhibition of

osteoclastogenesis (Tadaishi et al., 2011). It is possible, although not estimated in this study that a combination of antioxidants, from equol and lutein, decreased systemic inflammation during the kiwifruit and isoflavone treatment and this was associated with the increase in serum HDL-c. However, research is this area is limited and further studies are warranted to measure the correlation between serum equol, carotenoids and lipids in postmenopausal women supplemented with isoflavones.

HDL-c is also affected by particular dietary factors and nutrients. Specific foods, such as, oatmeal, legumes, and walnuts have been shown to increase HDL-c (Welty et al., 2007). Polyunsaturated fatty acids (PUFA) can increase HDL-c while trans-fatty acids tend to decrease HDL-c. (Mensink, Zock, Kester, & Katan, 2003). A high intake of low glycaemic carbohydrates can lead to a decrease HDL-c (Mensink et al., 2003). It is unclear whether intake of these dietary factors differed between study groups or over the intervention period, as they were not reported in this study. However, the changes in serum HDL-c seen here are consistent across the group according to equol production, which is directly related to isoflavone intake, and it is not likely that other dietary components had a major effect on this parameter.

5.5 Equol production and green kiwifruit consumption

Non-equol producers did not experience an increase in serum equol over the treatment periods; serum equol reached an average of 3.11 nmol/L in these participants. The equol producers reached an average serum equol concentration of 16.6 nmol/L after both treatments. In equol producers the equol concentrations were slightly higher after the kiwifruit/isoflavone intervention compared to isoflavone alone, but this difference did not reach significance.

From these results it is apparent that kiwifruit had no effect on the equol-producing capacity of daidzein-metabolising bacteria in equol producers. The log ratio of urinary daidzein to equol was used to delineate equol producers and non-producers: a ratio higher than -1.70 indicated an equol producer. This ratio is more accurate than measuring the absolute equol concentration because it accounts for the conversion of

a specified dose of aglycone daidzein (Levis et al., 2005). In this study, the log ratio of daidzein to equol did not change in the equol producers whereas the ratio declined in non-equol producers (table 4.5 and figure 4.5). There was a non-significant trend for the ratio to increase in equol producers following the kiwifruit/isoflavone treatment. In equol producers the consumption of kiwifruit did not have an additive effect on serum equol levels compared to the isoflavone treatment alone. However, this trend of increasing ratio of daidzein to equol indicates that kiwifruit could potentially modulate increased activity of equol-producing bacteria, but this would require further investigation. For future studies, faecal microbiota analysis would provide a more detailed investigation into the effects of kiwifruit on the activity and composition of equol-producing bacteria.

Although serum equol increased significantly amongst the equol producers following isoflavone supplementation, the current serum equol levels are relatively low compared to previous isoflavone interventions (Tousen et al., 2013; Wu et al., 2006). Equol producers had a serum equol concentration of 3.26 ng/ml after isoflavone treatment and 4.60 ng/ml after kiwifruit/isoflavone treatment. Equol produced by enteric daidzein conversion reaches peak serum concentration 24 hours after ingestion of daidzein (Yuan, Wang, & Liu, 2007). The blood samples in this study were taken ~24 hours after ingestion of isoflavone supplement and therefore indicate peak equol concentrations relative to the dose of daidzein administered. The low serum equol levels in this study may be due to the amount of daidzein in the supplements: the total dose of isoflavones received by participants was 50 mg/day aglycone isoflavones but the exact ratio of daidzein to genistein was not available (Otsuka Pharmaceutical Company Ltd., Tokyo, Japan). Serum daidzein increased from 10.9 nmol/L at baseline to 85.5 nmol/L at the end of treatment (results not shown). Wu et al. (2006) report that in participants receiving 75 mg/day isoflavone conjugates serum daidzein rose from 166.7 nmol/L to 888.8 nmol/L over the intervention period. Although participants from the study by Wu et al. (2006) already had high baseline serum daidzein, due to regular soy consumption, the final serum daidzein level well exceeded that achieved in the current study. This suggests that a much smaller daidzein dose was used in this study.

