Increased intake of vegetables, herbs and fruit: effects on bone in postmenopausal women

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ABSTRACT

Dietary approaches to address bone loss at midlife usually involve supplementation or fortification. We aimed to investigate a food based approach to reduce bone turnover in post-menopausal (PM) women in two studies. In the first study, we investigated whether daily inclusion of specific vegetables attributed with bone resorbing inhibiting properties was feasible. We hypothesised increased intake of fruit/vegetables to ≥9 servings/day would lower potential renal acid load (PRAL) significantly (~20mEq/day) and increase urine pH (0.5 pH units) sufficiently to affect bone markers. The results of the first study confirmed the feasibility of daily inclusion of specific vegetables, reduction in renal acid load and increased urine pH. The subsequent Scarborough Fair Study (SF) used a randomised, active comparator design to increase specific vegetable/herb/fruit intake in two groups (A and B) of 50 PM women, from ≤ 5 servings/day to ≥ 9 servings/day for 3 months while a control group consumed their usual diet (n=43). Primary outcome variables were plasma bone markers which were assessed at baseline, six weeks and twelve weeks. Secondary outcome variables were plasma inflammation markers including adiponectin, urinary electrolytes (calcium, magnesium, potassium and sodium) and dietary intake assessed at baseline and 12 weeks and urinary pH assessed twice weekly. Increased intake of vegetables/herbs/fruit reduced P1NP and CTX (osteopenia) in Group B (SF) and urinary calcium loss in both intervention groups A and B (SF) with reduced PRAL. Adiponectin, tumour necrosis factor, interleukin 6 and 10 reduced in all groups. This study showed the SF vegetables/herbs/fruit may influence bone turnover and inflammatory markers. Few human intervention studies demonstrate reduction in plasma bone resorption markers with diet. Even fewer studies demonstrate reduction without supplementation with calcium, vitamin D, alkaline substrates, concentrated extracts or consumption of large quantities of a single functional food. The SF vegetables/herbs/fruit may protect against high bone turnover and subsequent bone loss in women with osteopenia and may have possibilities as an adjuvant to pharmaceutical therapies or a holistic dietary approach to reduce bone turnover and bone loss. **Trial registration** ACTRN 12611000763943

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To my family and friends, your love and support was much appreciated.

Statement of originality

This is to certify that to the best of my knowledge, the content of this thesis is my own work. I certify that the intellectual content of this thesis is the product of my own work and that all the assistance received in preparing this thesis and sources have been acknowledged.

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ABBREVIATIONS

3DDD; 3 Day Diet Diary

AdipoR1; Adiponectin Receptor1

AdipoR2; Adiponectin Receptor2

AI; Adequate Intake

ALP; Alkaline Phosphatase

ANOVA; Analysis of Variance

AP-1; Activator Protein-1

APOSS; Aberdeen Prospective Osteoporosis Screening Study

ARE; Antioxidant Response Elements

ATF-4; Activating Transcription Factor 4

BAP; Bone Alkaline Phosphate

BMD; Bone Mineral Density

BMI; Body Mass Index

BMP; Bone Morphogenetic Protein

BMU; Bone Multicellular Unit

BRIFs; Bone Resorption Inhibiting Foods

BSA; Body Surface Area

BSP; Bone Sialoprotein

CAT; Catalase

CATK; Cathepsin K

CI; Confidence Interval (95%)

CL; Confidence Limit

Colla 1; Collagen Type 1 alpha

Colla 2; Collagen Type 2 alpha

CBFA-1; Core Binding Factor-1

COX-1; Cyclooxygenase (constitutive)

COX-2; Cyclooxygenase (inducible)

CRP; C-Reactive Protein

CSF1; Cytokine Stimulating Factor 1

μCT; Micro computed Tomography

CTX; C-Terminal Telopeptide of Type I Collagen

DASH; Dietary Approaches to Stopping Hypertension

DHA; Docasahexaenoic Acid

DKK-1; Dickkopf-1

DPD; Deoxypyridinoline

DXA; Duel-Energy X Ray Absorptiometry

EAR; Estimated Average Requirement

ECF; Extracellular Cellular Fluid

EPA; Eicosapentaenoic Acid

EpRE; Electrophile Response Elements

FFQ; Food Frequency Questionnaire

FOX01; Forkhead Box Protein 01

GLA; Gamma Linolenic Acid

GPCS; -L-Glutamyl–trans-S-1-Propenyl-L-Cysteine Sulfoxide

GPx: Glutathione Peroxidase

GR; Glutathione Reductase

GSH; Reduced Glutathione

GSSH; Glutathione Disulphide

GST; Glutathione S-Transferase

HFI; Hip Fracture Incidence

Hoxa-2; HomeoboxA-2

IGF-1; Insulin Like Growth Factor-1

IHH; Indian Hedgehog Signalling

IL-6; Interleukin 6

IL-10; Interleukin 10

IFCC; International Federation of Clinical Chemistry and Laboratory Medicine

IOF; International Osteoporosis Foundation

JAK-STAT; Janus Kinase-Signal Transducer and Activator of Transcription Pathway

KEAP1; Kelch like ECH-Associated Protein

LPR5; Lipoprotein Receptor 5

MAPK; Mitogen-Activated Protein Kinase

MCP-1; Monocyte Chemoattractant Protein-1

M-CSF; Macrophage-Colony Stimulating Factor

MMP; Matrix Metalloproteinase

MOH; Ministry of Health (NZ)

MSC; Mesenchymal Stem Cell

MC3T3-E; Mus Musculus Calvaria

NAE; Net Acid Excretion

NEAP; Net Endogenous Acid Load

NFATc-1; Nuclear Factor of Activated T-cells

NF-κB; Nuclear Factor kappa B

NO; Nitric Oxide

NQO1; NAD(P) H-Quinone Oxidoreductase-1

Nrf2; Nuclear Factor-Erythroid 2 Related Factor-2

NSAID ; Non-Steroidal Anti-Inflammatory Drug

NTX; Cross-linked N-Ttelopeptide of Type I Collagen

NZ; New Zealand

OA; Organic Acid

OHP; Hydroxyproline

OPG; Osteoprotegerin

OSCAR; Osteoclast Associated Receptor

P; Test statistic probability considered significant at the level 0.05

PI3K; Phosphatidylinositide 3-Kinases

PERK; Protein kinase RNA-like Endoplasmic Reticulum Kinase

PGE2; Prostaglandin-2

PKC; Protein Kinase C

P1CP; Procollagen Type I C Propeptide

P1NP; Procollagen Type I N Propeptide

PAI-1; Plasminogen Activator Inhibitor

pQCT; peripheral Quantitative Computed Tomography

PRAL; Potential Renal Acid Load

PYD; Pyridinoline

PTH; Parathyroid Hormone.

r ; Pearson's Correlation (2 tailed)

RANKL; Receptor Activator of Nuclear Factor kappa B Ligand

RCF; Relative Centrifugal Force

RDI; Recommended Daily Intake

RNA; RiboNucleic Acid

ROS; Reactive Oxidative Species.

RPM; Revolutions per Minute

Runx2; Runt Related Transcription Factor

SA-BMC; Size Adjusted Bone Mineral Content

SATB-2; Special AT-rich Sequence Binding Protein

SD; Standard Deviation

SDT; Suggested Dietary Target

SEM; Standard Error of Mean

SF; Scarborough Fair

SIRT-1; Sirtuin-1

SOD; Superoxide Dismutase

SPSS; Statistical Package for Social Sciences

SPARC; Sport and Recreation New Zealand

SWAN; Study of Women's Health Across the Nation

TA; Titratable Acidity

TAC; Total Antioxidant Capacity

TLRs; Toll-Like Receptors

TRAP; Tartrate Resistant Phosphatase

TREM-2; Triggering Receptor Expressed by Myeloid Cells-2

TNF; Tumour Necrosis Factor

WHI; Women's Health Initiative

Wnt/ catenin; Wingless/ Catenin Pathway

CHAPTER 1. INTRODUCTION AND PURPOSE OF THESIS

1.1 What is osteoporosis and its significance

Osteoporosis meaning "porous bone" derives from the Greek words osteoun (bone) and poros (pore) and is the term for inadequate bone mass. It is a global problem seen most often in the elderly and in women (80%) (National Osteoporosis Foundation, 2010). Bone loss is accelerated at early menopause resulting in bones become increasingly fragile and prone to breakage. This is attributed to low bone mass and consequent deterioration in bone tissue's micro-architecture (Kanis, Burlet, Cooper et al., 2008). World Health Organization (W.H.O) diagnostic criteria for osteoporosis include a bone mineral density (BMD) of more than or equal to 2.5 standard deviations below that of a normal young adult female (T score $\leq$ -2.5 S.D's) (World Health Organisation, 2004). Osteoporosis is considered one of the ten most important diseases affecting the world's population (Räth, Monetti, Bauer et al., 2008), and is particularly prevalent in developed countries with ageing populations and longer life spans. It is predicted to also become a problem in developing nations as their population's life span rapidly increases (Genant, Cooper, Poor et al., 1999). Osteoporosis poses a significant health and economic burden for New Zealand families and the public health system. As the number of older (>50 years) New Zealanders steadily increases, the cost of treating fractures and secondary illnesses related to osteoporosis is expected to rise from $330 million in 2007 to $458 million by 2020 (P. Brown, McNeil, Radwan et al., 2007; P. Brown, McNeill, Leung et al., 2011).

1.2 Diet, nutrition and bone loss in ageing

Bone loss associated with ageing involves a complex array of interactions. While family history and genetics appear to play a dominant role in bone health, this can be modified by diet, exercise (both present and past), lean body mass, vitamin D levels and lifestyle factors such as cigarette smoking, excessive alcohol intake and some medications (corticosteroid hormones and proton pump inhibitors)(Anderson, 2012).

Epidemiological studies attempting to identify dietary influences on bone health and bone loss can be confounded by the above factors. Therefore, determining the extent of dietary influences on bone loss due to ageing has posed a conundrum for researchers for several decades (Ilich & Kerstetter, 2000). Current estimates are that diet and physical activity may explain up to 25% of bone measurements throughout the life cycle, though the predominant influence is genetic (Anderson, 2012).

From a nutritional point of view, to prevent or ameliorate bone loss and subsequent osteoporosis the emphasis must be on overall nutrient adequacy (both macro- and micronutrients) (Anderson, 2012) due to many nutrients working synergistically. However, there are several important dietary factors strongly associated with building and maintaining bone health, which warrant closer scrutiny if potential prevention of age related bone loss is to be achieved and maximised through diet.

This important group of dietary factors includes not only the established macro- and micronutrients, fibre and food groups but also the contributions from a diet with a lower renal acid load and a variety of bioactive phytochemicals, which may reduce inflammation and oxidative stress. Differing strategies to prevent or reduce bone loss at midlife have utilised all the above dietary factors, though the evidence for some remains inconclusive and controversial (Pizzorno, Frassetto, & Katzinger, 2010; Fenton, Tough, Lyon et al., 2011) or is still relatively novel (Habauzit & Horcajada, 2008; Salminen, Kauppinen, & Kaarniranta, 2012).

The main nutrients linked with bone health include protein (Ilich et al., 2000; Bonjour, 2005; Heaney & Layman, 2008), calcium (Dawson-Hughes & Bischoff-Ferrari, 2007; Bronner, 2009), vitamin D (K. Zhu, Devine, Dick et al., 2008; Kruger, Schollum, Kuhn-Sherlock.B. et al., 2010), vitamin K (Bullo, Estruch, & Salas-Salvado, 2011), vitamin C (Sahni, Hannan, Gagnon et al., 2008), potassium (Dawson-Hughes, Harris, Palermo et al., 2009; Whiting, 2011) and sodium (Frassetto, Morris, Sellmeyer et al., 2008). The two food groups associated with bone health are dairy (Heaney, 2009) and fruit and vegetables (Lanham-New, 2006; Tobias, Turley, Sefanogiannis et al., 2006; Trzeciakiewicz, Habauzit, & Horcajada, 2009).

The fruit and vegetable food group have a long history of association with bone health due to their provision of essential bone micronutrients, fibre, which enhances dietary calcium uptake (Kruger & Coetzee, 2013) and contributes to reducing calcium excretion and renal acid load by provision of bicarbonate precursors (New, MacDonald, Campbell et al., 2004; Remer, Manz, Alexy et al., 2011; Shi, Libuda, Schonau et al., 2012; Moseley, Weaver, Appel et al., 2013). More recently, the focus has been on fruit and vegetables provision of the majority of known phytochemicals (Lister, Skinner, & Hunter, 2007; Dinkova-Kostova & Talalay, 2008; Salminen et al., 2012) which can directly affect the skeleton by reducing inflammation (Cashman, 2008) and lowering oxidative stress (Poljsak, 2011).

1.3 Purpose of thesis

Determining if a nutritional intervention can be used to forestall bone loss and prevent fragility fractures and whether it can feasibly be implemented in the wider population is increasingly important, as costs to the public health system rise due to an ageing population. Increased consumption of fruit and vegetables, which are nutrient and phytochemical rich as well as alkaline forming, could provide a straightforward strategy to reduce bone loss during ageing. In addition, increased intake of fruit and vegetables may reduce levels of inflammation, which also is considered protective against other chronic diseases associated with ageing (Calabrese, Cornelius, Mancuso et al., 2008; Salminen et al., 2012).

Recommended intakes of fruit and vegetable servings per day are being scaled upwards by several national and international organisations (Lock, Pomerleau, Causer et al., 2005; Tobias et al., 2006; World Cancer Research Fund / American Institute for Cancer Research, 2007; National Heart Foundation NZ, 2009; Whitney & Rolfe, 2009; USDA & USDHHS., 2010) as evidence accrues for the ability of fruit and vegetables to reduce the risk of chronic disease associated with ageing (Kang, Ascherio, & Grodstein, 2004; Bazzano, 2006; Dauchet, Amouyel, Hercberg et al., 2006; He, Nowson, & MacGregor, 2006; Carter, Gray, Troughton et al., 2010; Vanzour, Rodriguez-Mateos, Corona et al., 2010). The evidence is particularly strong for vegetables (Villegas, Shu, Gao et al., 2008; Carter et al., 2010).

Fruit and vegetables dietary intake recommendations range from a minimum of 5 to 10 servings /day with proposed public health goals of 600 grams (7.5 servings) as a population minimum target, which does not include starchy vegetables and fruit (World Cancer Research Fund / American Institute for Cancer Research, 2007). Only two thirds of the NZ population reach the Ministry of Health daily recommendations of at least 2 servings of fruit and 3 servings of vegetables, and this recommendation is inclusive of starchy vegetables such as potatoes, kumara and pumpkin (Ministry of Health (NZ), 2003; University of Otago & Ministry of Health, 2011). The New Zealand definition of a serving of fruit is defined as follows: one average banana, apple or pear etc. (approximately 130 grams) or half a cup of stewed fruit (135 grams) or 25 grams dried fruit or one cup of juice (250mls). Vegetables servings include half a cup of cooked vegetables (50-80 grams) or one potato/other root vegetable (135 grams) or half cup raw leafy salad vegetables (60 grams). Australian 2013 guidelines (http://www.nhmrc.gov.au/guidelines/publications/n55) specify 5 servings/day of vegetables for women and six for men in addition to two servings of fruit/day. Guidelines also differ with respect to quantities of raw salad vegetables, where the Australian guidelines specify that one cup equals one serving rather than the New Zealand guidelines where half cup of raw salad vegetables equals one serving.

In light of recommendations from other organizations and countries, it could be argued that the New Zealand's Ministry of Health fruit and vegetable intake recommendations for an adult population are low, particularly when compared with the New Zealand Heart Foundations recommendations for women over 50 years which is 8 servings a day (National Heart Foundation NZ, 2009) and Australian Ministry of Health recommendations of at least 5 servings of vegetables and 2 of fruit for women (slightly more for men) (Australian Government, Ministry of Healthy Ageing, & National Health and Medical Research Foundation, 2013).

This thesis therefore seeks to determine whether a significant increase in fruit and vegetables intake to 9 servings /day, emphasising vegetables, fruit and herbs high in fibre, phytochemicals associated with bone health, and low net endogenous acid load is a feasible target in a population of New Zealand

midlife women and whether this increase can improve bone health. The effect on bone will be indirectly accessed via markers of bone resorption and formation, inflammatory markers and urinary calcium excretion coupled with estimated dietary acid load. An increase in urine pH of ≥0.5 is reported to reduce bone marker of resorption C-Terminal Telopeptide of Type I Collagen (CTX) (Wynn, Krieg, Aeschlimann et al., 2009).

The first study will aim to determine the feasibility of increased intake of fruit (≤3 servings/day) and vegetables (≥6 servings/day) to 9 servings/day, the change in estimated net endogenous acid production (NEAP), potential renal acid load (PRAL) and urine pH and whether any observed changes in urine pH is sufficient to warrant further investigation with bone markers.

The second study aims to determine the effects of increased intake of fruit (≤3 servings/day) and vegetables (≥6 servings/day) to 9 servings/day, and herbs on bone health. Baseline bone mineral density analysis (DXA) will be done, but any effects of the dietary change will be determined by surrogate plasma markers of bone and inflammation and urinary mineral excretion. Dietary data will be collected to determine changes in estimated potential renal acid load (PRAL), fibre and macro and micronutrient intake for comparison with New Zealand dietary recommendations (Ministry of Health (NZ), 2006). Urine pH will be monitored to assess whether there is a change in alkalinity with increased alkaline provision through increased intake of fruit and vegetables.

To the author's knowledge, these two studies will be the first to emphasise vegetable intake rather than fruit and include a combination of specific vegetables, herbs and fruit with bioactive components known to influence bone health in animal models but as yet untested in humans (Mühlbauer & Li, 1999; Mühlbauer, 2006).

1.4 Study objectives

1.4.1 Study One: The Feasibility study

Using a human model comprised of 21 midlife women (40-65 years) who represent a group at increased risk of bone loss, to investigate the following objectives:

1) Is an increase in fruit and vegetable intake to ≥9 servings/day (including daily consumption of allium, cruciferous and green leafy vegetables) achievable, in a community based setting.

2) How does this dietary change affect estimated dietary NEAP/PRAL, overall nutrient intake and intake (servings/day) from other food groups.

3) Does this dietary change affect urine pH and if so, to what degree is there an increase in alkalinity.

1.4.2 Study Two: The Scarborough Fair Study

Using a human model comprised of 150 midlife women (50-70 years) to investigate the following objectives:

To determine the effects of:

1) Increased intake of vegetables/herbs/fruit on bone and inflammatory markers in post-menopausal women.

2) Increased vegetables/herbs/fruit intake including specific bone resorption inhibitory fruit, vegetables and culinary herbs (BRIFs) on bone and inflammatory markers in post-menopausal women.

3) A decrease in estimated potential renal acid load (PRAL) of the diet on urinary excretion of minerals especially calcium.

CHAPTER 2. LITERATURE REVIEW

2.1 Bone structure

The paradox of bone is that it is often viewed as inert whereas its activity is ceaseless; it affords stiffness and rigidity while being flexible and resilient. Its silent, immutable persistence for centuries after death belies its rapid response and coordination of homeostasis it carried out in life (Cooks, 1955). Bone is a composite material with an impressive strength to weight ratio (Dempster, 2004). Collectively, the skeleton comprised of bones and cartilage accounts for 17% of body weight (Arnett, 2003), however it not only supports the adjoining muscles' movement but can withstand the muscles' lifting of weights of more than 3 times a person's total body weight (Chidlovski, 2012). Apart from its roles in providing mechanical support, it facilitates movement as well as protects soft tissues and vital organs.

Bone also stores vital minerals such as calcium, phosphate and growth factors. Approximately 99% of the calcium in the body is stored in bones and teeth (McConnell & Hull, 2011). As a very dynamic structure there is a constant interplay between the body's requirements and the release or deposition in bone tissue of critical minerals and growth factors (Garner & Anderson 2012). The skeleton has been compared metaphorically to a giant ion exchange column (Barzel, 1995), with withdrawals and deposition of minerals highly dependent on regulatory mechanisms at cellular and serum levels. A consistent dietary supply of bone building minerals (calcium, magnesium, phosphorus, boron, zinc, copper and manganese) is required along with vitamins D and K to maintain reserves in the skeleton (Anderson, Garner, & Klemmer, 2012b). Additional functions are fat storage (yellow marrow in long bones) and production of many types of blood cells (red marrow) (Anderson et al., 2012b).

Mature bone is composed of two types of tissue: compact (cortical) bone, which comprises 80% of the adult skeleton and spongy bone (cancellous) with many small openings comprising 20%. These two types of bone tissue are shown in Figure 1 along with the covering articular cartilage.

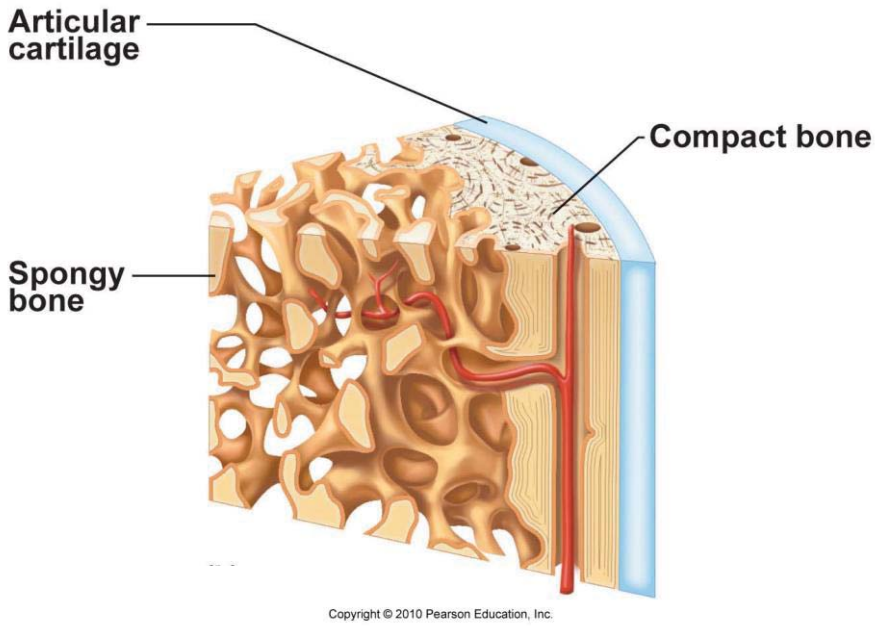

Figure 1 Compact and spongy bone

This figure shows compact (cortical) and spongy (cancellous) bone covered by the articular cartilage. Blood vessels are woven through trabecular spaces. Reproduced from Marieb, 2010 (with permission from Pearson Education Inc., 2010).

With its major functions being structural and mechanical, cortical bone is dense, with calcification contributing 80-90% of its volume and while it appears to have no obvious spaces, it does have microscopic pores (5-10%). The bony latticework that comprises spongy bone is termed trabeculae (little beams). Though appearing irregular in orientation these narrow columns of bone are precisely angled to contribute strength, while the spaces between them contribute lightness and protect the underlying red bone marrow responsible for most blood cell production.

The increased porosity of spongy bone (comprising 50-90% of its volume) is due to its metabolic activity with the principal function to provide a vast surface area for the exchange of ions. On a daily basis, up to half a gram of calcium can be lost or drawn in to the skeleton (Marieb & Hoehn, 2010).

While most bones in the skeleton are composed of both compact and spongy bone, osteoporotic fractures tend to occur where there is more trabecular rather than cortical bone. With ageing, osteoporosis affects mainly the spine and hip, with both areas becoming brittle and prone to fractures

(World Health Organization, 1994). Figure 2 shows a greater predominance of trabecular bone tissue at these sites (spine and hip).

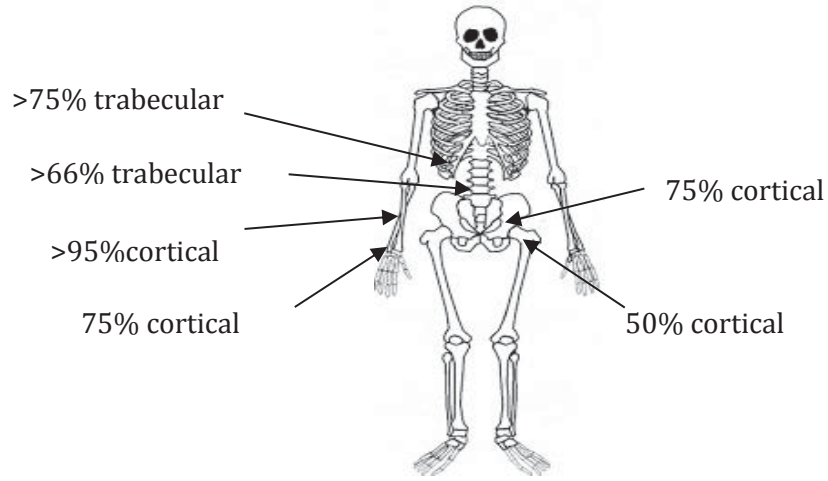

Figure 2 Trabecular and cortical bone sites

This figure demonstrates the proportion of trabecular and cortical bone at various body locations. Adapted from Baron (1999).

2.2 Composition

The major components of bone are cells (10%); organic matrix (30%) and minerals as crystalline hydroxyapatite (60%) with proportions of each of these varying over the life cycle (Weiner, Traub, & Wagner, 1999; Feng & McDonald, 2011). Bone contains relatively few cells leaving a preponderance of extracellular matrix that includes widely separated cells. The organic bone phase is predominantly protein with collagenous fibres (88%) and other proteins such as osteocalcin, osteonectin, proteoglycans (10%) and lipids and glycosaminoglycans (1-2%).

Around and inserted inside the collagen fibres are minerals of the inorganic phase. These are calcium, phosphate (PO_4^{-}) and carbonate in the form of hydroxyapatite [$Ca_{30}(PO_4)_6(OH)_2$] (Garner et al., 2012). The carbonated apatite crystals, thought to be the smallest crystals the body can form, are arranged in layers that cross over the collagen fibres (Weiner et al., 1999; Dempster, 2004). The remainder of the mineral phase consists of calcium phosphate $(Ca_3PO_4)^2$, calcium carbonate ($CaCO_3$), magnesium phosphate ($Mg_3(PO_4)_2$, calcium fluoride (CaF_2) and calcium or sodium chlorides ($CaCl_2$,

NaCl). The hardness of bone is dependent on the quality of the inorganic phase whereas flexibility is a product of the collagen fibres which provide tensile strength (Viguet-Carrin, Garnero, & Delmas, 2006; Garner et al., 2012).

2.3 Cell types and lineage

The three major cell types (Figure 3) with central roles in bone metabolism are osteoblasts (bone forming), osteoclasts (bone resorbing) and osteocytes (regulation of both osteoclasts and osteoblasts)(Bonewald, 2011).

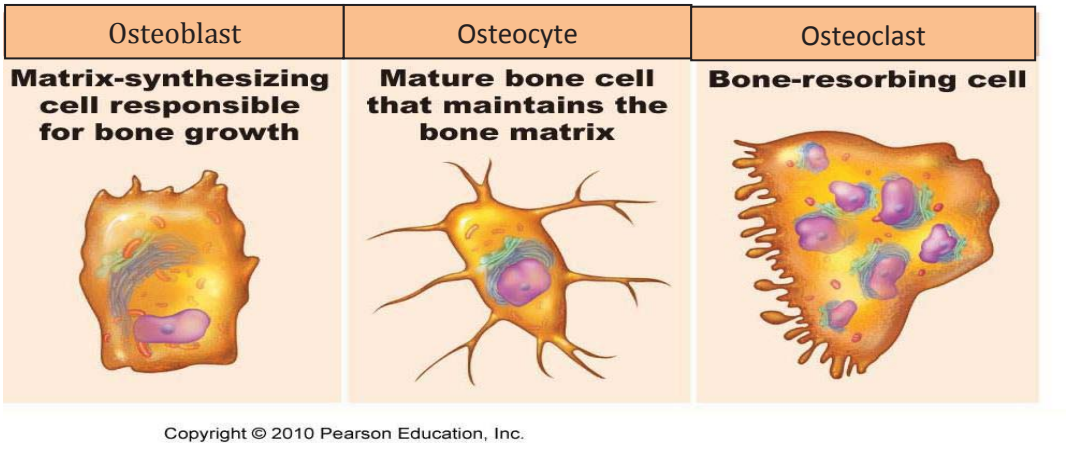

Figure 3 Bone cell types

This figure depicts the structure of the 3 bone cell types. Osteoblasts and osteocytes are both mononucleated while osteoclasts are large multinucleated cells with a ruffled membrane.
Adapted and reproduced with permission from Pearson Education Inc. 2010.

Osteoclasts are derived from haematopoietic stem cells and directly from monocytes/macrophage precursors, whereas osteoblasts are of mesenchymal stem cell (MSC) origin and share this common precursor cell (MSC) with adipocytes (McCormick, 2007). Osteocytes are a subpopulation of osteoblasts, which have become surrounded by osteoid (unmineralised bone matrix) and cocooned in lacunae during mineralisation (Raggatt & Partridge, 2010) and play a central role in bone homeostasis (Teti & Zallone, 2009; Teti & Eastell, 2010; Atkins & Findlay, 2012). Regulation of bone cell differentiation and commitment is tightly controlled, involving numerous signalling networks and

transcriptional regulation of gene expression (Boyce & Xing, 2008; Soltanoff, Chen, Yang et al., 2009).

The osteoblast cell lineage begins with the undifferentiated MSC which have the potential to become chondrocytes, adipocytes, myoblasts or osteoblasts (Figure 4). The differentiation of the MSC to the osteo-chondro progenitor is negatively influenced by Homeobox A-2 (Hoxa-2) and positively influenced by special AT-rich sequence binding protein 2 (SATB-2) (Soltanoff et al., 2009). The trigger for the development of bone forming cells is thought to be the Indian Hedgehog (IHH) signalling switch which allows for osteoblast progenitors to develop into runt-related transcription factor (Runx2) osteoblast precursors. Although crucial for osteoblast differentiation to commence, IHH appears to have no further role whereas Runx2 is considered the master regulator of bone formation and cell maturation resulting in terminal differentiation (Deschaseaux, Sensaobao, & Heymann, 2009). As with all key proteins, a cascade of other regulatory mechanisms works in concert with Runx2 (refer Figure 4). These mechanisms include parathyroid hormone (PTH) (both anabolic and catabolic actions), the gene Osterix, activating transcription factor 4 (ATF-4), SATB-2 and Wnt/ß catenin signalling. These proteins collectively direct differentiation of the progenitor cell under Runx2 control to the osteoblast precursor stage then via action of osterix (Osx) to the pre-osteoblast and finally to the fully differentiated and activated osteoid secreting osteoblast. Additionally, Runx2 has been shown in-vitro to increase production of bone matrix proteins such as osteopontin, bone sialoprotein (BSP), osteocalcin, fibronectin, collagen type 1 alpha 1 (Col1a1), Col1a2, matrix metalloproteinase 13 (MMP13) and osteoprotegerin (OPG), all of which enhance formation rather than degradation of bone tissue (Komori, 2005; Soltanoff et al., 2009).

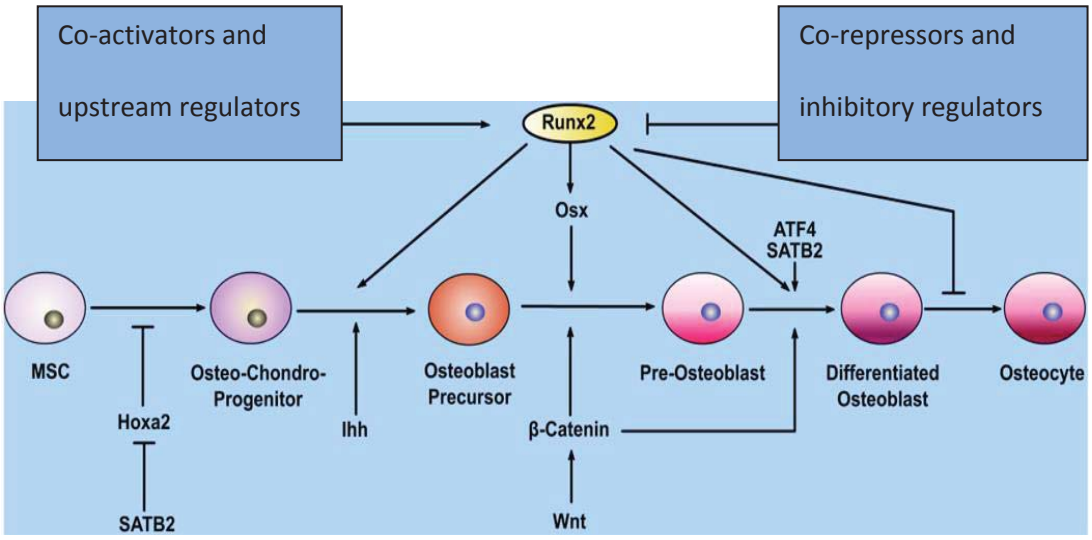

Figure 4 Transcription and signaling involved in osteoblast differentiation

A simplified schematic showing how osteoblasts and osteocytes are derived from a common mesenchymal stem cell (MSC) with Runx2 stimulating terminal differentiation in conjunction with a range of co activators and co repressors. Condensed from Soltanoff et al, (2009).

The main task of osteoblasts is to secrete collagen to form osteoid. Characteristically of cells involved in protein production, mature osteoblasts have well developed endoplasmic reticulum and multiple golgi apparatus. The rate and extent of collagen production depends on the type and location of the osteoblast (Holick & Dawson Hughes, 2004). When bone matrix is produced, osteoblasts also regulate the process involved in adding inorganic mineral crystals to the collagen fibres. The addition of 50-70% of the mineral crystals occurs within 5-10 days of the bone matrix being produced but the remainder of the mineralisation may take up to 2.5 years to complete (Garner et al., 2012). Osteoblasts also contribute to the control of calcium homeostasis and bone regulation by the major hormones involved (PTH and 1, 25-di-hydroxyvitamin D), which interact via receptors located on the cell's surface. Calcium cell surface receptors present on both PTH tissue and osteoblasts sense the level of calcium ions in the extracellular fluid and thus act as mediators of the negative feedback control on PTH hormone secretion (Garner et al., 2012).

Once their functions, to secrete collagen, control mineralisation and provide negative feedback to PTH hormone comes to an end osteoblasts have one of two fates. They will either line the surface of bone to help maintain blood calcium levels (bone lining cells), or they will become trapped within the

bone in lacunae where they differentiate into osteocytes, which will care for the newly formed bone by contributing to bone remodelling and mineral equilibrium (Teti et al., 2009).

Osteocytes are spider like cells with many long cellular extensions with gap junctions at the end of their processes which pass through canaliculi (refer Figure 5). The interconnecting canaliculi are passageways which ferry nutrients via diffusion from the extracellular matrix to the cells (Teti et al., 2009; Atkins et al., 2012).

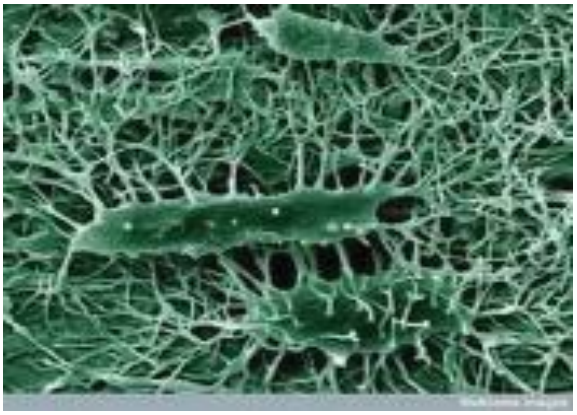

Figure 5 Osteocyte cell structure showing spider-like processes

This image shows the structure of osteocyte cells in the cortex of a mouse tibia bone. Bone is imbedded in resin, etched with perchloric acid to remove the entire mineral in the sample leaving a replica of the area. What is observed is the resin that filled the spaces in the bone and the spaces inside the cells. Scanning electron micrograph from http://www.flickr.com/photos/wellcomeimages/5814814672
Credit: Kevin Mackenzie, University of Aberdeen. Wellcome Images

Osteocytes are the most abundant bone cell accounting for 90% of all bone cells and collectively have a prodigious surface area (100 x trabecular bone surface area). As awareness of osteocytes varied roles in maintaining bone health is more fully appreciated, they are no longer considered the passive end stage cells of the active osteoblast as originally proposed. Osteocytes are central to the functioning of the Wnt/β catenin pathway, therefore osteocytes have a role in both formation and resorption activities (Bonewald, 2011; Nakashima, Hayashi, Fukunaga et al., 2011).

Due to their numbers and surface area, osteocytes are thought to contribute significantly to regulation of the body's reserves of calcium and phosphate stored in bone (Atkins et al., 2012). Osteocytes control the rate and extent of mineralisation of the collagen fibres which the osteoblasts have laid

down. Osteocytes are also the main cells to secrete sclerostin, which can inhibit osteoblast activity. Sclerostin acts via the Wnt/β-catenin pathway as an antagonist of lipoprotein receptor 5 (LPR5) which is a positive regulator of bone mass, therefore sclerostin is negatively associated with high bone mass and formation activities. Sclerostin and PTH (which inhibits sclerostin secretion) are now a focus for bone formation treatment (Bonewald, 2011; Atkins et al., 2012).

Exercise (loading) is vitally important for bone strength as it reduces both sclerostin secretion and cellular apoptosis. When strain is placed on bone e.g. through weight bearing exercise, osteocytes reduce their secretion of sclerostin, thereby enhancing bone formation (Atkins et al., 2012). Exercise also reduces osteocyte apoptosis whereas lack of loading on bone or complete immobility will cause apoptosis, largely due to diminished oxygen supply. Osteocytes, like all cells, require oxygen to survive; therefore they are dependent on the movement of fluids in their canaliculi created by moderate loading on bone to bring in oxygen by diffusion of interstitial fluid. Lack of exercise or weightlessness rapidly leads to apoptosis of osteocytes (Aguirre, Plotkin, Stewart et al., 2006; Mann, Huber, Koggiani et al., 2006; Salingcarnboriboon, Tsuji, Komori et al., 2006; Atkins et al., 2012). Osteocyte's cell death is also reported in pre-menopausal women to be induced by withdrawal of oestrogen with subsequent increased bone loss (Atkins et al., 2012).

In the adult skeleton, osteocytes are also intimately connected with osteoclast activity as they are receptor activator of nuclear factor kappa B ligand (RANKL) producing cells. Indeed osteocytes may be the major controller of all resorption activities (refer Figure 6) as more RANKL is secreted in-vitro from osteocytes than either osteoblasts or bone marrow stromal cells (Nakashima et al., 2011; Atkins et al., 2012). In the mature skeleton, micro-bone damage due to normal wear and tear leads to osteoclastogenesis and subsequent repair. The micro damage is proposed to cause cell death by apoptosis of osteocytes in the damaged area. The apoptosis of the osteocytes release apoptotic cell bodies, which express (RANKL) (Bonewald, 2011) and signals the activation of bone resorption and directs the osteoclasts to the damaged site (Atkins et al., 2012).

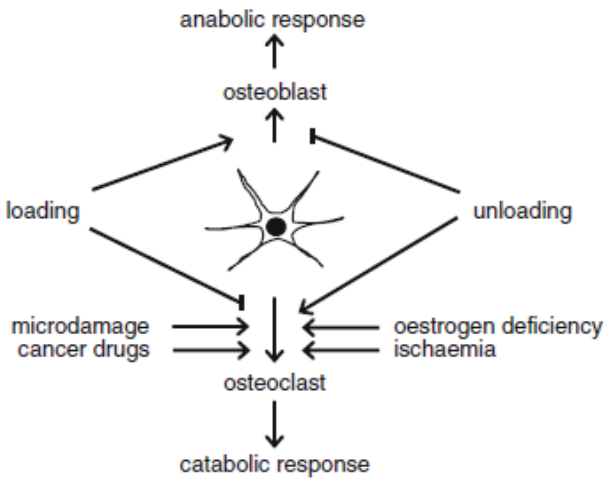

Figure 6 Central role of the osteocyte in bone anabolism and catabolism

This figure depicts the central influence of osteocytes on both osteoblast (anabolic) and osteoclast (catabolic) activity. Reproduced with permission from Atkins & Findlay (2012).

Osteoclasts are unique for being the only cell type in the body with the distinct role of degrading their own tissue (Karsenty & Oury, 2010). Because of this exclusive adaption for removal of the mineral component of bone, they have a critical effect on skeletal health ensuring damaged bone can be repaired (Soltanoff et al., 2009). Osteoclasts are large, short-lived cells, transiently present compared to osteocytes (Manolagas & Parfitt, 2010). Osteoclasts, being derived from monocytes/macrophage precursors, play a central role in inflammatory bone loss (Schett, 2011).

While oestrogen regulates bone homeostasis overall, there is a large variety of transcription factors involved in the differentiation of the osteoclast progenitors to mature bone resorbing cells. Regulation of bone resorption is centred on a master regulator transcription factor, nuclear factor of activated T-cells (NFATc1) and the cytokine colony stimulating factor 1(CSF-1) working with the cytokine RANKL and related proteins: tumour necrosis factor (TNF) and receptor (TNFR), osteoprotegerin (OPG) and receptor activator of nuclear factor kappa B (RANK) (McCormick, 2007; Boyce, Zhenqiang, & Lianping, 2009; Lacey, Boyle, Simonet et al., 2012). A combination of RANKL and macrophage-colony stimulating factor (M-CSF) expression on osteoblastic stromal cells coupled with expression of RANK by the osteoclast precursors is required for the osteoclast precursor to develop into its fully functioning role with ability to express tartrate resistant acid phosphatase (TRAP),

cathepsin K (CATK), calcitonin receptor and the β- integrins (Boyle, Simonet, & Lacey, 2003; Raggatt et al., 2010).

RANKL is assisted by TNF which not only induces osteoblastic expression of RANKL but can stimulate osteoclast formation directly. Expression of nuclear factor kappa B (NFκB) is required for RANKL and TNF to complete differentiation of the osteoclast progenitor cells. Aiding in this role are transcription factors c-Fos and the master regulator NFATc1. Both can substitute for NFκB and IL-1 can utilise them also to augment osteoclast formation in times of inflammation (Boyce et al., 2009).

Whenever RANKL expression is up-regulated, expression of its decoy receptor osteoprotegerin is reduced. While osteoblasts produce some OPG the majority comes from B cells (Boyce et al., 2009) and osteocytes (Bonewald, 2011). The extent of bone resorption is thought to be determined by the ratio of these 2 cytokines RANKL/OPG, which is controlled by PTH, Vitamin D and oestrogen (Kostenuik, 2005). Mature multinucleated osteoclasts develop as a result of the above cytokines and growth factors and begin their primary function of resorption.

Osteoclasts function by clustering around spongy bone, adhering to it and then breaking it down. Before breakdown begins the osteoclasts must first seal off the area to be resorbed with the ruffled surface of the osteoclast attached by actin filaments (podosomes) to the bone via integrins (Bruzzaniti & Baron, 2006; Boyce et al., 2009). This sealing zone is tight, so a low pH environment can be created and contained to breakdown bone's strong, composite structure. As active pumping of hydrogen ions from carbonic anhydrase II into the resorption pit via the osteoclasts ruffled border begins, so too begins the dissolution of bone inwards from the surface. The release of the minerals calcium and phosphorus and excess HCO_3^- follows, while lysosomal enzymes including collagenases, cathepsin K and other hydrolases continue to break down the matrix protein. While the bone surface in the resorptive trench is exposed to this harsh proteolytic environment, adjacent areas of bone are completely protected by the sealing zone (Boyce et al., 2009; Garner et al., 2012).