There was a main effect of equol production on plasma CTx and LDL-c levels. Plasma CTx and LDL-c were significantly lower in equol producers compared to non-equol producers: CTx was 0.426 μg/L versus 0.563 μg/L and LDL-c was 3.27 mmol/L versus 3.45 mmol/L in equol producers versus non-equol producers. The lower serum levels of CTx and LDL-c in equol producers may be associated long-term with lower rates of bone resorption (Vasikaran, 2008) and risk of CVD development (Navab et al., 2011). Further study is warranted to investigate habitual daidzein intake in NZ postmenopausal women and its association with equol production and markers of bone and cardiovascular health. Regular daidzein intake was not estimated in this study and will be much lower than in Asian women (Jackson et al., 2011).

5.6 Strengths and limitations of this study

5.6.1 Strengths

This is one of few studies that have measured the response of bone markers, ucOC, DPD and CTx, to isoflavone supplementation in postmenopausal Caucasian women. Also, the combined intervention of kiwifruit and isoflavones has not been previously examined. Covariates of bone turnover, BMI, nicotine, and alcohol consumption, were accounted for in this study during the screening phase of recruitment, by excluding participants who were smokers, and/or consumed more than two standard drinks per day. Additional factors that influence the level of bone turnover markers were also carefully controlled by exclusion criteria. Furthermore, equol production had not been examined in NZ postmenopausal women: the proportion of equol producing women this pilot study was 30% and is consistent with previous studies of Caucasian non-vegetarian women (Setchell et al., 2005).

5.6.2 Limitations

This current pilot study used an intervention to assess the effects of isoflavone supplementation on bone turnover markers and lipid profile in NZ postmenopausal women. As A pilot study by definition uses a relatively small sample size and duration to test the feasibility of a study design. Analysing the equol-producer subgroup required a larger sample size to detect a change in markers of bone turnover due to the low prevalence of equol-producing Caucasian women, which is between 25-30% based on both epidemiological and clinical research (Tousen et al., 2013). From sample size calculations, detecting a significant change in DPD required the largest equol-producer subgroup of 27 participants. Based on equol-producer prevalence in the target population, a sample size of at least 128 individuals would be required to obtain an equol-producer subgroup of this size. Moreover, a much larger geographical area was necessary to recruit postmenopausal women that fit the eligibility criteria. Obtaining this sample size was not financially for a short-term pilot study. Moreover, there was no placebo treatment group as it is not practical for a pilot study to run for a ~8 months, as would be required to fit this crossover design.

Physical activity was not measured over the intervention period and it is known that exercise can influence the levels of particular bone turnover markers (Garnero et al., 2000; Seibel, 2006). The BMI of participants was similar to healthy postmenopausal women of the same age. This gives a crude indication of normal activity levels and healthy lifestyle in these participants. However, a change in regular physical activity levels during the intervention may have masked a change in the bone resorption markers in this study. In addition, physical activity influences the lipid profile (Wu et al., 2006). A large variation in physical activity among the participants could have masked some changes to the lipid profile. Additional factors influencing lipid profile are dietary fat intake and type, which were not analysed from the food diaries (Mensink et al., 2003). However, the lipid profiles of the participants were similar both within and between groups at baseline. It is possible that lipid intake changed over the intervention period and an endpoint food diary would have accounted for this variation.

Analysis of faecal microbiota over the treatment period would have clarified whether isoflavone or kiwifruit consumption affects the composition or abundance daidzein-metabolising bacteria. Previous studies suggest that both isoflavones and kiwifruit have the potential to modulate the equol-producing microbiota (Bolca et al., 2007; Lee et al., 2012).

The intake of vitamin K was not estimated in this study and it is not clear whether participants met the NZ AI (60 μg/day) for 51-70 year old women. Nonetheless, previous studies suggest that the current national standard for AI of vitamin K is low in regards to producing serum vitamin K levels that maximise OC carboxylation for the potential reduction of fracture risk (Binkley et al., 2009; Booth et al., 2000).

Food diaries are highly variable in accuracy (Brunner, Stallone, Juneja, Bingham, & Marmot, 2001). It is well established that self-reporting of food intake is subject to participant behaviour and cooperation and 30-50% of participants will alter their intake when completing a food diary (Brunner et al., 2001). The use of a psychometric Likert scale would have helped to account for inaccurate reporting by comparing how dietary records compare to usual dietary intake in each individual.