The released minerals are then ferried out, using ion transport systems to the extracellular fluid and bloodstream where peptide fragments e.g. CTX from the bone matrix can also be found (Garnero & Delmas, 2004). As the resorptive function of the mature osteoclast comes to an end, cathepsin K is activated to induce senescence and apoptosis of the osteoclast (Dawson-Hughes, Harris, Rasmussen et al., 2004; Soltanoff et al., 2009; Raggatt et al., 2010; McConnell et al., 2011).

While bone resorption as part of normal bone remodelling has previously been seen as the principal role of osteoclasts it is becoming increasing clear that this is not their only focus. Osteoclasts have a pedigree in the innate immune system due to being derived from the monocyte line. This pedigree becomes evident during inflammation when there is increased induction of osteoclasts from not only their bone marrow precursors but also directly from monocytes (Schett, Saag, & Bijlsma, 2010). Increased bone loss is seen with chronic inflammation as well as a reduction in bone formation and is particularly evident in inflammatory bone disease (H. Zhu, Jia, Zhang et al., 2008; Boyce et al., 2009).

2.4 Bone turnover: changes at middle age

Bone formation in childhood and adolescence exceeds that of bone resorption and allows for bones to grow via modelling. Bone growth ceases in the adult skeleton and the cycle of bone repair and formation is termed “bone remodelling”. The adult skeleton is remodelled more than any other organ in the body and at any one time there are more than one million foci of remodelling units (Boyce et al., 2008). Complete renewal of the skeleton occurs every decade with spongy bone replaced every 3-4 years and compact bone within 10 years (Schett et al., 2010). If remodelling didn’t occur, the ageing crystalline calcium salts would make the bones very brittle and susceptible to fracture (Marieb et al., 2010).

In the younger adult, total bone mass remains relatively stable with the quantity of newly formed bone matching the amount resorbed (J. Brown, Albert, Nassar et al., 2009). At menopause, withdrawal of oestrogen and ageing, affects coupling of the dual processes required to maintain bone mass, these processes become less coordinated, resulting in loss of bone mass (refer Figure 7), and subsequent

development of conditions such as osteoporosis (Schett et al., 2010). Bone resorption occurs much quicker than formation (3 weeks as opposed to 3 months) and this kinetic difference exacerbates bone loss at menopause (Harada & Rodan, 2003).

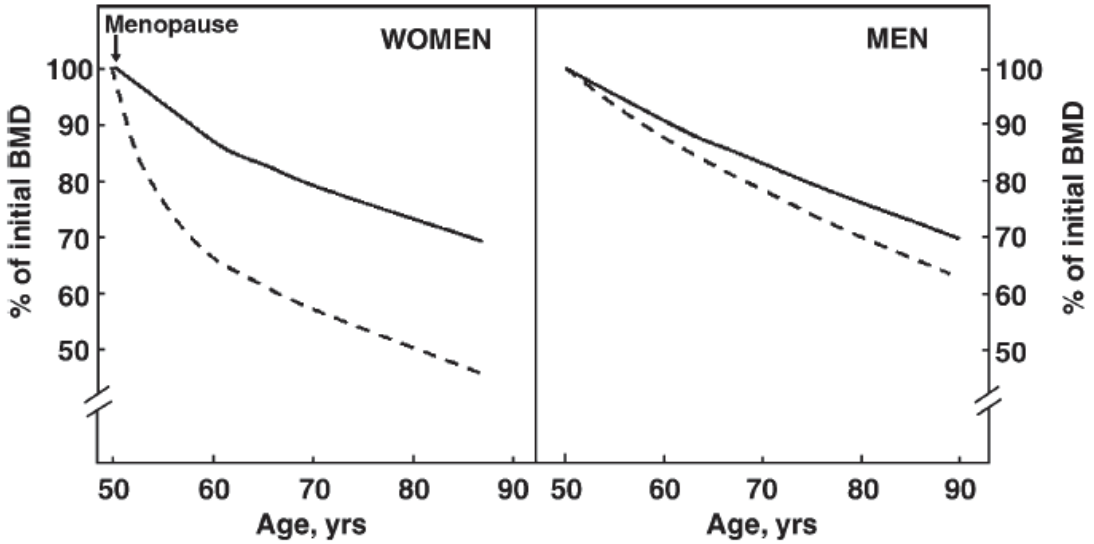

Figure 7 Age related bone loss

This figure depicts the patterns of bone loss in women and in men after 50 years of age. Dashed lines represent trabecular bone and solid lines, cortical bone. The figure is based on multiple cross-sectional and longitudinal studies using Dual-energy X ray absorptiometry. BMD (Bone mineral density).
Reproduced with permission from Khosla and Riggs, (2005).

2.5 Remodeling

As a dynamic entity, the skeleton uses remodelling to respond to a constantly changing internal and external environment. Normal wear and tear induces micro-damage requiring continual repair. Externally, the presence of force or exercise on muscles attached to bone will induce an increase in growth factors as will change in shape or weight. Internally the quantity of circulating hormones such as PTH, oestrogen, androgen and vitamin D_3 are key regulators in bone turnover (Boyce et al., 2008; Teti et al., 2010) and it is now widely accepted that the degree of inflammation present in the body affects regulation of bone resorption (Wauquier, Leotoing, Coxam et al., 2009; Schett et al., 2010).

Frost (1963) first proposed the concept of remodelling and used the term " bone multicellular unit " (BMU) to describe the structural compartment composed of participating cells within a tightly sealed canopy which creates the microenvironment necessary for the remodelling process (Raggatt et al.,

2010; Garner et al., 2012). Remodeling is mainly confined to the outer surface of the bone but can also occur adjacent to the marrow cavity on the inner surface of long bones (Elmendorf, 2012). Raggett and Partridge outline remodeling as five distinct phases and it is depicted below in Figure 8.

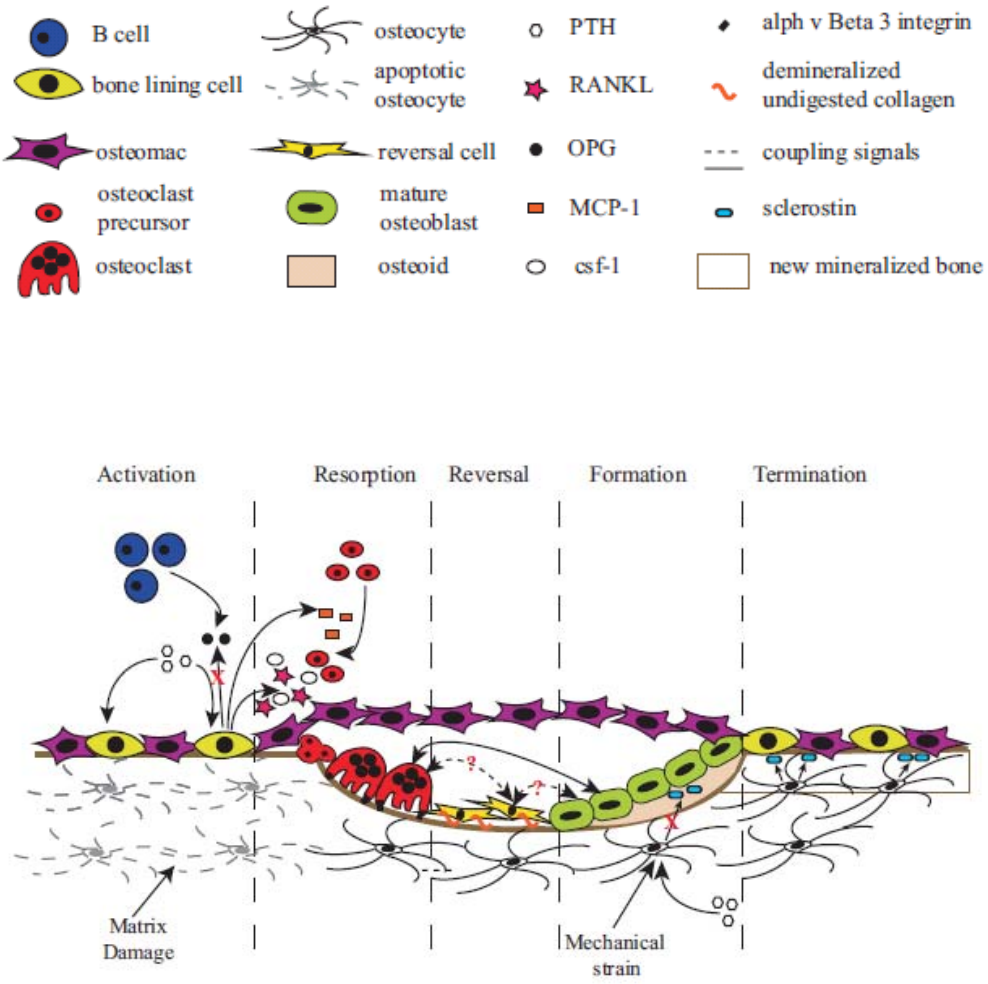

Figure 8 Remodeling Process

Bone lining cells and osteomacs on resting bone surface with B cells secreting osteoprotegerin (OPG) to suppress resorption. During Activation, parathyroid hormone (PTH) binds to a receptor on the pre-osteoblast. Damage to the mineralised matrix causes osteocyte apoptosis thereby reducing inhibition of osteoclastogenesis. Resorption: PTH signalling releases monocyte chemoattractant protein-1 (MCP-1) from osteoblasts and recruits immature osteoclasts to the site. Osteoblasts reduce secretion of OPG while receptor activator of nuclear factor kappa B ligand (RANKL) and cytokine stimulating factor -1(CSF-1) secretion is increased to enhance proliferation and differentiation of pre-osteoclasts to mature osteoclasts where they attach to binding sites to create the sealed zone for degradation of bone tissue. Reversal: Reversal cells are removing the demineralised undigested collagen from the cell's surface. Bone resorption is halted and formation is promoted. Formation: Degraded tissue and mature osteoclasts release formation signals and molecules. Sclerostin secretion is reduced by PTH and mechanically stimulated osteocytes allowing Wnt-directed bone formation. Termination: Bone formation ceases in conjunction with sclerostin returning. Newly deposited osteoid is mineralised and bone surface returns to the previous resting state.
Reproduced with permission from Raggatt, (2010).

2.5.1 Activation phase

Signalling begins by osteocytes responding to strain (mechanosensing), damage or immobilization. Additional signalling comes from oestrogen, cytokines, growth factors or PTH in response to PTH control of calcium homeostasis. Parathyroid receptors are found on osteoblasts and once bound, PTH activates protein kinases A and C and calcium intracellular signalling pathways, which culminate in osteoclast activation and bone resorption (Boyce et al., 2008; Raggatt et al., 2010; Feng et al., 2011).

2.5.2 Resorption phase

In response to osteocyte signals or PTH, the osteoblasts send a homing signal MCP-1 (monocyte chemoattractant protein1) to guide osteoclast precursors to the current site of remodelling. This chemokine also induces increased expression of cytokines cytokine stimulating factor 1 (CSF-1) and RANKL and decreased expression of OPG on the osteoblast. CSF-1 and RANKL work together by CSF-1 promoting increased numbers of osteoclast precursors and RANKL assisting this increase as well as directing maturation to multinucleate osteoclasts with enhanced longevity (Boyce et al., 2008; Soltanoff et al., 2009; Raggatt et al., 2010).

Osteoclasts also secrete matrix metalloproteins (MMPs) to breakdown unmineralised osteoid, making osteoclast attachment easier at adhesion sites (Bruzzaniti et al., 2006). Osteoclasts then attach to these binding sites and completely seal off the environment. The acidic pH created by hydrogen ions via carbonic anhydrase catalysing hydration of CO_2 to H_2CO_3 results in degradation and hollowing out of the bone tissue. The trenches are termed Howship's resorption lacunae. Remaining non-mineralised tissue is broken down by enzymes such as cathepsin K (Raggatt et al., 2010).

2.5.3 Reversal phase

During this phase the preparatory work for formation activities is accomplished by mononuclear cells (reversal cells). The surface is prepared by clearing away leftover matrix debris consisting mainly of collagenous fibres. The reversal cells are not yet clearly identified but it is likely that resident tissue macrophages termed "osteomacs" are responsible for removing the debris and possibly bone lining

cells for securing and cementing collagen at critical points within the lacunae (Raggatt et al., 2010; Garner et al., 2012).

2.5.4 Formative phase

Osteoclasts are thought to produce the signal to couple bone formation to their completed resorptive activities. However, because osteoblast recruitment and deposition of osteoid continue long after the osteoclasts have left the site, another cell type must also be involved. Osteocyte involvement is proposed for several reasons. Firstly, osteocytes respond to PTH and mechanical strain to induce bone formation (Teti et al., 2009) and secondly, when no strain on bone is present the osteocytes express sclerostin, which prevents Wnt signalling. The Wnt signalling pathway is central to establishing bone mineral density and Wnt is a potent inducer of bone formation. Mechanical strain decreases sclerostin secretion by osteocytes and promotes bone formation (Raggatt et al., 2010).

As the premature osteoblast cells are attracted to the resorptive pits they differentiate to begin their main role as mature osteoid secretory cells. The proteins are largely collagen type 1 but also proteoglycans, alkaline phosphatase and Gla (gamma-carboxyglutamic acid) containing proteins including osteocalcin and integrin binding ligand proteins. Once the osteoid is laid down, mineralisation with calcium (Ca^{2+}) and phosphate (Pi as $H_2PO_4^-/HPO_4^-$) to firstly form $Ca_3(PO_4)_2$ and then hydroxyapatite can begin (Raggatt et al., 2010; Feng et al., 2011; Sapir-Koren & Livshits, 2011).

2.5.5 Termination phase

As the amount of newly formed bone tissue nears the equivalent amount of resorbed tissue, signalling occurs to close down the remodelling in this BMU. This signal is thought to come from sclerostin. All that remains is for osteoblasts to differentiate to either osteocytes, bone lining cells or undergo apoptosis. Understanding of this final phase is still yet to be elucidated (Raggatt et al., 2010; Feng et al., 2011).

2.6 Bone markers

Researchers use markers of bone turnover found in serum and blood to determine effects of osteoporosis medications and for research purposes. These analytes of serum and urine proteins are not considered suitable for diagnosis of osteoporosis but their use in clinical studies and for monitoring purposes has enhanced understanding of the effects of menopause and ageing on bone health and fracture risk, as well as assisting knowledge of treatment effects of bone medications (Garnero, 2008; Eastell & Ebeling, 2009). With careful interpretation, they can also be used in diagnosis of some metabolic bone diseases (Siebel, 2005). They include a variety of direct and indirect products of the cells associated with bone turnover (Delmas, Eastell, Garnero et al., 2000) and are classified as either formation or resorption markers according to the metabolic process they are associated with (Siebel, 2005).

2.6.1 Resorption markers

Collagen type 1 is the most common protein in bone and its breakdown products can be measured in either urine or serum (Eastell & Hannon, 2008). Amounts of the breakdown products found in these fluids are said to be indicative of the level of resorptive activities (Civitelli, Armamento-Villareal, & Napoli, 2009). Breakdown products include deoxypyridinolene (DPD) and pyridinoline (PYD), cross linking components of collagen that are released during resorption. The peptide bound forms include CTX and cross-linked N-telopeptide of type I collagen (NTX). Release of both these peptide fragments from type 1 collagen are generated as a result of cathepsin K or metalloproteinase activity (CTX-MMP) in the BMU, therefore they are used as a measure of resorption activity (Szulc & Delmas, 2008).

Apart from collagen by-products, other bone proteins such as bone sialoprotein (BSP) and the enzymes associated with osteoclast activity, tartrate resistant acid phosphatase (TRAP) and cathepsin K can be measured and reflect the rate of resorption (Leeming, Alexandersen, Karsdal et al., 2006; Eastell et al., 2009; Vasikaran, Eastell, Bruyère et al., 2011). Higher levels of resorption markers are associated with increased risk of fracture (Talwar & Aloia, 2009).

While there is on-going investigation into newer resorption markers the collagen telopeptides are considered the most reliable measure, with serum CTX (s-CTX) the most commonly used measurement. S-CTX is considered the preferred marker of bone resorption for clinical studies recommended by the International Osteoporosis Foundation (IOF) and the International Federation of Clinical Chemistry and Laboratory Medicine (IFCC) (Vasikaran et al., 2011). The reasons for recommending the use of s-CTX in preference to other resorption markers are as follows: s-CTX has a well characterised standard in the assay allowing development of a clearly defined reference standard, most CTX is known to have been derived from bone resorption activity rather than other processes, it is well evaluated for fracture risk and drug monitoring in clinical studies and there is a readily available ELISA or automated immunoassay analysers (Roche, IDS). Automation has led to considerable evidence validating s-CTX's use in monitoring osteoporosis treatment and for research purposes (Vasikaran et al., 2011). Its utility was noted over a decade ago, with the combined effects of large changes seen with anti-resorptive therapies and significantly less spontaneous change over time compared to other peptide based assays. The above factors make s-CTX the obvious choice for monitoring bone turnover and effects of osteoporosis therapies (Rosen, Moses, Garber et al., 2000; Vasikaran et al., 2011).

Reported reference intervals for s-CTX in pre and post-menopausal women vary according to the study and country. A recent review of premenopausal women's reference ranges for s-CTX found values between 0.1- 0.659 μg/L with intra-individual coefficient of variation of 9.6 % (J. Brown et al., 2009). Post-menopausal (PM) mean values of s-CTX (μg/L) have been reported as low as 0.21 (Macdonald, Black, Aucott et al., 2008), through to 0.387 (SD ±0.197) (Martínez, Olmos, Hernández et al., 2009), 0.41 (95% CI 0.02-1.49)(Rogers, Saleh, Hannon et al., 2002) and as high as 0.57 (SD ±0.03) and 0.60 (SD ±0.04) in PM women in Indonesia and Philippines respectively (Kruger, Schollum, et al., 2010). However, in these latter populations, calcium and vitamin D deficiency was common, which could have contributed to the increased levels of CTX.

2.6.2 Formation markers

Markers of bone formation are related to the osteoblast function of forming new bone at the site where resorption has just occurred. Unlike resorption markers all bone formation markers have to be measured in serum or plasma (Siebel, 2005). One of the most common markers is procollagen type I N propeptide (PINP). This marker represents one of the propeptide fragments cleaved off the precursor of collagen, procollagen during the formation of bone matrix. Procollagen contains amino terminal end (P1NP) and C terminal end (P1CP) extensions which must be removed and subsequently released into the circulation where they can be detected. These propeptides are made by other tissues but the main source is bone (Singer & Eyre, 2008; Talwar et al., 2009; Vasikaran et al., 2011). Because P1NP primarily originated from bone tissue, is relatively insensitive to food and circadian rhythms and is particularly responsive in clinical trials, it is considered the preferred marker of bone formation for clinical studies recommended by the International Osteoporosis Foundation (IOF) and the International Federation of Clinical Chemistry and Laboratory Medicine (IFCC) (Vasikaran et al., 2011; Krege, Lane, Harris et al., 2014).

Two other bone formation markers are osteocalcin (OC) and alkaline phosphatase (ALP). Neither is related to collagen formation. Osteocalcin is a large vitamin K dependent peptide exclusively produced by osteoblasts and some chrondrocytes during bone formation and is one of the most abundant non-collagenous proteins. Osteocalcin binds to hydroxyapatite, and is deposited in the matrix but fragments are released during resorption and can be detected in the extracellular fluid (serum) and, due to its excretion by the kidneys, may also be measured in urine. Osteocalcin is a reflection of both resorption and formation activities (Singer et al., 2008; Szulc et al., 2008). Alkaline phosphatase was the first bone turnover marker in use (1929), and is still used today (Singer et al., 2008). During the mineralisation of the newly formed osteoid, alkaline phosphatase (ALP) is secreted by osteoblasts into the extracellular fluid and can be measured in serum. However, in adults, only about half of the total ALP activity is reflective of bone tissue; the other half will be from the liver. Assays are available that detect more specifically the bone derived isoform (BALP) (Szulc et al., 2008; Vasikaran et al., 2011).

During midlife, levels of OC and ALP can increase by 50-100%, whereas P1NP or P1CP increase by only 20%. Pre-menopausal women have increased levels of bone resorption markers s-CTX and osteocalcin (Eastell et al., 2008). It is unknown whether bone turnover markers remain at increased levels after menopause, though CTX has been shown to stay high for 40 years after menopause (Eastell et al., 2008).

The bone markers used in the study presented in this thesis are the preferred markers of bone turnover recommended by the IOF and IFCC, namely s-CTX for bone resorption and s-PINP for bone formation.

2.6.3 Variability in markers

Apart from the known circadian variation in markers of bone turnover (Leeming et al., 2006; Eastell et al., 2008) there is considerable inter-person variability as well as analytical differences, which must be compensated for or acknowledged when interpreting bone marker results (Siebel, 2005). Variations between individuals may be due to a range of factors including age, gender and ethnicity.

Age particularly affects bone turnover marker levels in women at the midlife transition in late pre-menopause with a decrease in bone formation and an increase in resorption, exacerbating bone loss (Garnero, Sornay-Rendu, Chapuy et al., 1996; Eastell et al., 2008). Men however, tend to show a decrease in bone formation markers but not the increase in resorption resulting in less bone loss.

The circadian variation in bone turnover markers makes it imperative to schedule blood tests at the same time of day. The maximal values are seen in the morning, and wane to their lowest in the evening (Szulc et al., 2008). Other factors that may affect the bone markers are exercise levels, medications and disease states, particularly those which may affect renal clearance of urinary bone resorption and formation markers. Prolonged bed rest e.g. stroke and Parkinson's disease will result in higher bone resorption and lower formation markers (Szulc et al., 2008).

Table 1 Markers of bone turnover

Marker	Full name	Origin	Assay	Comments
Resorption				
u-NTX	Urinary amino terminal cross linking telo -peptide of type I collagen	Osteoclastic hydrolysis of collagen type I	Automated Manual	Adjust for urinary creatinine (Cr) Specificity: collagen type I, Bone as highest contributor. Variability due to circadian rhythm
s-NTX	Serum amino-terminal cross-linking telopeptide of type I collagen	Osteoclastic hydrolysis of collagen type I, generated by cathepsin K	Automated Manual	Specificity: collagen type I, Bone as highest contribution probably from bone; variability due to renal function and circadian rhythm
u-CTX	Urinary carboxy-terminal cross-linking telopeptide of type I collagen	Osteoclastic hydrolysis of collagen, generated by cathepsin K	Automated Manual	Must be adjusted to levels of urinary creatinine (/Cr) Specificity: collagen type I, with highest contribution probably from bone u-CTX is isomerised (β) or non-isomerised (α). Isomerised if not otherwise specified. variability: circadian rhythm
s-CTX	Serum carboxy-terminal cross-linking telopeptide of type I collagen	Osteoclastic hydrolysis of collagen, generated by cathepsin K	Automated Manual	S-CTX is always isomerised (β) Specificity: collagen type I, Bone as highest contributor. Variability due to circadian rhythm, fasting, renal and liver function
s-ICTP or CTX-MMP	Carboxy-terminal cross-linking telopeptide of type I collagen	Osteoclastic hydrolysis of collagen by matrix metallo proteinases MMP	Manual	Specificity: collagen type I, Bone as highest contributor. Variability due to circadian rhythm, fasting, renal and liver function
u-DPD	Urinary deoxypyridinoline	Proteolytic hydrolysis of collagen, found in bone	Automated Manual	Adjusted for urinary creatinine (/Cr) Total or free (non-peptide-bound) Specificity: highest contribution from bone, mature collagen only. Variability: independent of dietary sources, influenced by UV radiation and circadian rhythm. Total or free (non-peptide-bound)
u-PYD	Urinary pyridinoline	Found in bone, cartilage, tendon, blood vessels	Automated Manual	Adjust for urinary creatinine (/Cr) Total or free (non-peptide-bound) Highest contribution from bone and cartilage, present in mature collagen only. Variability: independent of dietary sources; influenced by liver function, active arthritis and UV radiation, and circadian rhythm
s-TRACP	Serum tartrate-resistant acid phosphatase	Includes two isoforms: type 5a (platelets, erythrocytes and other sources) and type 5b (osteoclasts)	Manual	Variability: haemolysis and blood clotting, circadian rhythms Difficult to store; stable up to 2 years at −70 °C
Formation				
s-OC	Serum osteocalcin	Hydroxyapatite-binding protein exclusively synthesised by osteoblasts and odontoblasts	Automated Manual	Specificity: osteoblast function. Rapid degradation in serum leads to heterogeneity of OC fragments: usually measured as intact [1–49] or N-mid [1–43] fragment, or can be undercarboxylated (ucOC) Variability: renal function, circadian rhythms; large inter-laboratory variation
s-ALP	Serum alkaline phosphatase (total)	Ubiquitous, enzyme located on the outer cell surface of liver, bone, intestine, spleen, kidney and placenta	Automated Manual	Specificity: non-specific for bone (about 50% is liver isoform in healthy individuals)Multiple assay methodologies Source of variability: very small circadian rhythm

Marker	Full name	Origin	Assay	Comments
s-BALP	Serum bone-specific alkaline phosphatase	Ubiquitous, membrane bound tetrameric enzyme located on the outer cell surface of osteoblasts	Automated Manual	Specificity: specific for bone, but with some cross-reactivity with liver isoform (up to 20%) Multiple assay methodologies. Source of variability: very small circadian rhythm
s-PICP	Procollagen type I C propeptide	Precursor molecules of collagen type I synthesised by osteoblasts	Manual	Specificity: mostly derived from bone collagen type I (around 90%). Short serum half-life. Regulated by hormones (thyroid, IGF-1) variability: small circadian rhythm
s-PINP	Procollagen type I N propeptide	Precursor molecules of collagen type I synthesised by osteoblasts	Automated Manual	Specificity: mostly derived from bone collagen type I Assay: may recognise trimer alone (intact) or trimer and monomer (total PINP) Variability: small circadian rhythm

(Adapted with permission from Vasikaran et al., 2011)

2.7 Diet and bone health

2.7.1 Nutrients, non-nutrients and fruit and vegetables

Bone loss accelerates in women during midlife, leading to a loss of bone mineral density (Schett et al., 2010). The search for modifiable dietary factors to delimit the effects of ageing on bone loss has seen a variety of dietary components and food groups investigated. Two non-nutritive dietary factors associated with bone loss during ageing are: dietary acid-base balance and its effect on metabolic acidosis, and dietary intake of phytochemicals which affect inflammation directly and indirectly via cell signalling and antioxidants. Both dietary acid-base balance and phytochemical intake are directly associated with fruit and vegetables intake, and are therefore modifiable by diet. The effect of increased intake of fruit, vegetables/herbs, which reduces the dietary acid load and increases phytochemical intake, will be investigated for its effects on bone health, in two intervention studies in this thesis. A brief overview is given below of the nutrients and the non-nutritive dietary factors associated with bone health, prior to a more in-depth analysis of dietary acid-base balance and phytochemicals effect on inflammation, investigated in the subsequent trials.

2.7.2 Nutrients associated with bone health

The micronutrients calcium and vitamin D have a primary protective role against bone loss (Tucker, 2009) and maintenance of serum calcium levels. Adequate dietary intake of calcium is considered important for bone health and has been extensively studied (Heaney, 2009) although calcium's protective effect on hip fractures is by no means conclusive (Bischoff-Ferrari, Dawson-Hughes, Baron et al., 2007). While dietary calcium intake may vary, intestinal calcium uptake also varies and is controlled by vitamin D as 1, 25-dihydroxyvitamin D, along with PTH. If inadequate dietary intake is maintained and/or intestinal uptake is limited, calcium may be mobilised from bone to maintain serum levels. Likewise, if inadequate vitamin D is obtained, calcium uptake may be limited and bone will be called upon to supply the shortfall (Bischoff-Ferrari et al., 2007; Boonen, Lips, Bouillon et al., 2007; Cashman, 2007; Anderson, 2012; Avenell, Mak Jenson, & O'Connell, 2014)

Other micronutrients essential to bone health include potassium and magnesium, which help form the mineral component of bone (Tucker, Hannan, Chen et al., 1999; Demigne, Sabboh, Remesy et al., 2004; Lanham-New, 2008) and vitamins K, C and E. Vitamin K's role in maintaining bone health is by gamma carboxylation of bone's Gla proteins (osteocalcin). Carboxylation is needed for the bone proteins to attract calcium into their crystals and insufficient vitamin K is associated with reduced bone mineral density (BMD) and increased fracture risk (Bugel, 2008; Bullo et al., 2011). Vitamins C and E are noted for their antioxidant role in bone health (Maggio, Barabani, Pierandrei et al., 2003; Sahni et al., 2008). The omega 3 fatty acids eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) influence bone by increasing calcium uptake (Kruger, Coetzee, Haag et al., 2010). However, not all essential fatty acids have a positive role in bone health, with Omega 6 notable for its negative effect on bone resorption and formation (Genuis & Schwalfenberg, 2007; Watkins, Hannon, Seifert et al., 2012). Adequate intake of protein is essential for bone collagen formation. When protein intake is low, as can occur with ageing, bone loss is increased (Heaney et al., 2008; Kerstetter, 2009); however effects on fracture risk are yet to be conclusively determined (Darling, Millward, Torgerson et al., 2009).

2.7.3 Non-nutritive dietary factors and bone health

The first non-nutritive dietary factor, an imbalance in dietary acid-alkaline production, causes loss of mineral salts from bone (Barzel, 1995). In particular, a higher urinary calcium loss is seen with higher dietary acid load, and this increased loss of calcium is said to be derived from bone (Pizzorno et al., 2010; Adeva & Souto, 2011). Over time, continual urinary loss of the alkaline mineral constituents of bone, to buffer a high dietary acid load, leads to deterioration of bone tissue (Moseley et al., 2013) and may result in osteoporosis. This first mechanism is discussed in more detail next in the literature review.

The second non-nutritive dietary factor which may ameliorate bone loss and fracture risk at midlife is an increased dietary intake of phytochemicals. Bone loss is strongly influenced by inflammation levels which rise during ageing and with reduction in oestrogen at menopause (Barbour & Cauley, 2013). Pro-inflammatory markers increase the rate of bone turnover, particularly bone resorption (Schett, 2011). An increase in bone resorption over bone formation reduces bone mineral content and leads to bone loss (Cashman, 2008). Inflammation levels and inflammatory bone loss may be influenced by dietary intake of some phytochemicals in fruit and vegetables/herbs influencing NFκB and the RANK/RANKL/OPG pathway, or indirectly via cell signalling and antioxidant defence mechanisms (Son, Camandola, & Mattson, 2008). This second mechanism involving phytochemicals and their effect on inflammation is discussed later in the literature review and will include an overview of specific fruits, vegetables/herbs associated with bone health.

2.8 Dietary acid-base balance and how it affects bone health

2.8.1 Diet, urinary pH and calcium excretion

An acidic diet is one where predominantly acid is created during the metabolism of the daily food intake. All diets contain some acid producing foods. Many are common nutritious components of the western style diet and include protein sources such as meat, fish, cheese, eggs, dairy and grains

(Remer & Manz, 1995). Vegetables and fruit are considered alkaline because of their higher mineral content of mainly cations.

2.8.1.1 The kidneys

The kidneys function as the main regulatory system for acid-base imbalances that result from dietary load, disease or metabolism. Usually, as a result of dietary acid load and metabolism there is an excess of hydrogen ions which must be eliminated when blood pH levels drop (Marieb et al., 2010). The kidneys excrete all of the acid produced daily, which is estimated to be 50-100 mEq/day as ammonium salts and hydrogen phosphate. The kidneys must also regenerate the same amount of bicarbonate to maintain acid-base balance; otherwise an imbalance will result in metabolic acidosis or alkalosis (Remer, 2000; Welch, Mulligan, Bingham et al., 2008).

The combination of net gain of new bicarbonate ions through use of ammonium ions and phosphate buffers to excrete hydrogen ions, coupled with reabsorption of most of the existing bicarbonate maintains the extracellular pool of bicarbonate ions. If replenishment didn't occur the daily generation of non-volatile acid (50-100mEq) would rapidly deplete the total ECF pool of 350 mEq HCO_3^- (Seldin, 1989).

However, with ageing, the kidney becomes less able to excrete hydrogen ions and therefore produce new bicarbonate and the kidney tubules allow less reabsorption of existing bicarbonate. In later life, the consumption of a western diet, considered largely acid producing, may lead to a state of chronic, mild, metabolic acidosis (Pizzorno et al., 2010; Adeva et al., 2011).

2.8.1.2 When renal acid excretion is less than endogenous acid production.

An imbalance between renal acid excretion, dietary acid intake and net endogenous acid production can occur through increased dietary acid intake, production in the body or impaired elimination as happens with kidney disorders or functional decline due to ageing (Frassetto, Morris, & Sebastian, 1996; Berkemeyer, Vormann, Gunther et al., 2008; Shi et al., 2012).

Renal disorders such as renal tubular acidosis have demonstrated the body protects against possible fatal acidemia by utilizing the buffering capacity of bone. Children suffering from renal tubular acidosis previously experienced stunted linear growth, due to bone minerals being sequestered to maintain blood pH. However, with the addition of bicarbonate to the children's diet, bone minerals were not needed to maintain blood pH and stunting was overcome (Goodman, Lemann, Lennon et al., 1965).

A mild, systemic metabolic alkalosis resulting from predominant alkaline based foods is considered the optimal state for human beings (Sebastian, Frassetto, Sellmeyer et al., 2002). When dietary intake of acid forming foods is higher than an average intake (1 mEq/kg/day), there is a shortfall between net acid production and excretion (Lemann, 1965; Gannon, Millward, Brown et al., 2008). A chronic low grade metabolic acidosis is said to ensue, with bone proposed to buffer the shortfall in excretion, and this is thought to be particularly relevant as renal function declines with age (Adeva et al., 2011).

2.8.1.3 Composition of urine

Urine consists of water (95%) plus solutes (5%) e.g. urea, sodium, potassium, phosphates, sulphates, creatinine and uric acid. Normally urine is slightly acidic (pH 6.0) (Tietz, 1970) but this can vary from 4.0 to 8.5 (Marieb et al., 2010). Testing of early morning urine will most likely give the lowest pH reading of the day as overnight the kidneys have processed the acid load of the previous day and made the readjustments necessary, particularly the excretion of the non-volatile (fixed) acids produced by metabolic processes (Tietz, 1970).

It has been widely accepted that intake of different types of foods will cause urine pH to vary (Wachman & Bernstein, 1968; Michaud, Troiano, Subar et al., 2003). Remer and Manz determined the relationship between renal net acid excretion (NAE) and urine pH using 24 hour urine samples from 63 male volunteers (16-24 years) consuming diets ranging from lacto vegetarian, normal mixed to high protein diets of young bodybuilders (refer Figure 9).

Correlation between urine pH and renal NAE was assessed using Pearson's correlation coefficient, and simple linear regression analysis was used to produce the regression equation (Remer et al., 1995).

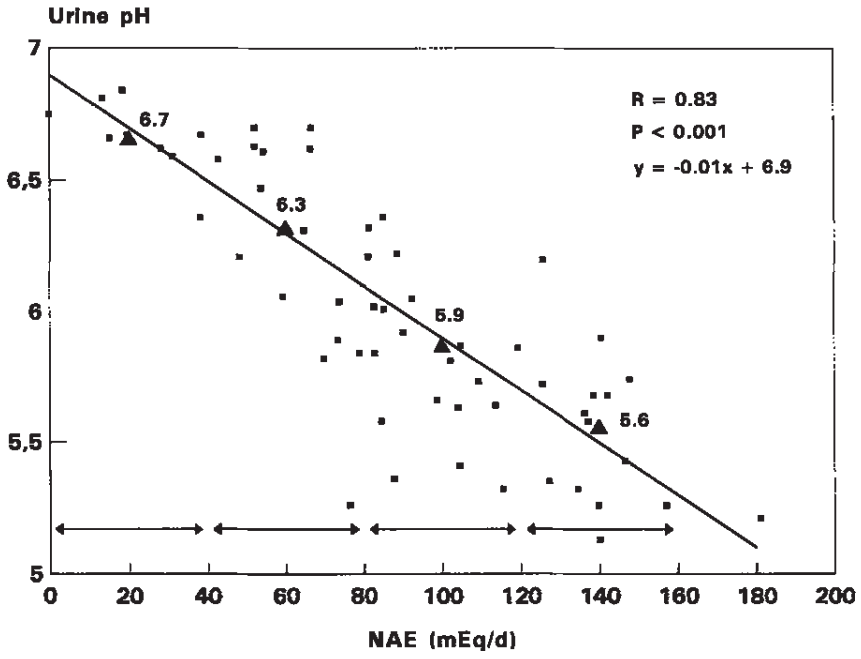

Figure 9 Association between urine pH and renal net acid excretion

The values above the triangles represent urine pH means for the Net Acid Excretion (NAE) intervals (40mEq). NAE is calculated from the rates of excretion of ammonium and titratable acid (TA) and amount of HCO^-_3 lost in the urine (negligible). NAE= [(U_{NH4+} x V) + (U_{TA}xV)]-(U_{HCO3} x V). Reproduced with permission from Remer and Manz, 1995.

With large population sizes, urine pH can be predicted using dietary data and body size (Michaud et al., 2003; Welch et al., 2008). Other contributors in this area have tried to quantify the amount of acid produced and released in the urine as a result of metabolism of the acid load in food.

2.8.1.4 Net endogenous acid production (NEAP) and Potential renal acid load (PRAL)

Frassetto and colleagues, estimated the net endogenous acid production (NEAP) using the potassium and protein ratio.

Estimated NEAP (mEq/d) = 54.5x protein (g/d)/ potassium (mEq/d) - 10.2

Protein and potassium collectively, have been demonstrated to account for 71% of the variability in urine acidity (Frassetto, Todd, Morris et al., 1998). Variables affecting urine pH including smoking, medications (thiazide diuretics), exercise levels, age, BMI and race (Michaud et al., 2003; Taylor & Curhan, 2007; Welch, 2008).

A large population based study (EPIC) by Welch (2008) demonstrated that a more alkaline diet (low PRAL), high fruit and vegetable intake and lower consumption of meat, is associated with a more alkaline urine pH even after adjusting for confounders and excluding study participants with high blood pressure, urinary protein, ketones and glucose (Welch et al., 2008). In a sub-study of the main study (EPIC), further investigation was carried out using a food frequency questionnaire (FFQ) and 7 day diet diary (7DDD) (first day done by interview), casual and 24 hour urine pH, plasma vitamin C and a lifestyle questionnaire was completed. PRAL was calculated from both FFQ and the 7DDD. Good correlations (0.22) were obtained between both casual urine samples pH and 24 hour urine samples pH in the sub-study ($p < 0.001$) and the dietary acid load estimated by either FFQ or 7DDD. Vitamin C intake was also significantly and positively related to urine pH. The difference between PRAL in the high and low pH categories were 4.2 mEq/day (Welch et al., 2008). PRAL was calculated as follows:

PRAL (mEq/d) = 0.49 x protein (g/d) +0.037 x phosphorus (mg/d) - 0.021x

potassium (mg/d)-0.026 x magnesium (mg/d) – 0.013 x calcium (mg/d)

Estimated NEAP (mEq/d) = PRAL (mEq/d) + OA_{est} (mEq/d)

OAest (mEq/day) = individual body surface area x 41/1.73

Body surface area was calculated according to the method of du Bois and du Bois

BSA = (W 0.425 x H 0.725) x 0.007184 (Dubois & Dubois, 1916).

http:/www-users.med.cornell.edu/~spon/picu/calc/bsacalc.htm

(Frassetto, Lanham-New, Macdonald et al., 2007).

If long-term dietary intake is to be measured, the usual means is by FFQ or 4-7 day diet diary taken at several points over the time interval required.

The feasibility of using laboratory measured urine samples to determine dietary intake acid load is attractive as it avoids the human error inherent in reported food intake data as well as the significant time factor in data collection. A recent study by Fenton et al., (2009) determined a single casual morning urine pH and urine mineral excretion is not a stable variable over a 5 year interval that would have utility as a risk factor for osteoporosis. Their study examined whether within-subject (n=200) urine ions (calcium, chloride, magnesium, phosphate, potassium, sodium, sulphate) and urine pH were stable five years apart. Comparing between subject variance with total variance over five years using intraclass coefficients, the authors found only sodium and chloride had moderate stability. They concluded that urine measures taken 5 years apart were inferior to food intake measurements and urinary measures of these ions and pH is not a better way to calculate dietary acid load.

A number of factors would have contributed to the variability found in this study including length of time between sampling and dietary change that may have occurred (dietary intake not assessed), storage of samples (years) and additionally the measurement of NAE was indirect rather than direct so ammonium and bicarbonate ions were not considered. If urinary measures were to be used to estimate dietary intake over long term then more frequent sampling and correlation with dietary intake data would be needed.

2.8.1.5 Calcium excretion and bone

One of the main reasons bone health is said to be improved with a diet high in alkaline precursors is because of reduction in calcium loss in urine (Doyle & Cashman, 2004; Jajoo, Song, Rasmussen et al., 2006; Fenton, Eliasziw, Lyon et al., 2008; Pizzorno et al., 2010). Less urinary calcium loss may be

due to either, more alkaline forming fruit and vegetables contributing higher amounts of base precursors, or lower protein intake contributing less hydrogen ions (New & Millward, 2003).

Fenton showed a positive correlation between urinary net acid excretion and urinary calcium, with an estimated change in urine calcium excretion of 1.6 mmol/d (66 mg/d) associated with a change of 47 mEq of net acid excretion (Fenton et al., 2008). This linear increase in urinary calcium excretion with increasing net acid excretion (NAE) could amount to a loss of nearly 50% of the calcium in the skeleton over two decades (Fenton et al., 2008). However, conflicting study results have meant the source of the increased calcium lost via the urine with a higher protein diet and therefore higher acid load remains unconfirmed (Cao & Nielsen, 2010).

The mechanism by which increased calcium loss occurs with higher protein intake involves an increase in glomerular filtration rate and decreased reabsorption (Hegsted, Schuette, Zemel et al., 1981). A 100-200% increase in protein intake increases the glomerular filtration rate up to 20%, while decreasing fractional tubular reabsorption by 2% (Hegsted et al., 1981). However, increased protein intake from food items such as meat and dairy products as opposed to purified proteins will generally provide increased phosphorus and other nutrients which may limit the hypercalciuric effect of the protein (Massey, 2003; Roughead, Johnson, Lykken et al., 2003; Dawson-Hughes et al., 2004).

It is yet to be determined conclusively, where the calcium is derived from, when hypercalciuria occurs with a high protein and or high acid forming diet. While some calcium may come from bone to buffer an acid load and compensate for ageing kidneys (Kerstetter, O'Brien, & Insogna, 2003), there is also an increased intestinal absorption of calcium with increased protein from meat (Bonjour, 2005; Kerstetter, Wall, O'Brien et al., 2006) but not soy protein (Kerstetter et al., 2006). Fenton suggests an almost complete compensation of calcium loss in the urine occurs by increased intestinal absorption in healthy adults (Fenton, Lyon, Eliasziw et al., 2009), while Shi working with children, found hypercalciuria can be compensated for by increased intestinal absorption but only when a high alkali equivalent diet is habitually consumed. A low intake of fruit and vegetables with a corresponding high

dietary acid load does not allow increased intestinal absorption or increased reabsorption in the kidney tubules (Shi et al., 2012). Previously, the use of potassium bicarbonate as a dietary supplement (to mimic the effect of increased fruit and vegetables), was found to be necessary to increase intestinal calcium uptake (Ceglia, Harris, Abrams et al., 2009). Calvez's recent systematic review however, determined calcium loss from bone would happen only when there is calcium insufficiency or renal dysfunction (Calvez, Poupin, Chesneau et al., 2012).