The use of a pure daidzein supplement would have been more appropriate to delineate the association between equol and markers of bone and cardiovascular effect to eliminate confounding with the genistein present in the supplement. The best option would be a pure S-equol supplement, as this would have eliminated daidzein effects. No pure daidzein or S-equol supplements were available for purchase in NZ and due to government restrictions it was only possible to import supplements containing a mix of isoflavones.

5.8 Implications for human health and future research

Green kiwifruit may represent an ideal food to increase vitamin K intake and reduce plasma ucOC in postmenopausal women. It is possible that serum vitamin K increased with green kiwifruit consumption, and modulated the decrease in plasma ucOC but

this must be verified in future studies. Serum ucOC is related to bone health and a decrease in this bone marker may reduce future fracture risk, given that plasma ucOC is suppressed long-term. Green kiwifruit and equol improved serum HDL-c levels in equol producers, which could have long-term benefits reducing the risk of CVD development. In future studies, the use of a pure equol supplement would enable the direct assessment of equol on serum HDL-c in the non-equol producer group.

The results of this study suggest that isoflavones, and equol production, have no effect on bone resorption, shown by the lack of change in the biochemical markers of bone resorption, urinary DPD and plasma CTx. Nonetheless, this should not deter further research regarding the potential bone-protective effects of equol. The undersized equol producer subgroup was insufficiently powered to detect significant changes in these bone markers. A higher dose of aglycone daidzein or the use of pure S-equol supplements will achieve perhaps more physiological relevant levels of serum equol.

5.9 Conclusions

The supplementation of isoflavones, daidzein and genistein (50 mg/day), had no effect on bone turnover in postmenopausal Caucasian NZ women. In addition this study found no effect of equol production on bone turnover. Kiwifruit and isoflavone treatment benefited participants by decreasing their plasma ucOC levels, potentially by increasing vitamin K intake and subsequent OC carboxylation. In equol producers, isoflavones inhibited a decline in serum HDL-c, while the addition of green kiwifruit to isoflavone supplementation increased serum HDL-c. Equol and some bioactive component/s of green kiwifruit, i.e. carotenoid (lutein), synergistically modulate HDL-c metabolism.

The current pilot study suggests that further research is required concerning the potential effects of equol on the inhibition of bone loss and improvement to the lipid profile. The role of equol in bone and cardiovascular health will be depicted more accurately by studies with a longer duration and a larger sample size.

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Appendix 1

Consent form

MASSEY UNIVERSITY

Are soy isoflavones and kiwifruit good for your bone health?

CONSENT FORM

.

I have read the Information Sheet and have had the details of the study explained to me. My questions have been answered to my satisfaction, and I understand that I may ask further questions at any time.

I agree to participate in this study under the conditions set out in the Information Sheet.

Signature: ..... **Date:** .....

Full Name - printed .....

This project has been reviewed and approved by the Massey University Human Ethics Committee: Southern A, Application (13/15). If you have any concerns about the conduct of this research, please contact Dr Brian Finch, Chair, Massey University Human Ethics Committee: Southern A, telephone 06 350 5799, 84459.
email humanethicsoutha@massey.ac.nz.

Appendix Two

Participant information sheet

MASSEY UNIVERSITY

Do soy isoflavones and kiwifruit help to keep your bones healthy?

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.

Why are we doing this trial?

Women, especially postmenopausal women over 50 years old, constitute the most vulnerable population to osteoporosis due to estrogen loss when menstrual cycles end. Estrogen Replacement Therapy (ERT) has been used effectively in protecting bone mineral density and preventing vertebral fractures. However, data from the Women's Health Initiative study (WHI) indicated that taking estrogen long-term may cause breast cancer and other undesired side effects in postmenopausal women. Thus the search for alternatives is ongoing.

Some plants such as soybeans and red clover produce Phytoestrogens which are estrogen-like molecules and it is thought they may be an alternative to ERT. Isoflavones, which are the most studied phytoestrogens related to bone health, have been shown in studies of older Asian women to be protective against fractures, especially hip fractures.