Increased quantities of non-digestible fermentable carbohydrates such as resistant starch, inulin and fructo-oligosaccharides provide another rationale for the increased intestinal calcium uptake seen with higher consumption of fruit and vegetables. Non-digested fibre reaching the large intestine provides colonic bacteria with fermentative material from which they produce organic acids (short chain fatty acids) which lower the luminal pH and increase calcium uptake. While the mechanisms responsible for increased uptake of calcium have yet to be fully elucidated, several likely options involve both active and passive diffusion. Firstly, an increase in the solubility of calcium salts due to lower pH in the lumen, may allow for increased uptake of the dissolved calcium via passive diffusion through paracellular uptake or via a cation exchange mechanism. Additionally, active uptake may be enhanced as increased levels of calcium transporter calbindin are also seen with increased fibre intake (Kruger et al., 2013).

The association between fruit and vegetable intake and intestinal uptake of calcium may be one of the reasons why improved bone status has been widely reported with higher fruit and vegetable intake (Macdonald, New, Golden et al., 2004; Tylavsky, Holliday, Danish et al., 2004; Ashwell, Stone, Mathers et al., 2008; Hardcastle, Aucott, Reid et al., 2011). However, whether increased loss of calcium in the urine with a diet low in fruit and vegetables or high in PRAL results in increased bone loss is still undetermined.

2.8.2 Fruit and vegetables, acid load and bone

Bone is said to respond to an acid load created by food choices with high PRAL and/or a diet low in fruit and vegetables (low PRAL), by releasing alkaline buffering mineral salts of potassium and chloride firstly, then magnesium, sodium, citrate and carbonate from the hydration shell surrounding bone (Barzel, 1995; Barzel & Massey, 1998). With chronic acid stress, bone will then mobilise calcium and phosphate (Barzel et al., 1998). Over time, repeated calls on bone for buffering components has been proposed to lead to reduction in the mineral content of the bone and a reduced and more fragile bone tissue (Buclin, Cosma, Appenzeller et al., 2001; Muzylak, Arnett, Price et al., 2007; Frassetto & Sebastian, 2012).

2.8.2.1 Early theories

The earliest concepts of acid ash theory and alkaline and acid forming foods were based around the mineral remains of food once it had been metabolised. Acid forming food contains the minerals sulphur, phosphorus and iodine and alkaline forming foods contain potassium, calcium and magnesium (Remer, 2001).

A link between the acidity of the diet and bone loss was first proposed in 1968 (Wachman et al., 1968), when it was noted the pH of a herbivore's urine is normally alkaline and carnivore's is normally acidic. Wachman and Bernstein, proposed a more plausible explanation for the loss of bone mass over time, is the body's response to a continual dietary acid load by liberating salts from bone to increase serum pH. Over time, this continual loss of the basic salts from bone results in the development of osteoporosis. Estimates are for 2 mEq calcium per day being lost, as a result of the body endeavouring to buffer 1mEq fixed acid produced from the metabolism of acid forming foods in the daily diet. This amount of calcium lost per day would, over 10 years amount to a loss of 15% of inorganic substance in an average individual (Wachman et al., 1968).

Much of the groundwork for the acid ash theory was established by human experiments Lemann and co-workers did in the sixties (Lemann, 1965). They demonstrated that when large quantities of

ammonium chloride (acid) were given to the subjects there was a corresponding increase in the amount of calcium and phosphorus released into the urine. This is thought to be due to the retained acid being buffered initially in the tissues by an exchange of cations (Na^+ and K^+) and then further long-term exposure to excess hydrogen ions in the extracellular tissue creating the need for the breakdown of bone, in particular the alkaline bone salts to make bicarbonate (Lemann, 1965; Vormann & Remer, 2008).

The change from eating mainly plant based foods 10,000 years ago, to diets based around cereal grains and nutrient poor but energy dense foods, is said to have contributed to a significant change in net endogenous acid production (NEAP) (Sebastian et al., 2002). Higher intakes of sulphur-containing amino acids from protein rich foods is also considered a contributing factor in the daily surplus of acid, as their metabolism generates acid which the kidneys must excrete (Remer & Manz, 1994; Busque & Wagner, 2009).

Net endogenous acid production has been estimated to have changed from a value of -88 mEq/d in preagricultural or Paleolithic times to an estimated + 48 mEq/d based on the third US National Health and Nutrition Examination survey (Sebastian et al., 2002; Pizzorno et al., 2010). Other authors have concluded similarly, with estimates of approximately 1 mEq/kg/day of non-volatile acids produced with a typical western diet (Vormann et al., 2008). Non-volatile "Fixed acids" include phosphoric, uric, lactic and sulphuric acid. Amino acids which generate sulphuric acid during their metabolism include both cysteine and methionine. Phosphoproteins and chloride salts in some foods also generate acid (Seldin, 1989).

The following chemical equations outline the result of metabolism of acid forming amino acids (Bushinsky, 2004).

Methionine or cysteine $\rightarrow$ glucose + urea + SO_4^{2-} +2 H^+

Arginine$^+$ $\rightarrow$ glucose + urea + H^+

$R\text{-}\ H_2PO_4 + H_2O \rightarrow ROH + 0.8\ HPO_4^{2-}/0.2H_2PO_4 + 1.8\ H^+$

Not all amino acids produce acid, some produce bicarbonate HCO_3^- (aspartate and glutamine) which can offset non-volatile acid production to a small degree.

The generation of bicarbonate from dietary sources is solely reliant on the fruit and vegetables food group (Lanham-New, 2008). Fruit and vegetables contain organic acids (which can combine with hydrogen ions) and the salts of organic anions such as citrate, succinate and proprionate. These also can generate bicarbonate as follows:

$glutamate\text{-} + H^+ \rightarrow$ glucose + urea

$lactate^- + H^+ \rightarrow$ glucose + CO_2

$citrate^- + 4.5\ O_2 \rightarrow 5\ CO_2 + 3\ H_2O + HCO_3^-$

(Bushinsky, 2004)

2.8.2.2 Dietary acid production from common foods

Common western type foods have had their corresponding potential renal acid load (PRAL) values determined after examining what happens during and after digestion of the food (Remer et al., 1995).

Food Group	PRAL value
Cheese with high protein content	23.6
Meat and meat products	9.5
Bread	3.5
Milk and non-cheese products	1.0
Vegetables	- 2.8
Fruits	- 3.1

The potential renal acid load (PRAL) calculated for various types of food show high protein foods have a higher positive PRAL than grains or fruit and vegetables. Fruits and vegetables are said to be alkaline forming and therefore contribute negative PRAL values. They act as the major buffer in the diet for the acid components through the supply of minerals (Na, K, Ca, and Mg) and their base forming potential as carbonate ions. Chemical salts have been used experimentally, to mimic the dietary alkaline effects of fruits and vegetables which resulted in favourable effects on bone loss (Dawson-Hughes et al., 2009).

2.8.2.3 Population based studies of fruit and vegetables intake and bone health

The significant association between bone health and higher consumption of fruit and vegetables was noted in the literature over forty years ago. Researchers in the field suggested a means of decreasing the attrition rate of bone was to "emphasise fruit and vegetables, vegetable protein and moderate amounts of milk" in the diet (Wachman et al., 1968). Since then, several population based studies (New, Bolton-Smith, Grubb et al., 1997; Frassetto, Todd, Morris et al., 2000; Prynne, Mishra, O'Connell et al., 2006) have also concluded fruit and vegetable intake is associated with bone health.

In 2000, a cross-sectional study by Frassetto and co-workers (Frassetto et al., 2000) looked at consumption of vegetable foods and animal protein intake in 33 countries worldwide to assess hip fracture incidence (HFI) in women aged 50 years and older. Their findings were an extension of work done previously by Abelow (1992), who demonstrated a significant positive correlation between

animal protein and hip fractures in 16 countries. Frassetto investigated vegetable protein as being representative of a vegetable based diet which carries surplus organic anions and allows for increased endogenous base production. Their study demonstrated increased vegetable consumption was an independent predictor of reduced HFI. Based on this association they have proposed the higher hip fracture incidence in post-menopausal women in the western world may be partially explained by the higher net renal acid load conferred by a diet rich in acid producing precursors from animal protein and grains and low in base producing foods (vegetables). This study has been criticised because it doesn't account for potential confounders between the various populations such as genetics, sun exposure and exercise levels, which can each have a large influence on bone health. Differing life expectancies invariably mean countries with longer living populations will incur higher levels of morbidity (Calvez et al., 2012) and this is evident, with the countries with the highest hip fracture incidence having the longest life expectancy. Another criticism is while hip fractures tend to predominate in one group (elderly women), the estimate of protein intake was determined from the population as a whole (Bonjour, 2005; Calvez et al., 2012).

With a large number of potential confounders, it is not surprising therefore, that epidemiological studies assessing effect of protein intake on hip fractures have resulted in conflicting evidence. A meta-analysis concluded that while protein levels in the diet are a positive influence on bone health, the effect is small (Darling et al., 2009) and while some have found a negative relationship between fracture risk and higher protein (Munger, Cerhan, & Chiu, 1999; Misra, Berry, Broe. K. et al., 2010) overall, the weight of the evidence is not strong (Darling et al., 2009).

Grains are another food group with high consumption levels in the Western world, and implicated in bone loss due to effects on dietary acid-base balance. Heaney and Weaver (2005), proposed the doubling in bone remodelling seen during the menopausal period, which increases to 3 times the premenopausal rate by the mid-sixties, is not for structural repair but for calcium homeostasis (Heaney et al., 2005). Providing adequate calcium and vitamin D will reduce bone remodelling but most of the remodelling is proposed to be due to high dietary energy intake from grains. Grains are

low in calcium and potassium which are alkaline, and high in sulphur containing amino acids which generate acid, therefore, a high grain diet creates a surplus of hydrogen ions which bone has to buffer to maintain homeostasis (Shewry & Halford, 2002).

Heaney and Weaver (2005), compared resorption levels among women eating western type grain based diets with hunter gatherer populations who typically derived a mere 5% of their energy needs from seeds (grains). They suggest the change in diet over the pre and post-agricultural periods, along with the shift to regions of higher latitudes and therefore lower vitamin D exposure as both being responsible for the approximate doubling of remodelling noted by Abbott across this time period in history termed the Agricultural Revolution (Abbott, 1996; Heaney et al., 2005).

A UK population-based study by Prynne and co-workers (2006), determined significant associations between actual fruit and vegetable intakes and bone mineral density status (BMD) in 5 female cohorts, ranging from adolescents to older women. The significant associations between BMD of older women and higher fruit and vegetable intake, especially at the spine could not however, be drawn between size adjusted bone mineral content (SA-BMC) and the renal acid load of the diet. This study was exceptional in that 7 day food diaries were obtained rather FFQ's, as is more usual in population based studies. The use of 7 day food diaries is considered a more accurate analysis of the usual diet compared to FFQs (Day, McKeown, Wong et al., 2001; Whitney et al., 2009) and therefore, gives more credence to the association with fruit and vegetables. Size adjusted bone mineral content (SA-BMC) was used as a measure of bone mineral status rather than bone mineral density (BMD) because it was thought that BMD did not adequately represent adolescent bone and body size (Prynne et al., 2006).

Another population-based study, The Aberdeen Prospective Osteoporosis Screening Study (APOSS), of 994 women aged 45-49 years, found fruit and vegetable intake correlated positively with bone mass (New et al., 1997). Positive associations were found between BMD and the nutrients associated with fruit and vegetables such as fibre, potassium, magnesium, zinc and vitamin C (New et al., 1997).

A retrospective study also found higher reported intakes of fruit and vegetables in childhood were associated with higher femoral neck BMD in healthy women aged 45-55 years (New, Robins, Campbell et al., 2000).

These studies lead to speculation that there are two periods in the lifecycle when the diet plays a crucial role in influencing the skeleton and BMD, adolescence (when bone mass is accumulating) and in later life (>60years) when bone loss is greater (Prynne, Ginty, Paul et al., 2004; Prynne et al., 2006). The women in the APOSS group were tested 7 years later and of those women still menstruating (to avoid confounding with oestrogen decline), there were still positive associations between nutrients associated with fruit and vegetables and greater BMD and reduced bone loss (Macdonald et al., 2004).

The Framington Osteoporosis Study, a longitudinal cohort study of 907 participants from the original Framington study, compared bone mineral density measurements and dietary intake data (FFQ) from 345 men and 562 women. The participants were aged 69-97 years at the time of the first bone measurement and the data demonstrated both cross sectional as well as longitudinal correlations between fruit and vegetable consumption and BMD in older men and women (Tucker et al., 1999). This study was noteworthy in that, although a strong cross sectional association between potassium and magnesium (mainly found in fruit and vegetables) and BMD was found; this association was lost in the longitudinal analysis for women but not men. This was possibly due to loss of muscle tissue (magnesium) seen in later life or other hormonal effects, which may mask the bone protective effects of these two minerals (Tucker et al., 2001, Tucker et al., 1999).

The relationship between fruit and vegetable intake and BMD of the non-dominant forearm and dominant heel was explored by McGartland and colleagues (2004), in a large cross sectional epidemiological study of 1345 Irish adolescents aged 12-15 years. They utilised a robust and validated FFQ for ascertaining usual food and beverage intake and collected information on other factors which had the potential to confound such as physical activity, weight and smoking and found a significant

association between high fruit consumption and heel BMD in 12 y-old girls. Although there was no significant difference noted in the forearm BMD measurements and fruit and vegetable intake, it is difficult to compare as the heel is composed predominantly of trabecular bone while the forearm is predominantly cortical bone. Therefore the heel is possibly more influenced by the diet as it is more metabolically active (McGartland et al., 2004).

A diet high in fruit and vegetables (>3 serves/day) was found to have significant effects on urinary calcium excretion and parathyroid hormone concentration (PTH) which were both lower and a corresponding larger bone size (whole body) than seen in those consuming <3 serves fruit and vegetables/day (Tylavsky et al., 2004). As sodium intake is known to have a significant effect on calcium urine excretion, not only in adults (Ilich et al., 2000), but also children (O'Brien, Abrams, Stuff et al., 1996), it was noted that sodium intakes between the high and low fruit and vegetable intake groups did not differ, despite calcium excretion being reduced with the higher fruit and vegetable intake group. A possible reason for this could be that a higher potassium content of the diet (via fruit and vegetables) acted as a calcium sparing nutrient (Sellmeyer, Schloetter, & Sebastian, 2002, Sebastian, 1994, Devine, 1995) or increased intestinal uptake of calcium (Ceglia et al., 2009; Pizzorno et al., 2010).

In conclusion, the majority of population based studies show better bone mineral density in those groups who consume more fruit and vegetables. Whether this relationship is due to individual nutrients associated with fruit and vegetables, phytochemicals, acid-base load, effects on calcium uptake and excretion or a combination, is however, not clear.

2.8.2.4 Animal studies, fruit and vegetables and acid-base balance

Rats have proven to be a suitable animal model to study the relationship of food and its effects on the skeleton (Kalu, 1991). Patterns of bone loss in the rat parallel that of post-menopausal women, with accelerated bone loss at a certain point in their life cycle. The initial rapid loss of bone is then followed by a phase of slower bone loss. Additional similarities with humans are that cortical bone is

lost at a slower rate than trabecular bone, calcium uptake from the intestine reduces, along with bone formation not keeping up with an increased resorptive rate (Kalu, 1991). These similarities make the rat a useful model when assessing the impact of various food or phytochemical substrates on bone health.

Muhlbauer and Li (1999), used ovariectomised rats to ascertain whether consumption of various common vegetables had an effect on bone resorption. The rats, aged 9-9.5 weeks were either bilaterally ovariectomised (OVX) or sham-operated (SHAM) under ether anaesthesia. In the SHAM rats, the ovaries were bilaterally exposed and handled, but not removed. To verify complete removal of the ovaries in the OVX rats, at the end of the experimental period the uterus was excised and the weight was determined. Ovariectomy causes a significant increase in bone resorption compared to sham- operated rats (32%±3% ($P<0.001$) but this increase in resorption was found to be reduced significantly (↓ 25%) by feeding the rats 1 gram of onion per day (approximately 7% of diet by weight). The onion exerted a rapid inhibitory effect on resorption in a similar manner seen in the rats that were exposed to calcitonin (daily injected doses of 1.25 or 2.5 IU per kg body mass at the optimal time). The researchers tested a variety of vegetables e.g. garlic, parsley, dill, cucumber, rocket, tomato and lettuce which also had similar but lesser inhibitory properties on bone resorption. This effect on resorption was not noted with milk powder despite its calcium content or with consumption of any foods of animal origin (Mühlbauer et al., 1999).

The effect of onions on bone resorption was further investigated in a later series of studies to determine whether it was the alkaline excess of the vegetable diet or some unknown phytochemical present in onion itself which reduced bone resorption. The rats were divided into two groups and fed either a vegetarian diet which consisted of 20% soy flour, 10% potato protein and 2% casein as protein plus a mixture of onion or 14 different vegetables consisting of equal parts of arugula, broccoli, cucumber, Chinese cabbage, red cabbage, dill, garlic, leek, lettuce, onion, parsley and tomato or they were fed an animal protein diet with casein as the sole source of protein. Both diets had similar raw protein content, calcium and phosphate. Rats consuming a vegetarian diet plus

additional onion had a significant decline in bone resorption (13%) after a 10 day dietary intervention, while additional onion with an acid forming diet (casein) led to an even greater reduction (18%). The effect on bone resorption was measured by urinary excretion of tritium released from bone which had been prelabelled with tritiated tetracycline and the amount of onion in the diet varied between 0.03 and 1.5 gram/rat/day in a dose dependent manner. Consumption of one gram of onion per/day by the rats, significantly reduced their bone resorption markers. Using varying quantities of potassium citrate to buffer the metabolic acid load from the casein diet, Muhlbauer and colleagues (2002), were able to demonstrate that the reduction in bone resorption shown by the onion was not due to its base excess. These findings suggest a phytochemical may be present in onion which inhibits bone resorption (Mühlbauer et al., 2002). This compound was later identified as γ-L-glutamyl–trans-S-1-propenyl-L-cysteine sulfoxide (GPCS) (Wetli, Brenneisen, Tschudi et al., 2005).

The above animal studies demonstrated common vegetables and fruit can affect bone metabolism, most likely via the mechanisms of action of the phytochemicals they contain rather than their base excess. Confirmation of the action of particular fruit, vegetables and herbs on bone turnover markers in humans has yet to be carried out.

2.8.2.5 Intervention studies and reduced dietary acid load

There is a dearth of intervention studies available that have looked at the effect of increased consumption of fruit and vegetables, whether it was to increase micronutrient or phytochemical intake or decrease acid load in any population, not just midlife women. The few available studies have focused on increased fruit and vegetable intake (Dragsted, Pedersen, Hermetter et al., 2004; Macdonald et al., 2008) sometimes in conjunction with a lowered sodium intake such as the DASH study (P. Lin, Ginty, Appel et al., 2003; Nowson, Patchett, & Wattanapenpaiboon, 2009). Several studies looking at acid load have relied on alkaline supplements including mineral water to mimic the effect of fruit and vegetables in the diet (Sakhaee, Maalouf, Abrams et al., 2005; Dawson-Hughes et al., 2009).

The DASH study is one of the better known dietary intervention studies involving increased fruit and vegetable intake (9 servings/day). This United States study was a randomised feeding trial intervention involving middle aged men and women with borderline hypertension who were provided free fruit and vegetables and low fat dairy products to assess effects on hypertension. An ancillary study of 186 men and women aged 23–76 years, assessed effects of the dietary intervention on bone and calcium metabolism. Participants were randomised to one of two dietary patterns (Control or DASH diet) and 3 levels of sodium intake (high, medium and low) with participants one month at each level of sodium intake and 2 weeks in between each level. The DASH group participants consuming 9.5 servings of vegetables and fruit compared to Control group participants 3.6 servings, had significantly reduced calcium excretion, which is associated with the reduced acid load in the diet (P. Lin et al., 2003). A decrease in bone resorption marker serum CTX (16-18%) and serum osteocalcin (OC) (8-11%) was demonstrated at each sodium level in the DASH diet compared with the control diet. Within the DASH diet sodium levels; there was an additional 3% reduction in serum OC from the high to low intake of sodium groups in the DASH diet (P. Lin et al., 2003).The hypercalciuric effect of sodium itself on the body has previously been confirmed (Ilich et al., 2000).

The significance of these findings on bone mass and fracture risk is still to be determined though it is proposed that the combined effects of the increased potassium intake, lower sodium intake and lower acid load of the DASH diet conserved calcium and significantly reduced bone turnover (P. Lin et al., 2003). One important variable that is difficult to gauge from the results of the DASH study is whether bone markers were reduced in the women who were post-menopausal. In this study, age was recorded but not menopausal status, therefore women could have been pre, peri or post-menopausal. Bone markers are highly variable depending on menopausal status (Eastell et al., 2008).

In a short term study (Buclin et al., 2001), involving a cross over design and a 4 day dietary intervention with 8 volunteers on four separate occasions, foods high in acid forming components (bread, pasta ,noodles and cheese) were alternated with those high in base forming components (potatoes, fruit and vegetables). The acid forming diet increased urinary excretion of calcium

significantly (74%), despite a similar consumption of calcium in both regimens. Urinary CTX excretion also increased with the acid forming diet by 19%. The authors suggested there is a strong likelihood that the increased osteoclast activity demonstrated by the CTX increase, results in the increased calcium excretion and its source is bone (Buclin et al., 2001)

As considerable dietary change is needed to achieve an alkali-producing diet, many interventions increase alkaline components via supplementation with substrates such as potassium citrate or bicarbonate, or mineral water, rather than increasing vegetable and fruit intake to assess effects of potassium and/or alkaline substrates on bone health. The effects of potassium on calcium excretion had been thought to be bone sparing (Dawson-Hughes et al., 2009) and translate into positive effects on bone resorption and turnover and subsequent BMD, however the evidence has been conflicting (Dawson-Hughes, Harris, Palermo et al., 2001; Sakhaee et al., 2005).

In a double blind crossover intervention study with 18 post-menopausal women, Sakhaee and colleagues (2005), demonstrated supplementation with potassium citrate (mimicking fruit and vegetables' alkali provision) reduced urinary calcium loss and increased urinary pH but did not affect bone markers of resorption and turnover, whereas calcium citrate did (Sakhaee et al., 2005). Calcium citrate reduced serum CTX (bone resorption) but had no effect on bone formation markers (GLA and BAP), however, the combined regime of both potassium citrate and calcium citrate, caused PTH to decline significantly as well as all three markers of bone resorption (hydroxyproline (OHP) by 9.5%, NTX by 18.1% and CTX by 20.4%) but had no effect on markers of bone formation. However, this study was only 2 weeks duration and previous work has demonstrated bone formation markers require longer to change (2-3 months as opposed to 2 weeks) compared to resorption marker change (P. Lin et al., 2003; Sakhaee et al., 2005).

Dawson-Hughes and co-workers (2001) added a supplement of bicarbonate to the typical acid-forming diets of 171 men and women aged 50 years and older. They found bicarbonate reduced urinary N-telopeptide and calcium excretion, but unlike Sakhee and colleagues, found potassium

supplementation didn't affect either calcium excretion or bone resorption markers (Dawson-Hughes et al., 2001). More recently, Macdonald and co-workers (2008) conducted a randomised controlled trial with 276 post-menopausal women (aged 55-65 years). Participants were randomly assigned to one of four groups: high potassium citrate (55.5 mmol/d) supplement, low dose (18.5 mmol/d) potassium citrate supplement, placebo or an additional intake of 300 grams fruit /vegetables in their daily diet for 2 years. Calcium excretion was found to be lower in the high dose supplement group at one time point during the 2 years, but no appreciable difference in bone turnover and resorption markers or BMD was found. They concluded the benefit of fruit and vegetables on bone may not be attributable to their alkaline forming components. Their study also included a group increasing fruit and vegetables. This group was instructed to increase consumption daily by 300 grams and participants were given a financial contribution for this purpose. Dietary analysis at the end of study showed while fruit intake had increased (295 grams) vegetable intake was only slightly increased (39 grams) and no difference in bone turnover and resorption markers or BMD was found in this group (Macdonald et al., 2008). As a result of their findings, the authors suggested, specific vegetables associated with bone health (Mühlbauer et al., 2002) should be emphasised in further intervention studies as well increased amounts of vegetables, as their study may have had too small a vegetable intake increase to have had any effect on bone indices (Ashwell et al., 2008; Macdonald et al., 2008). It has also been suggested the amount of potassium citrate supplemented was too low to appreciably affect calcium balance. In addition, adherence to the supplementation or increased intake of fruit/vegetable has been questioned when only one group (high potassium citrate supplement) showed lowered urinary calcium excretion and at only one time point (first 6 months) but not at later times during the study (Moseley et al., 2013).

Results from an American based study "The Women's Health Initiative" (WHI) Dietary Modification trial showed little difference in bone indices after an increased fruit and vegetable intervention (McTiernan, Wactawski-Wende, Wu et al., 2009). McTiernan and colleagues followed 500 post-menopausal women for 8 years with a dietary intervention involving increased fruit and vegetables (≥5 servings) and grains (≥6 servings) and reduced fat intake (≤20% fat). The 215 women in the

intervention group showed no change in risk of osteoporotic fractures after the eight years, slightly lowered hip BMD and a modest reduction in the risk of falls compared to the 285 women in the comparison group. This study, unlike the previous fruit and vegetable study (Macdonald et al., 2008), did not assess vegetable intake apart from fruit, and the total fruit and vegetable increase was relatively small (~1.2 serves) while a higher level of grains (higher acid load) was advocated (McTiernan et al., 2009) thereby neutralising any effect on dietary acid load. Including fruit and vegetables as one item/food group is a drawback seen in some fruit and vegetable interventions (Chapman & Armitage, 2012) as is the lack of increase in vegetables compared to fruit.

2.8.2.6 In-vitro studies and effects of acidosis

Despite conflicting results from in-vivo bone studies investigating PRAL and dietary acidity effects on bone, evidence from in-vitro studies appears much clearer. Cell cultures of calvariae in medium stimulating physiological metabolic acidosis show a highly stimulatory effect on osteoblasts and osteoclast cells and their subsequent homeostatic processes (Arnett & Spowage, 1996; Bushinsky, Smith, Gavrilov et al., 2002; Arnett, 2003; Frick & Bushinsky, 2003).

Cell culture studies initially used osteoclasts derived from neonatal rat long bones cultured on cow bone wafers/slices to provide another means of elucidating effects of change in pH on bone. By influencing the pH of the extracellular fluid (ECF) the osteoclast cells were bathed in, changes in resorption and specifically the resorption pits forming on the bone slices were seen (Arnett et al., 1996). It is unknown what the pH of the interstitial fluid bathing bone cells is, due to technical difficulties, however, we do know skin interstitial fluid has a pH closer to 7.1 and cells in tissues will generally have a lower pH than that of arterial blood (7.4), and venous blood (~7.36) (Arnett, 2008). More recently, Arnett and co-workers cultured peripheral blood mononuclear cells on dentin (ivory) in the presence of RANKL and M-CSF and found the osteoclasts to be almost inactive at pH 7.4 (normal blood pH) but activity greatly increased as the pH dropped.

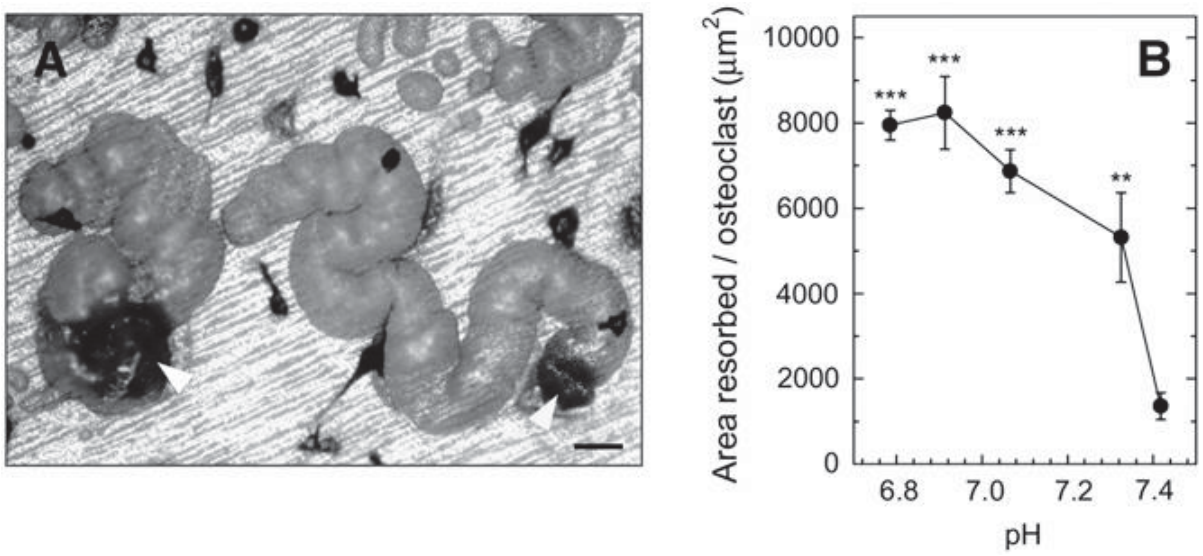

Figure 10 Acid activation of human osteoclasts

This figure depicts (A) Osteoclasts (white arrowheads) and their resorption trails (darker grey features); osteoclasts were activated by acidification to pH 7.0 for 2 days Scale bar = 20 µM. (B) Acidification dose-response curve for resorption pit formation by human osteoclasts generated at pH 7.45 and then cultured for a further 2 days at the indicated pH. Values are means ± SEM; significantly different from pH 7.42 control: **p < 0.01, ***p < 0.001 (ANOVA). Adapted with permission from Arnett, 2008.

Resorption pit formation by the osteoclasts increased progressively as the pH became acidic with osteoclasts most sensitive to changes around pH 7.1 and maximal activity near 7.0 (Arnett, 2008). This in-vitro resorption is depicted in Figure 10 above and is similar to that seen in-vivo involving creation of pits and trails caused by the removal of mineral (hydroxyapatite) and organic components (collagen). Initially thought to be a passive physiochemical response to an acidic environment, research in this area is now showing multiple effects of the extracellular hydrogen ion concentration on bone functioning with osteoclasts detecting reductions in pH of <0.1 unit and doubling resorption pit formation (Arnett, 2008).

One of the first changes occurring in bone when exposed to an acidic medium is a sodium and potassium efflux then loss of the mineral carbonate. The buffering of the proton influx is however, not uniform. Using a high resolution scanning ion microprobe with secondary ion mass spectroscopy Bushinsky and co-worker's (2002), showed the surface and interior of bone respond differently. Carbonate located on the surface of bone in the hydration shell, where it readily comes in contact with the ECF medium is depleted, along with phosphate from the centre of the bone. This would indicate

that bone buffers an acidic medium by dissolution of surface carbonate and loss of phosphate from the interior along with calcium, sodium and potassium (Bushinsky et al., 2002).

Krieger and co-workers reduced bicarbonate concentrations in mouse calvariae and noted a calcium efflux from the calvariae and prostaglandin 2 (PGE2) increasing in the medium. They determined PGE2 was mediating the acid induced bone resorption (Krieger, Frick, & Bushinsky, 2004). Cyclooxygenase (COX-1) has been determined as the rate-limiting enzyme in the production of PGE2 and can be either in the constitutive form (COX-1) or the inducible form (COX-2). Osteoblasts regulate production of COX-2 and this is highly sensitive to acidosis (Krieger, Frick, La Plante Strutz et al., 2007).

The longer the cells are in an acid medium the more pronounced will be the increase in RANKL RNA expression. So although the original efflux of calcium from the calvariae was induced by the presence of acid in the medium and therefore had a physiochemical cause, the ongoing presence of the acid induces a cell mediated response firstly with the PGE2 being released, which stimulates RANKL to cause pre-osteoclasts to mature as well as mature osteoclasts to increase their resorptive capacity (Bushinsky et al., 2002; Frick et al., 2003; Frick, LaPlante, & Bushinsky, 2005). A reducing pH in the ECF is the main stimulus for bone resorption by osteoclasts (Brandao-Burch, Utting, Orriss et al., 2005; Arnett, 2008).

Another effect of calvariae being incubated in acidic medium is reduction in osteoblast alkaline phosphatase activity as pH declines from physiological normal of 7.4 to 6.9 (Arnett, 2008). This reciprocal effect with osteoblasts was demonstrated by Brandao-Burch and colleagues using mineralised bone nodule-forming primary osteoblast cultures exposed to an acidic medium (Brandao-Burch et al., 2005). Despite there being no changes in osteoblast proliferation and collagen synthesis, there was reduced mineralisation of bone nodules, which ceased completely at pH 6.9 with a corresponding increase in matrix GLA protein. Osteoblast alkaline phosphatase activity was particularly compromised with a nearly eight fold reduction at pH 6.9 (Arnett, 2008).

In summary, in-vitro experiments have demonstrated cells in an acidic medium showed reduced alkaline mineral deposition by osteoblasts, a concomitant release of calcium from bone, and the increase in COX-2 enhancement of PGE2 production to stimulate osteoclast resorption. Whether these events parallel the in-vivo skeletal response to an acidic diet remains to be proven. However, conflicting results in differing models may reflect very different compositions of in-vitro and in-vivo extracellular fluids and indicates a need for cautious interpretation or extrapolation of any results in cell culture models to the animal/human model (Halliwell, 2012). A diet low in renal acid load and high in fruit and vegetables, may result in reduced calcium loss in the urine, and lower resorption markers however, as yet there is still long term evidence needed to determine this definitely improves bone health or protects from osteoporosis.

2.9 Dietary phytochemicals and their effect on inflammation and bone health

The term phytochemical is used to describe the wide variety of plant derived naturally occurring compounds which may affect cellular activities including polyphenols, isoflavonoids, anthocyanins and catechins (Son et al., 2008; Biesalski, Dragsted, Elmadfa et al., 2009). Plant-derived phytochemicals became a major focus of research in the last decade due to effects on inflammation and cell signalling (Williams, Spencer, & Rice-Evans, 2004; Bakker, Van Erk, Pellis et al., 2010; Fraga & Oteiza, 2011), positive effects on ageing (Gopalakrishnan & Tony Kong, 2008), bone turnover markers and bone mineral density in the animal model (Habauzit et al., 2008; Hunter, Skinner, & Lister, 2008).

2.9.1 The link between inflammation and bone

Osteoimmunology is the term coined to encompass the connection between the immune and bone systems (Boyce et al., 2009), with the principal cells involved in bone resorption, the osteoclasts, deriving from the same haemopoietic stem cells as those involved in immune function (Ginaldi, DiBenedetto, & DeMartinis, 2005). Transcription factor signalling from NFκB is now known to not only have a potent effect on bone's RANK/RANKL/OPG system and therefore all bone cells, but also

B cell maturation and lymph node development (Cashman, 2008). Mediators of inflammation can precipitate bone loss by uncoupling the balance between bone resorption and formation through their effect on the RANK/RANKL/OPG system. This system is highly susceptible to circulating levels of various pro and anti-inflammatory cytokines such as interleukin-1 (IL-1), interleukin-6 (IL-6), interleukin-10 (IL-10) and TNF (Park-Min, Ji, Antoniv et al., 2009; Ivashkiv, Zhao, Park-Min et al., 2011). The cytokines TNF and IL-6 both play a generic role in many age related disorders (Bruunsgaard & Pedersen, 2003) and increased activity of these two key inflammatory cytokines as well as IL-1 is critical to age related bone loss (Ginaldi et al., 2005).

Inflammatory bone loss (refer Figure 11) is promoted mainly due to TNF driving increased osteoclastogenesis through the RANKL pathway and increased T-cell activation (Yun & Lee, 2004; De Martinis, Mengoli, & Ginaldi, 2007). The decoy receptor of RANKL, osteoprotegerin (OPG) is suppressed by the pro inflammatory cytokines IL-1, IL-6 and TNF, while RANKL activity is enhanced. Activated T-cells also express RANKL and this adds to the already increased rate of osteoclastogenesis. The monocytes/macrophage cell line development is reduced while the precursor osteoprogenitor cells are channelled into becoming osteoclasts. Chronic stimulation of T-cell RANKL is thought to overwhelm interferon-γ, which usually modulates its activation. As interferon-γ becomes overwhelmed, and unable to control the stimulation of the T-cell RANKL, osteoclastogenesis increases leading to increased bone loss (Ginaldi et al., 2005).The increase in bone resorption is coupled with a blunting of the anabolic bone formation response, once again via action of the cytokine TNF (refer Figure 11). This cytokine induces the inhibitors of bone formation, sclerostin and Dickkopf-1 (Dkk-1). Dkk-1 acts as an antagonist on the Wnt pathway while sclerostin from osteocytes reduces signalling necessary for bone formation (Schett et al., 2010). Increased apoptosis of bone osteoblasts has also been noted when TNF levels increase (Ginaldi et al., 2005).

The cumulative effect of the pro-inflammatory cytokines on RANKL activity leads to bone loss due to the increased resorptive activity by osteoclasts and uncoupling of the formation and resorption cycle (Cashman, 2008).

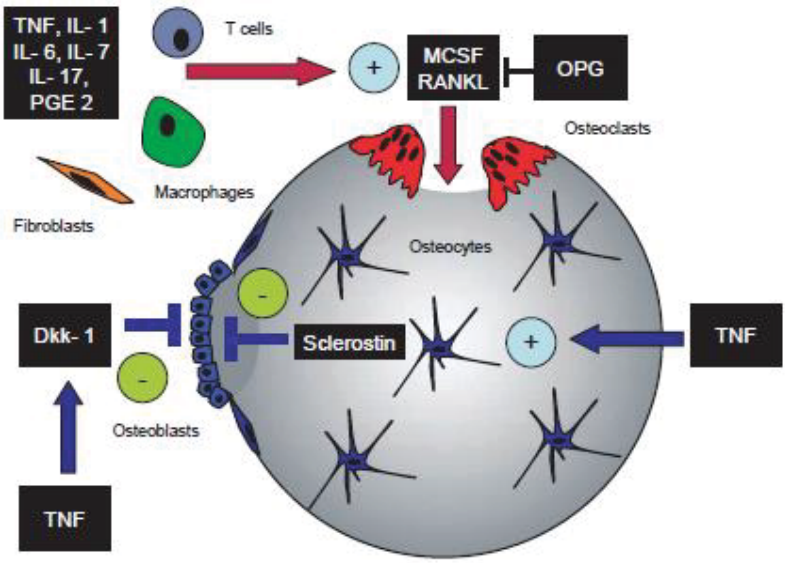

Figure 11 Inflammation and bone remodeling

This figure shows inflammatory mediators from macrophages, T-cells and fibroblasts stimulating osteoclast formation and bone resorption by inducing expression of RANKL and MCSF while TNF also blocks bone formation via sclerostin and Dkk-1. TNF;Tumour necrosis factor, IL;Interleukin,PGE2; Prostaglandin2,MCSF;macrophage colony stimulating factor, RANKL; Receptor activator of nuclear factor kappa B ligand, OPG;Osteoprotegerin,Dkk-1;Dickkopf-1. Reproduced with permission from Schett, (2010).

The bone loss due to increased production of pro- inflammatory cytokines can however, be suppressed by anti-inflammatory cytokines such as IL-10. IL-10 has been shown to potently inhibit NFATc1, the master regulator of osteoclastogenesis by suppressing both osteoclast associated receptor (OSCAR) and triggering receptor expressed by myeloid cells (TREM-2) which are required for osteoclast differentiation. IL-10 along with other anti-inflammatory cytokines induced by toll-like receptors (TLRs) can directly influence the Janus kinase-signal transducer and activator of transcription pathway (JAK-STAT) thereby reducing production of pro-inflammatory cytokines. This inhibition of pro-inflammatory cytokine production attenuates bone resorption leading to decreased inflammatory bone loss (Ivashkiv et al., 2011).

2.9.2 Ageing and bone

During ageing a progressive deterioration is seen in mechanical and physiological functioning of body tissues (Sarkar & Fisher, 2006). This slow deterioration in function may eventuate in differing organs expressing differing disease states. Bone tissue is progressively eroded during ageing, leading to fragility fractures and osteoporosis (Ginaldi et al., 2005). Inflammation levels, measured by

inflammatory cytokines, increase with age (Singh & Newman, 2011) particularly pro-inflammatory cytokines TNF and IL-6 (Bruunsgaard et al., 2003; Krabbe, Pedersen, & Bruunsgaard, 2004). Not surprisingly, ageing is noted for an increase in bone loss and other diseases with an inflammatory component such as asthma, Alzheimer's disease, cardiovascular disease and cancer (Miranda, Maier, & Stevens, 2001; Ginaldi et al., 2005; De Martinis et al., 2007). The increase in inflammation levels with age has been attributed in part to the increase in obesity during ageing (Halade, Jamali, Williams et al., 2011). Adipocytes are a considerable source of several important cytokines and fat derived peptides: IL-6, CRP, TNF and plasminogen activator inhibitor (PAI-1) (Licastro, Candore, Lio et al., 2005; Hotamisligil, 2006). The Free Radical theory of Ageing (Harman, 1955, 2006), suggested a global mechanism to explain the effects of ageing. This theory proposed that ageing and disease processes are due to the accumulating effects of damage to the cellular constituents, caused by free radicals (Harman, 2006). The antioxidant defences that counterbalanced the production of reactive oxygen and nitrogen species (ROS and RNS), were unable to be maintained and transcription factors such as NFκB, which are sensitive to redox states, are activated. NFκB activation stimulates a state of chronic inflammation, with increased production of pro- inflammatory cytokines which in turn, further increase production of ROS. Ageing therefore, has been attributed to a combination of higher inflammation levels, free radical production and lowered antioxidant defence with higher production of pro-inflammatory cytokines such as interleukin 6 (IL-6), c- reactive protein (CRP) and tumour necrosis factor (TNF) as a result of NFκB being exposed to higher redox states (Sarkar et al., 2006). However, conclusive evidence is lacking from systematic reviews and meta analyses, that increased antioxidant defence reduces the risk of diseases associated with ageing (Bjelakovic, Nikolova, Simonetti et al., 2004).

2.9.3 Oxidative stress and bone

Oxidative stress is characterised by increased reactive oxidative species (ROS). ROS production influences the immune response and is negatively linked with bone health (Wauquier et al., 2009). ROS include superoxide anion ($\bullet O_2^-$), hydroxyl radical ($\bullet OH$), hydrogen peroxide (H_2O_2), alkoxy and peroxy radicals, hypoclorous acid or peroxynitrite and reactive sulphur species. ROS are continually

produced in small amounts as a result of the body's metabolic processes. This is a normal and essential part of cell functioning as ROS protect cells from microbial invaders, act as secondary messengers and are involved in cell signalling (Kodiha & Stohacj, 2012). Because of ROS ability to also cause damage, healthy cells are always repairing or replacing their oxidant damaged biomolecules (Halliwell, 2012). This delicate balance between essential production of ROS and their clearance from cells is maintained in the healthy adult by a variety of defensive strategies the cell can use to deactivate or remove ROS. Several enzymatic systems such as superoxide dismutase (SOD) and glutathione/glutathione disulphide (GSH/GSSG) act as primary defence systems to maintain the intracellular redox state (Kodiha et al., 2012).

An increased production of ROS is seen with ageing, inflammation and environmental hazards such as UV radiation. This increase can also be coupled with a decline in production of antioxidant enzymes: superoxide dismutase (SOD), glutathione peroxidase and catalase enzymes, which usually counterbalance ROS production and thereby control the amount of damage that occurs in cells (Finkel & Holbrook, 2000). The increase in ROS production and oxidative stress seen in post-menopausal women is attributed in part to oestrogen loss, which lowers thiol antioxidant status particularly catalase and glutathione peroxidase antioxidant systems. Any increases in H_2O_2 levels would have previously been counterbalanced by the enzyme catalase (Kodiha et al., 2012).