To be effective, one of the components of the Isoflavones, Diadzein, has to be broken done in the intestines by gut bacteria to a product called equol. Only 30-50% of all populations are able to break Daidzein down to equol, and this percentage is less in Caucasians as Caucasian women do not seem to be able to digest the phytoestrogens as completely as Asian women.

Recent research in animals has indicated that the digestion of the isoflavones can be enhanced by changing the bacterial population that is in the large intestine. It is possible that specific New Zealand products could affect the intestinal bacteria population (prebiotic effect) and therefore affect the breakdown of the isoflavones, resulting in possible benefits for older women in New Zealand. These foods include green or gold kiwifruit. A pilot study completed at Massey in 2012 showed promising results.

The purpose of this study is to evaluate the effects of soybean isoflavones, in combination with green kiwifruit, on equol production and bone health in post menopausal women.

This 16 weeks study will have 60 female post menopausal participants, aged 50 to 65 years old, who have gone through natural menopause, where menopause is defined as at least 12 months beyond the last menstrual cycle. In this 16 week period you will have a 2 week lead-in, followed by a 6 weeks of taking 2 tablets daily, containing 46 mg of soy isoflavones, with or without kiwifruit, another 2 week wash out period and conclude with another 6 week period taking 2 tablets containing 46 mg of soy isoflavones with or without the kiwifruit.

We will require a **total of 4 fasted morning blood and spot urine samples** from you over this 16 week period, as well as **4 24 hour urine collections**. We will also ask 30 of the participants to provide us with 2 faecal samples during the trial, one before you start intervention of soy isoflavones and kiwi fruit, and the other after the intervention is completed 6 weeks later. During the 16 week period we will ask you not to eat any soy products, yoghurt or kiwifruit other than what we will give you. Blood, urine and faecal samples from this study will be sent to Japan

for analysis by the partners in the joint venture undertaking this study. The study is funded by a Ministry of Business Innovation and Employment grant "Functional Foods" under a bilateral agreement between NZ and Japan.

Would you like to take part?

We would like to invite 60 women, aged 50 - 65 years to join our study. To fit in to our study you should:

- be a non smoker
- be able to swallow a capsule
- be at least 12 months beyond your last menstrual cycle
- not have osteoporosis
- have no history of bone disease or any systemic disease that may affect bone density
- have not been diagnosed with diabetes
- have not been diagnosed with high blood cholesterol (have a total cholesterol of 6mmol/L or less)
- not have had gastrointestinal diseases other than appendicitis
- have not had a gut infection within the last month
- not have an endocrine disease
- not be taking any fibre supplements
- have not taken antibiotics or laxatives during the month before the study
- have not had unexplained diarrhoea in the last month
- not have kidney impairment
- not have any liver impairment
- have not had recent major surgery
- not be taking any vitamin, mineral or probiotic supplements or be willing to stop 4 weeks before the study begins
- not have had a bone fracture in the last 6 months

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

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What is involved?

This is a 16 week food study. We will exclude people taking medications which may interfere with our tests. We will ask you to agree to avoid the consumption of kiwifruit, other than those we give you, fermented milk, yoghurt, or other pre or probiotic dietary supplements for the16 weeks. We will ask you to take 2 tablets containing 46mg of the Isoflavones daily for 2 six week periods.

We will ask you for blood and urine samples on a **total of 4 occasions** during the trial. We will give you the collection kit for the urine samples and explain the procedure to you.
We will also ask 30 of the participants to provide us with 2 faecal samples, one before you start taking the kiwi fruit and soy Isoflavone tablets and the other six weeks later.

The total amount of your time that the whole trial will take is approximately 6 hours. In regard of the time and commitment involved in this study we will give you vouchers to the value of $150.

What are we going to measure?

Medical assessment for study inclusion

We will ask you to fill in a form about your health and current medications and to have a health screening blood sample taken at Medlab, for glucose and cholesterol levels and kidney and liver function. Those falling outside normal healthy parameters will be excluded for the study at this point.
We will also ask you come to Massey University for a DEXA scan to assess your bone density. Volunteers with osteoporosis as defined by the WHO criteria (T score < -2.5 at the hip or spine) will be excluded.