The increase in ROS production and decline in cell antioxidant systems promotes onset and pathogenesis of several diseases. In bone it is thought to activate an immune response that disturbs bone remodelling. This is mainly through the action of molecules such as TNF which increase resorption leading to bone loss and osteoporosis (Basu, Michaëlsson, Olofsson et al., 2001; Hinoi, Fujimori, Wang et al., 2006; Schett et al., 2010). ROS may increase osteoblast apoptosis and senescence and increase expression of RANKL which leads to increased resorption and bone mineral loss (Weaver, Alekel, Ward et al., 2012). Due to their ability to reduce oxidative stress, antioxidants have been suggested as a potential method of reducing bone loss in post-menopausal women (Wauquier et al., 2009) with some antioxidants activating pathways and enzyme production

associated with reducing the inflammatory response (Salminen et al., 2012). There is however, still much debate about antioxidants and ROS role in ageing, inflammation and cell signalling, with some researchers (Viña, Borras, Abdelaziz et al., 2013; Stuart, Maddalena, Merilovich et al., 2014) suggesting ROS may act to enhance longevity and delay the ageing process via effects on cell signalling. While there is emerging evidence in this debate, this discussion is out of scope of this thesis.

2.9.4 Nuclear factor-erythroid 2 related factor-2 (Nrf2) and bone

Nuclear factor-erythroid 2 related factor-2 (Nrf2) is a transcription factor with a central role in the inflammatory process and associated with bone loss (Hinoi et al., 2006; Kodiha et al., 2012). Nrf2 regulates production of antioxidant and phase 2 detoxifying enzymes and stress response proteins, in response to an increase in the redox state of the cell. When Nrf2 is released from its sequester, kelch like ECH-associated protein (KEAP1) in response to inducers (phytochemicals/signalling molecules) in the cytoplasm, it translocates to the cell nucleus. Once in the nucleus, it can bind to a region of DNA encoding for antioxidant response elements (ARE) or electrophile response elements (EpRE) (Baird & Dinkova-Kostova, 2011). This action confers protection on the cell by the release of anti-inflammatory proteins including macrophage antioxidants (H. Zhu et al., 2008). Different agents are known to stimulate Nrf2 to activate the signalling pathways; among them are dietary plant phytochemicals (Salminen et al., 2012).

In Nrf2 deficient mice, pro-inflammatory cytokines such as TNF and IL-6 are overproduced compared to wild type mice (Kim, Cha, & Surh, 2010). Both these cytokines increase bone resorption and reduce the bone formation response. Similarly, genes encoding for matrix metalloproteinase's (MMP-9 and MMP-7) involved in digestion of bone matrix during resorption are also known to be up-regulated by the inflammatory cytokines and their expression down-regulated in macrophages by Nrf2 activation (Kim et al., 2010). In-vitro studies using neonatal mouse tibia and calvariae found Nrf2 negatively regulated osteoblastogenesis most likely via a mechanism involving Runx2, the main regulator of osteoblast differentiation. Runx2-dependent osteocalcin promoter is entirely inhibited by

Nrf2 overexpression. Additionally, collagen type 1 and induction of alkaline phosphatase activity is repressed in cell culture lines (MC3T3-E1 and ATDC5) with Nrf2 activation (Hinoi et al., 2006). However, a recent study using the ovariectomised mouse model has shown induction of Nrf2 by sulforaphane inhibited osteoclast activity with CTX and RANKL/OPG levels reducing but had a positive effect on osteoblast differentiation with increased levels of osteocalcin and ALP (Ibanez, Brines, Cuadrado et al., 2011).These conflicting results in differing models demonstrate evidence of different compositions of in-vitro and in-vivo extracellular fluids which may affect phytochemical action on Nrf2 activity due to rapid oxidation of auto-oxidisable compounds (Halliwell, 2012).

Small doses of many phytochemicals directly stimulate cell defence and apoptosis with isothiocyanates, flavonoids, polyphenols, stilbenes, anthocyanidins and procyanidins shown to cause G2/M cell cycle arrest in various cell lines (Gopalakrishnan et al., 2008). More recently, the focus has included the regulation and modification of inflammation by plant compounds (Son et al., 2008; Birringer, 2011; Salminen et al., 2012). Osteoporosis is associated with increased levels of pro-inflammatory cytokines particularly TNF-and IL-6 (Bruunsgaard et al., 2003; Krabbe et al., 2004). However, this inflammatory response can be suppressed by specific plant phytochemicals which work to activate pathways and enzyme production associated with reducing the inflammatory response (Salminen et al., 2012).

The main features in the ability of phytochemicals to affect inflammation include suppression of the NF-kB pathway, up-regulation of transcription factor Nrf2 via nuclear factor erythroid 2 related factor2/ antioxidant response elements (Nrf2/ARE) pathway and the enzyme AMP-activated protein kinase. The resulting defence of the cell ensues by suppression of pro-inflammatory marker production such as TNF and IL-6 and increased production of phase 2 cytoprotective enzymes such as found in macrophages (H. Zhu et al., 2008). Examples of these antioxidants and phase 2 enzymes include glutathione S-transferase (GST), NAD(P)H:quinone oxidoreductase (NQO1), superoxide dismutase (SOD), catalase, reduced glutathione (GSH), glutathione reductase (GR) and glutathione peroxidase (GPx)(H. Zhu et al., 2008).

Increased production of detoxifying enzymes affords greater protection to cells against ROS. These toxic compounds are quickly converted to less harmful intermediates upon conjugation with the detoxifying enzyme, with added protection provided by increased antioxidant capacity due to production of the non-protein sulfhydryl compound GSH (Lewis, Mele, Hayes et al., 2010). Phytochemicals increase genetic transcription of detoxifying enzymes by several mechanisms depicted in Figure 12. They disrupt binding of the Nrf2-Keap-1 complex in the cell cytoplasm allowing Nrf2 to phosphorylate and enter the nucleus where it binds to antioxidant response elements (ARE) in the promoter region of phase 2 detoxifying genes. Phytochemicals also prevent the breakdown of Nrf2 and up regulate signalling cascades such as mitogen-activated protein kinase (MAPK), protein kinase C (PKC), phosphatidylinositide 3-Kinases (PI3K) and protein kinase RNA-like endoplasmic reticulum kinase (PERK) which help translocate the Nrf2 into the nucleus and lead to increased genetic transcription of the detoxifying enzymes (Gopalakrishnan et al., 2008).

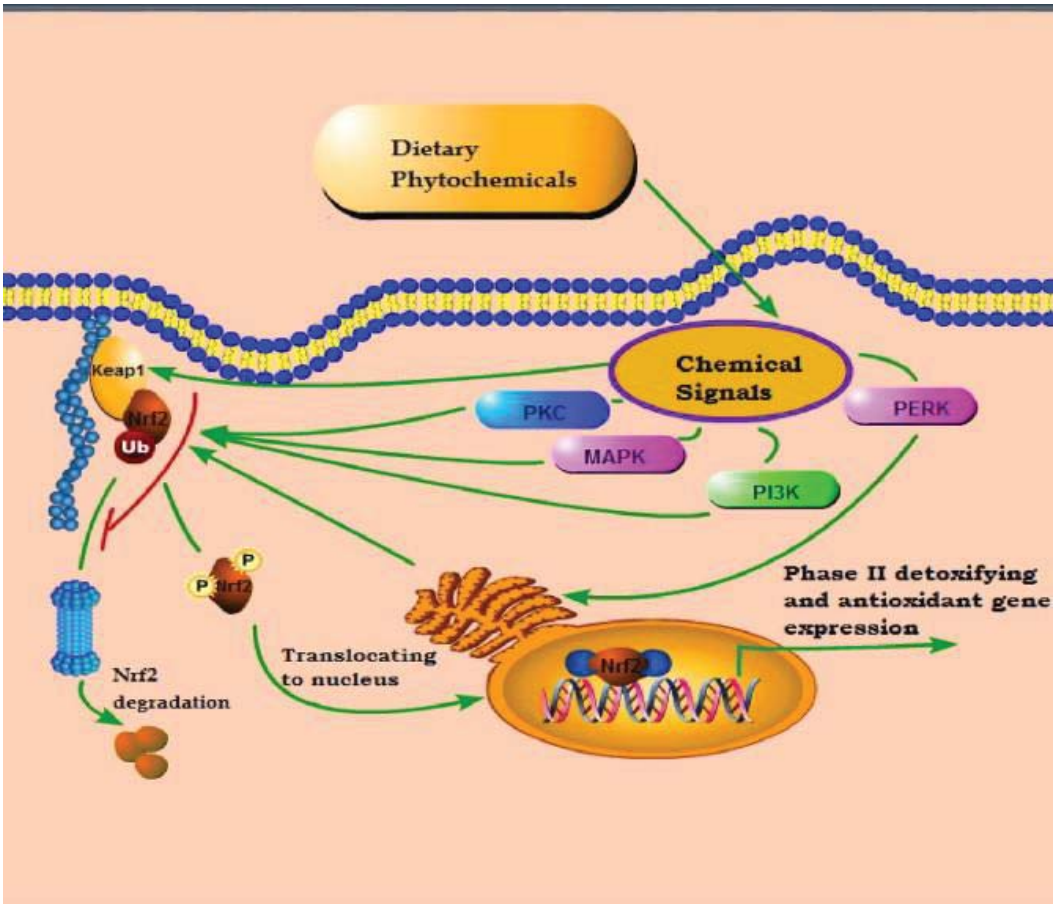

Figure 12 Effects of phytochemicals on Nrf2

This figure depicts how dietary phytochemicals promote the regulation of detoxifying enzymes which provide cytoprotection. Under normal conditions, Nrf2 is found in the cytosol attached to Keap-1. Dietary phytochemicals and their metabolites can disrupt the association between Keap-1 and Nrf2. Once Nrf2 is phosphorylated it translocates into the nucleus, where it binds to antioxidant response elements present in the promoter region of phase II detoxifying genes. This binding of Nrf2, increases gene expression of the detoxifying enzymes. The other ways phytochemicals promote Nrf2 activity is by both preventing its proteosomal degradation which increases its half-life, and by activation of upstream signalling cascades MAPK, PI3K, PKC and PERK which increases production of phase II detoxifying enzymes due to increased nuclear translocation of Nrf2. PI3K;Phosphatidylinositide 3-Kinases,PERK; Protein kinase RNA-like Endoplasmic Reticulum Kinase,PKC; Protein Kinase C, Nrf2; Nuclear Factor-Erythroid 2 Related Factor-2,KEAP1; Kelch like ECH-Associated Protein,MAPK;Mitogen-Activated Protein Kinase.
Adapted with permission from Gopulakrishnan and Kong, 2008.

An interesting aspect of some phytochemical's action is its opposing action in different cells. Vesprik (2011), recently demonstrated some phytonutrients, which previously were shown to inhibit oestrogen signalling in breast cancer cells, may enhance bone formation via action on the Nrf2/ARE system (Veprik et al., 2011). Some phytochemicals act at a systemic level and may reduce bone loss due to decreased levels of inflammation (Biver, Chopin, Coiffier et al., 2012). This may be mediated at a local level by the influence on cell signalling to increase bone formation activity while reducing resorptive activity thereby improving bone mass or reducing bone loss (Cashman, 2008; Habauzit, Offord, & Horcajada, 2010).

2.9.5 Adiponectin

Adiponectin is a recently discovered anti-inflammatory cytokine associated with fat cells (adipokines) as well as being a hormone regulating energy homeostasis and glucose and lipid metabolism (Yamauchi, Kamon, Minokoshi et al., 2002), Structurally similar to tumour necrosis factor (TNF), adiponectin is found in human plasma in several different forms: as a trimer, hexamer, a high molecular weight form and as cleavage products. Initially adiponectin was thought to be produced exclusively by fat cells, but is now known to also be produced by and have receptors (AdipoR1 and AdipoR2) on bone forming cells (Berner, Lyngstadaas, Spahr et al., 2004). While adiponectin has been shown in in-vitro studies to be both insulin sensitising and anti-inflammatory it is also associated with higher rates of bone loss (Barbour, Zmuda, Boudreau et al., 2012) and fracture risk (Biver, Salliot, Combescure et al., 2011) as well as increased mortality in older people (Kizer, Arnold, Jenny et al., 2011; Murphy, Register, Shively et al., 2014). The negative relationship between adiponectin and BMD is most noted in post-menopausal women where higher adiponectin levels were associated with lower BMD independent of levels of adiposity or measurements at load or non-load bearing sites (Richards, Valdes, Burling et al., 2007). Recently, Karsenty's group established the unique ability of this adipokine to regulate bone mass both negatively at a local level by decreasing FOX01 activity, and positively through peripheral control of sympathetic tone (Kajimura, Lee, Riley et al., 2013).

Higher adiponectin levels have been associated with increased dietary intake of plant based factors (Cassidy, Skidmore, Rimm et al., 2008), antioxidants (Detopoulou, Panagiotakos, Chrysohoou et al., 2009), lower glycaemic (Neuhouser, Schwarz, Wang et al., 2012) and Mediterranean type diets (Fragopoulou, Panagiotakos, Pitsavos et al., 2010). Intervention studies have found higher levels of adiponectin resulting from increased fatty acids, fish or fish oil supplementation, lower fat (15-24% energy from fat), carbohydrate restriction (20-30% energy from carbohydrate) and dietary fibre (soluble corn-based fibre) and plant extract supplementation as well as increased dairy and alcohol consumption (beer and whiskey)(Silva, de Almeida, & Feoli, 2011). There is a dearth of intervention studies investigating the effects of dietary pattern changes on adiponectin and in particular none involving increased intake of fruit and vegetables. As higher adiponectin levels are now accepted as

being central to bone loss, particularly in postmenopausal women, the link between the positive association of higher fruit and vegetable intake with higher adiponectin levels pose a conundrum which has yet to be investigated. Understanding how adiponectin levels change during dietary interventions may help elucidate possible associations with actions of other inflammatory markers and lead to a fuller understanding of this hormone's multiple mechanisms' of action.

2.9.6 Specific fruits and vegetables, their phytochemicals and effects on bone

2.9.6.1 Oranges

Oranges were first investigated for their bone resorption inhibiting properties by Muhlbauer (2003). Mühlbauer fed young rats 1 gram/day of orange peel and using an extensively validated method, found lowered urinary excretion of tritium-labelled tetracycline, reflecting lowered bone resorption. Since then, the active flavanone hesperidin (hesperetin-7-O-rhamnoglucoside) found in citrus fruit has been identified as having a positive effect on bone health and is one of the most bioavailable polyphenols (Vallejo, Larrosa, Escudero et al., 2010). The anti-inflammatory properties common to many polyphenol compounds such as flavanols found in cocoa (Vazquez-Agell, Urpi-Sarda, Sacanella et al., 2013), and green tea (Siddiqui, Shukla, Adhami et al., 2008) and particularly flavanones (oranges) is via inhibition of the NF-kB pathway (Horcajada & Offord, 2013). Several pro-inflammatory markers such as the cytokines, IL-6, TNF and nitric oxide (NO) are influenced by the key signalling factor NF- kB. When levels of NF- kB increase, pro-inflammatory markers may up-regulate osteoclast proliferation and differentiation leading to increased bone resorption and subsequent bone loss (Horcajada et al., 2013). TNF and NO are both strong activators of osteoclastogenesis (Wimalawansa, 2010; Schett, 2011). Older rats demonstrated decreased inflammatory markers IL-6 (↓ 81.0-87.9%) and nitric oxide (NO) (↓ 34.7-39.5%) compared to control after 3 month supplementation with either 0.5% hesperidin (Hp), 0.5% naringin (Nar) another flavanone in oranges, or combination of both flavanones (Hp+Nar, 0.25% each)(Habauzit, Sacco, Gil-Izquierdo et al., 2011). Increased levels of IL-6 are also thought to lead to osteopenia (Schett, 2011). Hesperidin may not only reduce resorption via reduced inflammation but may also promote bone formation as seen with an increase in bone mass in 3 month old rats (intact) fed hesperidin (0.5% in

diet) and reduced bone loss was seen in ovariectomised 6 month old rats (Horcajada, Habauzit, Trzeciakiewicz et al., 2008). However, in a later randomised, placebo controlled human study, supplementation with 500 mg/day hesperidin for 2 years failed to reduce bone loss in healthy post-menopausal women (50-65 years) compared to placebo group (1-2% bone loss in both groups), but the supplemented group did demonstrate a more favourable balance of bone markers (Horcajada et al., 2013).

2.9.6.2 Onions

Other bone friendly phytochemicals are polyphenols found in the flavonoid class, including quercetin/rutin (onions), kaempferol (grapes and red wine), (+)-catechin and epigallocatechin gallate (black and green tea respectively) (Habauzit et al., 2010). The flavonoid quercetin found in onions, has been investigated extensively for its effect on bone metabolism. Mühlbauer first identified a reduction in bone resorption in ovariectomised rats fed 30-1,500 mg onion/day (Mühlbauer et al., 1999). The effect was dose dependent with a higher amount of onion reducing resorption by 25 % ($p < 0.01$) (Mühlbauer et al., 2002). Further studies assessing urinary excretion of tritium released from bone of 9-week-old rats prelabelled with tritiated tetracycline, determined significantly lower bone resorption in those rats fed one gram of onion/day. The active phytochemical was identified as gamma-L-glutamyl-trans-S-1-propenyl-L-cysteine sulfoxide (GPCS) (Wetli et al., 2005). The effects of this phytochemical as an antioxidant, anti-inflammatory or cell signalling compound has not been determined, however rutin, another flavonoid found in onion which is broken down by rutinose to quercetin, was originally attributed with at least some of the bone resorptive properties of onions (Horcajada-Molteni, Crespy, Coxam et al., 2000). In an animal study, rutin (quercetin-3-O-glucose rhamnose) consumed by rats at 0. 25% of diet, reduced bone loss in ovariectomised (OVX) rats compared to sham-operated rats. Additionally femoral failure load was higher in rats consuming rutin and their bone markers were positively affected with urinary deoxypyridinoline (DPD) excretion (a marker for bone resorption) and calciuria both reduced while plasma osteocalcin (OC (a marker for osteoblastic activity) was higher compared to the sham operated rats without rutin. This study (Horcajada-Molteni et al., 2000) however, cannot explain the effect of onion on bone resorption found

in Mühlbauer's study because the rat's intake of rutin was much greater compared to dietary intake in Mühlbauer's study (1999). A pharmacological dose of 0.25% of rutin was in the rat's diet where 14.5 grams/day of total food was consumed. This gives the equivalent of 36.2 mg of rutin per day for the rat. However, only 1 gm/day of onion was used in the Mühlbauer rat's diet supplying one sixth of the dietary rutin intake of the rats in Horcajada-Molteni's study. Additionally, other Allium vegetables such as leeks and garlic demonstrated similar bone resorption inhibiting properties in Mühlbauer's study and these vegetables/herbs have only a small fraction of the rutin compared to onions. Rutin has however, been attributed with reducing TNF and ROS levels in bone marrow macrophage cells. This reduction was due to inhibition of RANKL-induced NF-kB activation (Kyung, Lee, Shin et al., 2008). A rat study showed similar effects on ROS and positive effects on bone biomechanical quality and micro-architecture with dietary intake of quercetin (5 mg/kg/day, 30 mg/kg/day and 50 mg/kg/day) for 8 weeks (Liang, Luo, Ge et al., 2011). Higher levels of quercetin significantly lowered the oxidative DNA damage level, up-regulated the total serum antioxidant capability, while increasing bone turnover (Liang et al., 2011). It is yet to be determined whether an increase in bone turnover in the animal model would be a favourable outcome for postmenopausal women with already increased bone turnover. Other investigators have found a decrease in bone turnover (Woo, Nakagawa, Notoya et al., 2004). A cell culture study using RAW 264.7 cells in the presence of receptor activator of NFκB ligand (RANKL), and a human model consisting of differentiating peripheral blood monocytic cells (PBMC) isolated from peripheral blood in the presence of RANKL and M-CSF demonstrated quercetin reduced the transcription factors NFκB and activator protein-1 (AP-1), thereby reducing osteoclastogenesis in a dose dependent manner with quercetin (0.1–10 μM) (Wattel, Kamel, Prouillet et al., 2004).

2.9.6.3 Dried plums

Dried plums (Prunus domestica L) contain several polyphenolic compounds including flavonoids, coumarins and phenolic acid derivatives such as neochlorogenic acid crytochlorogenic acid and chlorogenic acid (Arjmandi, Khali, Lucas et al., 2002). The total combined polyphenol content of dried plums is estimated at 184 mg per 100g (Sacco, Horcajada, & Offord, 2013) and as dried plums

rank high in oxygen radical absorbance capacity, their role in bone health has been attributed to the protective effect of reducing oxidative damage (Arjmandi et al., 2002). A combination of both anti-oxidant and anti-inflammatory properties are likely mechanisms for their bone protective effect (Weaver & Hohman, 2013). Dried plums (prunes) affect markers of bone turnover, and this has been demonstrated in two clinical studies (Arjmandi et al., 2002; Hooshmand, Chai, Saadat et al., 2011). The first randomised study with postmenopausal women, demonstrated three months intake of dried plums (100 grams = 12 plums/day) significantly increased indices of bone formation compared to women consuming 75 grams/day of dried apple, though no effect on bone resorption markers was seen (Arjmandi et al., 2002). A similar but longer dietary intervention (1 year) with post-menopausal women consuming dried apple and plums in the same quantities as the previous study, found BMD increased at the ulna and spine sites in women eating the dried plums compared to the women consuming apple. Unlike the previous study, bone formation and bone resorption markers were reduced in the dried plum group compared to the dried apple group (Hooshmand et al., 2011). However, this later study also gave all participants supplements of calcium (500mg/day) and vitamin D (400 IU/day) with known bone resorption lowering effects (Sakhaee et al., 2005). The mechanism of action of the bioactive compounds in dried plums are said to be via the down-regulation of RANKL, which reduces bone resorption, and increases production of Insulin Like Growth Factor-1 (IGF-1) (controlled by the transcription factors Runx2 and Osterix) which stimulates bone formation (Franklin, Bu, Lerner et al., 2006; Habauzit et al., 2008; Bu, Hunt, & Smith, 2009). The active constituent in dried plums affecting bone markers has been attributed to caffeic acid esters i.e. neochlorogenic acid, cryptochlorogenic acid and chlorogenic acid (Bu et al., 2009) and rutin (Horcajada-Molteni et al., 2000). All these phenolic compounds are found in appreciable quantities in dried plums. The chlorogenic acid isomers are found in quantities as high as 174.2 mg/100 g, which represents more than 94% of total phenolics whereas rutin (which breaks down to its aglycone, quercetin), is estimated to be 3.3 ng/100 g. These phenolic compounds reduce bone resorption either through inhibiting the osteoclast cell formation and activity (quercetin) (Woo et al., 2004) or a combination of anti-inflammatory and antioxidant properties (chlorogenic acid isomers) (Bu, Lerner,

Stoecker et al., 2008; Bu et al., 2009). The presence of high levels of phenolic compounds in dried plum may explain the unique effect of this dried fruit in preventing bone loss.