Blood, urine and faecal samples:

During the study we will ask for fasted samples at a total of 4 time points during the trial; after a 2 week lead in period, during week 8, during week 10, and during week 16. The blood samples will be used to assess **bone markers**; C telopeptide of type 1 collagen and undercarboxylated osteocalcin, **Isoflavone metabolites**; genistein, diadzein, and equol, **hormone levels**; Follicle Stimulating Hormone (FSH), Thyroid Stimulating Hormone (TSH) and Estradiol (E2), and **lipid levels**; total cholesterol, HDL cholesterol, and Triacylglyceride.
The urine samples will be used to assess **bone markers**, Deoxypyridinoline, and **isoflavones**, genistein, diadzein and equol. We will also ask 30 of the participants to take 2 faecal samples during the study.
Before you start the study we will ask you to take a soy isoflavone tablet at night and collect a urine sample from your second void the next day. This urine will be sent to Japan to be assessed for equol, the breakdown product of soy isoflavone.

Blood, urine and faecal samples will be sent to the Japanese collaborators in this study, the blood for the soy isoflavone analysis, the urine for soy isoflavones and bone markers Deoxypyridinoline, DPD, and the faeces for possible changes in the microflora population using an analysis of the Bacterial DNA.

If anything untoward is found in your tests you will be contacted by Dr 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.

A schedule of measurements is given below:

Time	Measurements
Before the study starts for inclusion or exclusion	Dexa bone scan at Massey University, whole body, hip and spine Screening blood sample at Medlab for cholesterol, glucose, kidney and liver function. **Inclusion or exclusion**
At the start of the trial Week 0-2 Wash out period	Ingestion of a Isoflavone tablet at night, followed by a second void urine sample the following morning. Fasted blood and urine sample at week 2. 24 hour urine collection Faecal samples from 15 participants.
Weeks 3 – 8 Take 2 capsules of soy isoflavones with or without kiwifruit daily (depending on crossover)	Fasted blood and urine samples at week 8 24 hour urine collection Faecal samples from 15 participants.
Weeks 9 - 10 Wash out period	Fasted blood and urine samples at week 10 24 hour urine collection Faecal samples from 15 participants.
Weeks 11 – 16 Take 2 capsules of soy isoflavones with or without kiwifruit daily (depending on crossover)	Fasted blood and urine samples at week 16 24 hour urine collection Faecal samples from 15 participants.

Are any of the procedures harmful or painful?

This study involves routine clinical and laboratory testing procedures, which are widely used around the world. We will also ask you to come to the Nutrition Studies Laboratory to assess your bones, while wearing surgical scrubs, on our Hologic Dexa machine used for estimating bone mineral density and bone mineral content of your hip and femur. The DEXA has X-ray beams at 2 different energies and while no dose of radiation is harmless this dose is very low and unlikely to cause harm. The total effective dose of radiation to which you will be exposed to is 10 microsieverts (μSv), which is much lower than the range normally used in medical diagnostics. To place in perspective, the amount of radiation you are exposed to during a flight to the United Kingdom return is 100 μSv and from a dental Xray 50 μSv. 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, Plant and Food Research. No names will be used, just the designated numbers. We will give you a summary of the findings of our research if you would like one.

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.

Who is funding this research?

Ministry of Business Innovation and Employment grant "Functional Foods" under the bilateral agreement between NZ and Japan.

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.

Will I get any financial ion?

We will give you retail vouchers of $150 to compensate you for your inconvenience and time.

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 at any time;*
- *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 0800 0800 28

This study has been reviewed and approved by the Massey University Human Ethics Committee:Southern A, Application (13/50). If you have any concerns about the conduct of this research, please contact Dr Brian Finch, Chair, Massey University Human Ethics Committee: Southern A, telephone 06 350 5799 x 84459, email humanethicsoutha@massey.ac.nz

Appendix Three

Screening questionnaire

MASSEY UNIVERSITY

HUMAN NUTRITIONAL STUDIES PROCEDURE

Screening Questionnaire

Name……

DOB……

Telephone #……
Cell phone#……

Address……

Email address.....

Have you ever had any of the following:	√ if yes or X if no
A history of gastrointestinal disease other than appendicitis or irritable bowel syndrome	
Diabetes or persistent sugar in the urine	
Endocrine disease (hormone trouble)	
Thyroid disease (eg goitre)	
Kidney problems	
Disorders of the liver	
Recent major abdominal surgery	
A bone fracture in the last 6 months	
A gut infection within the last month	
Osteoporosis or a bone disease	
Any chronic or recent constipation	
Diarrhoea in the last 1 month	

Do you smoke?

How many drinks containing alcohol do you have a day?

Have you ever been told you have a high alcohol consumption rate?

Are you allergic to green kiwifruit

Are you allergic to any soy products?

What was the date / year of your last menstrual period?

Are you allergic to plasters?

Do you have any blood borne infections?

Do you take any of the following	√ if yes or X if no
Vitamin supplements	
Mineral supplements	
Iron supplement	
Other health foods	
Extra calcium	
Pills for anaemia	
Pills for gastric ulcer	
Pills for diabetes	
Pills to lower the blood fat levels	
Fibre products for your bowels	
Blood thinning pills eg warfarin or aspirin	
Probiotic, prebiotic or fibre supplement eg lactulose	
Antibiotics within the last 1 month	
Water pills (diuretics)	

Please list medications and supplements you are currently taking below

Do you have any plans for any activity or such, which could cause a change of diet, or difficulty in keeping to the requirements for this trial over the 16 week trial period, eg sporting events, holiday, surgery etc?

Appendix Four

3-Day food diary

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 Chris Booth on (06) 3505901 or email C.L.Booth@massey.ac.nz

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)
Example 7:55am	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

Recipes (Day 1)

Recipes (Day 2)

Date: **DAY 2**

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

Date: 21st November 2010 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 5

24-hour urine collection

Methods of 24-hour urine collection

1) Period :

On the … October, first urine is not collected. You collect 24-hour urine from second urine of the … October to first urine of the … October.

※Please carry the unit on you for all the urine of 24 hours.

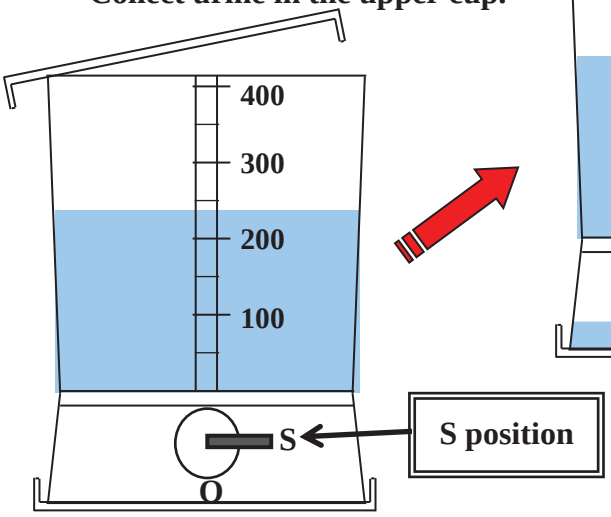
2) Urine collection

1. Confirm whether the bottom lid is securely fitted and cock is set to the **S** position. Collect your urine in jug supplied. Remove the upper lid and then pour urine in the upper cup.
2. Place the unit **upright** and **measure urine volume**. If the urine is filled in the upper cup, the volume is 400mL.
3. Confirm that urine has been channeled into the thin pipe. The urine is completely channeled to the top within 10 seconds.
4. Turn the cock to the **O** position. The urine in the thin pipe drops into the lower cup. **Don't attach the top lid.**
5. Confirm that all the urine in the thin pipe has dropped into the lower cup and turn the cock back to the **S** position.
6. Tip out the remaining urine in the upper cup, attach the top lid, and store the unit ready for the next urine collection. **Don't wash the unit.**
7. After you finish urine collection, please bring the unit with urine in the lower cup to the lab at Massey University.

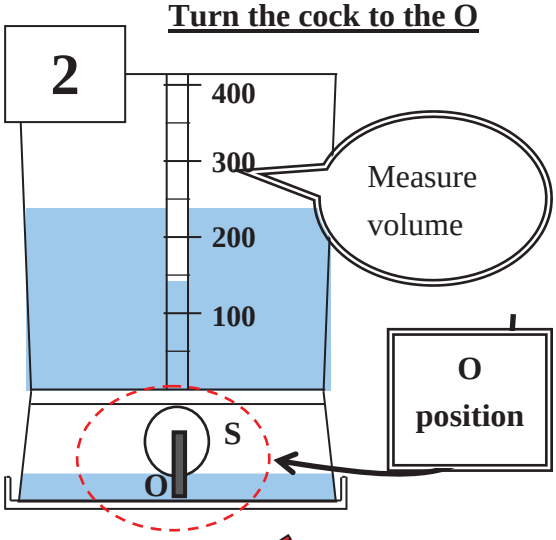
8. Please record the urine volume each time below:

Procedure

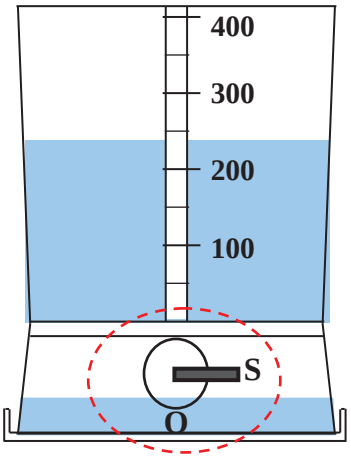
1 **Set the cock to the S position.**
Remove the upper lid.
Collect urine in the upper cup.



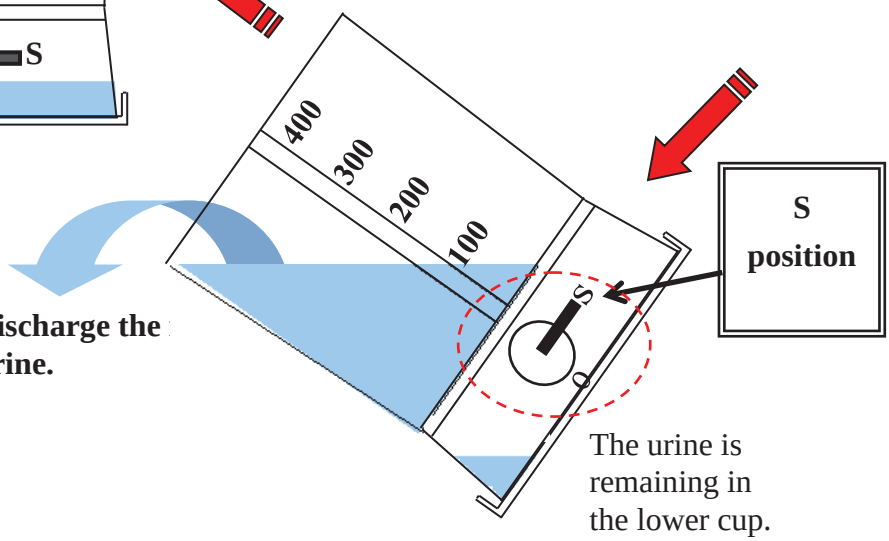
2 **Place the unit <u>upright</u> and <u>measure urine volume.</u>**
<u>Turn the cock to the O</u>



3 **After you confirm that all the urine in the thin pipe drops into the lower cup,**

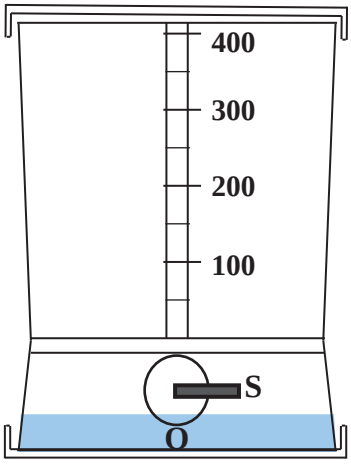


4 **Discharge the urine.**



The urine is remaining in the lower cup.

5 **Attach the top lid and store the unit ready for the next urine collection. Don't wash the unit.**



Appendix 6

Compliance chart

ID	day	date	medication taken	different diet eaten	capsules taken
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				

ID	day	date	medication taken	different diet	capsules taken
Week 5	Monday				
Week 5	Tuesday				
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 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				