2.9.6.4 Tomatoes

Other phytochemicals associated with bone health include carotenoids (Sugiura, Nakamura, Ogawa et al., 2008)) particularly lycopene (L. Rao, Krishnadev, Banasikowska et al., 2003; Mackinnon, Rao, Josse et al., 2011) found predominantly in tomatoes. Among the carotenoids, lycopene is attributed with the highest singlet oxygen quenching capacity (L. Rao et al., 2003; L. Rao, Mackinnon, Josse et al., 2007). Lycopene's action on bone has been ascribed to its ability to reduce oxidative stress, thus reducing bone loss in midlife due to oxidative stress (Mackinnon, Rao, et al., 2011). The urinary bone resorption marker NTX, reduced in a study of post-menopausal women, along with a reduction in oxidative stress when the women were supplemented with lycopene over a four month period (Mackinnon, Rao, et al., 2011). Oxidative stress parameters were determined by total antioxidant capacity (TAC), which significantly increased from baseline in the lycopene supplemented groups compared to placebo group. A significant decrease was also demonstrated in both lipid and protein oxidation in the supplemented groups (Mackinnon, Rao, et al., 2011). Conversely when lycopene intake was restricted for one month, lowered activity of both the antioxidant enzymes superoxide dismutase (SOD) and catalase (CAT) was demonstrated in postmenopausal women alongside increased resorption marker NTX (Mackinnon, venket Rao, & Rao, 2011).

2.9.6.5 Olives

Olives are rich in phenolic compounds and one of the main phenolics oleuropein, has been investigated for a protective effect on bone mass in the presence of increased inflammation using an ovariectomised rat model (OVX) (Puel, Quintin, Agalias et al., 2004). Three groups of rats received either a control diet with 25 g peanut oil and 25 g rapeseed oil/kg (OVX), the control diet with 50 g olive oil/kg or the control diet with 0·15 g oleuropein/kg for 3 months. Talcum powder (magnesium silicate) was injected prior to the end of the experiment to induce inflammation and subsequently the OVX rats without supplementation with olive oil or oleuropein, had decreased bone mineral density

(metaphyseal and total femoral mineral density) and higher plasma concentrations of a-1-acid glycoprotein, a marker of inflammation compared to OVX rats with supplementation (Puel et al., 2004). Antioxidant effects of olives have also been investigated in humans, with 98 healthy male volunteers ingesting 2 ml of olive mill wastewater (OMWW). After one hour had elapsed from the time the OMWW was ingested, there was a significant increase in total plasma glutathione concentration (both reduced and oxidised forms) whereas no difference was observed in plasma antioxidant capacity. The difference in plasma glutathione concentration has been attributed to the increase in phase II enzyme expression controlled by antioxidant response element (ARE). The enzymes with increased expression are likely to include γ-glutamylcysteine ligase and glutathione synthetase and provide an indirect antioxidant effect of polyphenols which is now considered a more likely mechanism of action (Visioli, Wolfram, Richard et al., 2009). There is yet to be any human study demonstrating the benefits of olive oil polyphenols on bone parameters (Sacco et al., 2013).

2.9.6.6 Reversatrol

Stilbenes are another subclass of polyphenols whose main dietary source is reversatrol found in red wine, grapes, herbs, berries, red cabbage and spinach (Crozier, Jaganath, & Clifford, 2009; Raj, Lieben Louis, Thandapilly et al., 2014). Reversatrol has powerful antioxidant and anti-inflammatory properties (Durbin, Jackson, Ryan et al., 2014). In-vitro studies (bone cell culture), with reversatrol demonstrated inhibition of RANKL induced NF-κB activation as well as activation of bone formation via transcription factors core binding factor-1(Cbfa-1) and sirtuin-1 (Sirt-1) (Shakibaei, Buhrmann, & Mobasheri, 2011). Supplementation in aged rats prevented demineralisation of bone and deterioration in micro-architecture (Durbin et al., 2014). Reversatrol has been studied for its anti-inflammatory and cardioprotective effect (Raj et al., 2014), and recently evidence of an effect on bone in human subjects has been demonstrated (Poulsen, Ornstrup, Harsløf et al., 2014). High dose supplementation of resveratrol (500mg) in 24 young, obese men assigned resveratrol or placebo demonstrated a significant increase in bone-specific alkaline phosphatase (BSAP) after four weeks. There were no changes in other markers of bone, inflammation or calcium metabolism and the change in BSAP on bone metabolism and in particular osteoblastogenesis, is still to be elucidated (Poulsen et al., 2014).

2.9.6.7 Drawbacks of in-vitro studies of phytochemical actions in-vivo

Animal studies are necessary to determine a broad range of factors associated with mechanisms of action of plant compounds on bone health, particularly bone micro-architecture and strength, however, there are several drawbacks of animal studies. One of the main criticisms of phytochemicals and animal studies is the high doses of bioactive compounds often given to the animals which cannot be feasibly replicated in humans due to differences in size, metabolism or toxicity levels. Cell culture studies, are also valuable in determining the beneficial effects of phytochemicals on target tissue such as bone but the compound used may not be representative of that which results in the plasma or target tissue (Visioli, Lastra, Andres-Lacueva et al., 2011). Aglyclones or high concentration extracts of the glycoside forms of polyphenols are often used rather than the conjugated derivatives (Trzeciakiewicz et al., 2009). The aglycone forms of polyphenols isolated from plants are metabolised by gut microflora and/or their enzymes (Williams et al., 2004), to their conjugated form in-vivo. Conjugated forms of polyphenols should therefore, be the preferred form in cell culture, however few studies have been able to determine biological activity of conjugated forms of polyphenols due to difficulty obtaining commercially available metabolites (Habauzit et al., 2008; Trzeciakiewicz et al., 2009). Further metabolism of flavonoids also occurs in the liver (Williams et al., 2004). This extensive metabolism is thought to affect redox potential as metabolised forms of polyphenols (e.g. glucuronides) will have less ability to be a hydrogen donor. Recent evidence suggests polyphenols have indirect interaction effects with intracellular signalling cascades (Williams et al., 2004) rather than direct antioxidant effects (Visioli et al., 2011). Another shortcoming of the studies is the concentration of the polyphenol used in the experiments which may be well in excess of what is biologically attained in human plasma after eating a polyphenol rich food (Habauzit et al., 2008).

In summary, phytochemicals affect biological processes via inflammation, oxidative stress and cell signalling pathways, though the direct antioxidant effects of polyphenols is now thought to be less likely (Visioli et al., 2011). Several plant compounds including quercetin/rutin and trans-reversatrol suppress the activation and up-regulation of redox sensitive transcription factors NF-κB and AP-1. NF-κB and AP-1 increase inflammation levels and bone loss therefore modulation of their activity by

plant phytochemicals is protective of bone loss. Another mechanism of action of phytochemicals on bone loss may be via phase II antioxidant enzymes and Nrf2. Polyphenolic compounds can activate transcription factor Nrf2 that binds to the antioxidant response element in the promoter region of genes encoding antioxidant enzymes (Crozier et al., 2009; Visioli et al., 2009). Several phenolic compounds affect Nrf2 including gallic acid, *p*-coumaric acid (tomato), curcumin, caffeic acid phenethyl ester (dried plum), lycopene (tomato) and reversatrol (grape) (Crozier et al., 2009; Veprik et al., 2011). The MAPK signalling pathway is activated by pro-inflammatory cytokines such as TNF and IL-1, 2 and 17. Catechin and quercetin modulate some of the signalling proteins in the MAPK signalling family (Crozier et al., 2009). Downstream this may result in less ROS formation due to increased antioxidant defence (Weaver et al., 2013). Figure 13 below summarises some of the current thinking on the direct and indirect actions of polyphenols on bone.

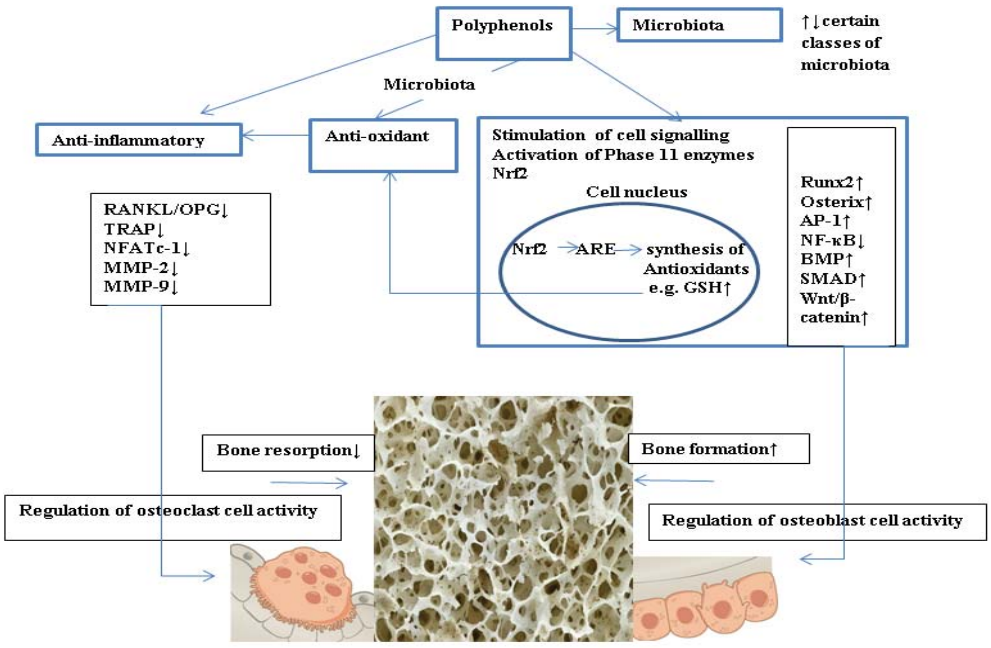

Figure 13 Direct and indirect action of dietary polyphenols on bone

Action of known dietary fruit and vegetable polyphenols on bone turnover. Effects include cell signalling, antioxidants and inflammation. RANK Receptor Activator of Nuclear Factor kappa B Ligand, OPG Osteoprotegerin, TRAP Tartrate Resistant Phosphatase,NFATc-1 Nuclear Factor of Activated T-cells, MMP Matrix Metalloproteinase,Nrf2 Nuclear Factor-Erythroid 2 Related Factor-2,ARE Antioxidant Response Elements, GSH Reduced Glutathione,Runx2 Runt Related Transcription Factor,AP-1 Activator Protein-1, NFκB Nuclear Factor kappa B, BMP Bone morphogenetic protein SMAD Wntβ-catenin Modified from Shen, 2012, Trzeciakiewicz, 2009, and Visoli 2014

2.9.7 Known phytochemicals in intervention study diets

The amount of phytochemicals consumed in a typical daily diet has been estimated at around 1.5 grams/day (Habauzit et al., 2008). The two intervention study diets to be compared were both high in fruit and vegetables with 9 servings/day, including 6 servings of vegetables and 3 servings/day of fruit. Daily consumption of herbs (usual culinary quantities) was also part of the diet. Group recommendations are included in the table below and although some choice in the vegetable/fruit options was allowed, a representative sample diet has been used for gross estimation of phytochemical variety in the diet (refer Tables 2&3) and allows for one serving of each vegetable/herb. The total number of phytochemical groups present in the list of vegetables (12 vegetables and additional herbs) was higher in Group B (97) compared to Group A (77). Key differences in groups of phytochemicals include Group A was higher in number of carotenoids present in the list of vegetables/herbs (25) compared to Group B (20). Group A had one less vegetable with beta-carotene but Group A's vegetables would contain a much greater concentration of beta-carotene than Group B's because Group A had vegetables with particular high levels (e.g. carrots and pumpkin) of beta-carotene, however only Group B contained lycopene. A higher number of terpenoids were present in Group A (11) compared to Group B (8) however flavonoids, sulphur containing compounds and "other phytochemicals" (non-phenolic) were all higher in Group B (20, 9, 2) compared to Group A (16, 3, 19) respectively.

The group B diet was emphasising the range of fruit and vegetables/herbs with proven anti- resorption inhibiting properties demonstrated in animals (Mühlbauer et al., 2002). Many of this range contain phytochemicals which have not as yet been identified as the active principle. The fruit eaten daily included oranges and prunes (suggested one serving of each or more if desired but not greater than 3 servings/day in total for fruit). The known phytochemicals associated with prunes (*Prunus domestica* L.) include several flavonoids and coumarins. Estimates of 184 mg/100 grams of total phenolics for this dried fruit has been reported in the literature (Habauzit et al., 2008). Assuming a minimum number of servings of one/day, and a serving size of approximately 50 grams/day of dried prunes, an intake of ~ 90 mg of total phenolic phytochemicals from this fruit alone is probable. The most

common phenolic compounds will be chlorogenic acid isomers such as neochlorogenic acid, cryptochlorogenic acid and chlorogenic acid (Habauzit et al., 2008). The other important phytochemical available in Group B includes the flavanone glycoside hesperidin from oranges. Hesperidin is broken down by the microbiota in the large intestine to release the aglycone hesperitin which is then able to be absorbed through the walls of the colon. Flavanone intake values of hesperitin have ranged from population levels of 15 mg/day (Knekt, Kumpulainen, Jarvinen et al., 2002) up to 28 mg/day, but importantly, to have a physiological impact the intake should be sustained (Manach, Morand, Gil-Izquierdo et al., 2003). Assuming one orange per day (rather than juice) an intake of 15 mg/day of hesperitin is possible. The two fruits recommended in Group A included bananas and apples. While bananas do not appear to contain many readily identified phytochemicals (most are non-extractable), apples do contain flavonols. Estimates of flavonol concentration in fresh produce are generally low (~15–30 mg/kg fresh weight) therefore an apple weighing ~200 grams would contain 4-8 mg of flavonols such as quercetin, kaempferol and myricetin if the skin is consumed (Manach, Scalbert, Morand et al., 2004). A study of the protective effects of apple on bone metabolism found that a flavonoid exclusively found in apple, phloridzin (Phlo), prevented ovariectomy-induced osteopenia in ovariectomised (OVX) or sham-operated rats with inflammation by attenuating inflammatory status demonstrated by reduction in inflammation and bone resorption markers (Puel, Quintin, Mathey et al., 2005).

Vegetables listed in Group B which contain flavonols are onion, broccoli, beans (green) and tomato. Onion is considered a particularly rich source of the flavonol quercetin with an average serving (50-80 grams) supplying 35-120 mg of this flavonol, while broccoli supplies 8-20 mg, beans 8-10 mg and tomato 0.4–3.0 mg in an average serving (50-80 grams). Tomato also contains lycopene which is a carotenoid with powerful antioxidant properties which may prevent bone loss in post-menopausal women via reduction in bone resorption markers (L. Rao et al., 2007; Mackinnon, Rao, et al., 2011). A variety of common herbs has also been investigated for their effects on bone formation and resorption. Parsley, sage, rosemary and thyme have demonstrated antiresorptive properties (Mühlbauer et al., 2002). Parsley contains apigenin and luteolin in quantities ranging from 1.2-9.2 mg

in a single culinary servings of 5 grams (Manach et al., 2004). A similar culinary and medicinal herb *Rosemary officinalis* L., is also known for its food flavouring as well as anti-inflammatory properties. The anti-inflammatory constituents in Rosemary herb essential oil have been identified as monoterpenes (Takaki, Bersani-Amado, Vendruscolo et al., 2008) with two terpene constituents: myrcene (24.6%) and 1,8-cineol (19.8%) attributed with anti-inflammatory effects due to inhibition of prostaglandin synthesis or other endogenous mediators (Takaki et al., 2008). Sage oil has also demonstrated some antiresorptive properties in the rat model (Mühlbauer, Lozano, Palacio et al., 2003).

Total carotenoids in intervention Group A were higher than in Group B due to their higher concentration in some of the vegetables Group A emphasised. A positive association between carotenoid intake and bone mineral density was demonstrated in several studies of post-menopausal women (Wattanapenpaiboon, Lukito, Wahlqvist et al., 2003; Sahni, Hannan, Blumberg et al., 2009), while a Japanese study of post-menopausal women, demonstrated the carotenoid β-cryptoxanthin, was significantly associated with radial BMD (Sugiura et al., 2008). Another carotenoid studied for its bone protective effect is lycopene. Lycopene has considerable antioxidant potential but also is a powerful singlet oxygen quencher (A. Rao & Rao, 2007; Mackinnon, Rao, et al., 2011). While there would likely be some intake of the carotenoid lycopene from other fruits and vegetables in Group A, only Group B included tomatoes which are estimated to contribute over 80 % of lycopene in the diet (Shen, von Bergen, Chyu et al., 2013). Cruciferous vegetables were eaten by intervention groups in the form of cabbage (Group A) and broccoli/ bok choy (Group B). There was a considerably greater intake of sulphur compounds in Group B (9) compared to Group A (3), most likely attributable to broccoli and onions eaten in this group. Broccoli contains high concentrations of one type of glucosinolate (glucoraphanin), which breaks down to an isothiocyanate (sulphurofane) and indole-3 carbinol which are thought to be the major phytochemical constituents protective for cancer (Brauer, Libby, Mitchell et al., 2011). It is unclear whether the glucosinolate is the active principle in the cruciferous vegetable broccoli which reduced bone resorption markers in animals (Mühlbauer, Lozano, Reinli, et al., 2003). One known mechanism of action of isothiocyanates and indole-3

carbinol involves increased oxidative defence by inducing the enzymes quinine reductase and glutathione S-transferase (Brauer et al., 2011; Tomofuji, Ekuni, Azuma et al., 2012) however, there are other phenolics in broccoli which also may be involved in cellular antioxidant defence (Song, Derito, Liu et al., 2010), and therefore may also have contributed to the reduction in bone resorption markers in animal studies.

Mühlbauer and co-workers (2003) determined a minimum effective dosage of bone resorption inhibiting food (BRIFs) would be 6.2 grams of fresh fruit/vegetables per kilogram of body weight which corresponded with a rats food intake of BRIFs of 170 mg/per day. The recommended serving numbers of BRIFs was calculated for an average 65 kilo woman (65 X 6.2gms = ~ 400 grams)) of BRIFs. Assuming a serving size of 80 grams, five servings/day of BRIF fruit/vegetables was estimated to be required to demonstrate an effect on bone resorption markers. Group B had 4-6 servings/day of BRIF specified which was composed of 3-4 servings of BRIF vegetables and 1-2 servings of BRIF fruit. Herbs were considered additional to all vegetable servings for both groups for the following reason. Herb intakes would be in very small quantities, therefore could not replace vegetable servings as this may have influenced nutrient intakes, PRAL and therefore possibly calcium excretion also.

In conclusion, a sample of 12 common vegetables plus herbs, consumed by the two intervention groups demonstrates there was a greater amount of phytochemicals in Group B (97) compared to Group A (77). There was also a higher intake of several key groups of phytochemicals associated with bone health in Group B compared to Group A. Groups of phytochemicals that were higher in Group B included flavonoids, sulphur compounds and other non-phenolic phytochemicals. While carotenoids were higher in Group A only Group B contained the carotenoid lycopene.

Table 2 Intervention Groups diet

Group A fruit/vegetable/herb dietary recommendations

	Herbs	Fruit			Vegetables Green leafy	At least 5 servings/day of any vegetable (non SF group). Examples of commonly consumed vegetables
	Basil, mint, oregano	**Apple**	**Banana**	**Other fruit (not citrus or prunes)**	**Spinach Silverbeet, White/green, Cabbage**	Carrot, Pumpkin, Peas, Cauliflower, courgettes,
No. of servings	**1 culinary serve**	**1**	**1**	**1**	**1**	

Group B fruit/vegetable/herb dietary recommendations

	Herbs	Fruit			Vegetables Green leafy	Other vegetables (≥2-3 servings from this category) and ≤2 servings self-selected
	Parsley Sage, Rosemary Thyme Garlic	**Prunes**	**Oranges/ other citrus**	**Other fruit (not banana or apple)**	**Chinese cabbage, e.g. Bok Choy, Red cabbage, Lettuce, Rocket**	Onions, Broccoli, Tomatoes, Mushrooms, Cucumber, Leeks, Green beans
No. of servings	**1 culinary serve**	**1**	**1**	**1**	**1**	

Table 3 Phytochemicals present in intervention groups fruit

	Beta-carotene	Alpha-carotene	Lutein/zeaxanthin	Beta-cryptoxanthin	Lycopene	Anthocyanins	Flavonols	Flavones	Flavanones (hesperidin)	Flavan-3-ols	Isoflavonoids	Proanthocyanidins	Phenolic acids	Ellagic acid	Lignans	Resveratrol	Saponins	Lipoic acid	Chlorophyll	Phytosterols	Glutathione	Fructoologosaccharides /inulin	Glucosinolates	Limonoids
Group A																								
Apples	S	S				S	Y			Y		Y	Y											
Bananas	T											T								Y		Y		
Group B																								
Citrus fruits	Y		Y	Y	S				Y											Y	Y			Y
Plums	Y					Y	Y			Y		Y	Y											

Key: Y= Yes present, T= trace, S= small amount

Table 4 Phytonutrients present in Group A and B intervention vegetables/herbs

	Carotenoids					Other terpenoids					Flavonoids							Other Phenolics				Sulphur compounds		Alkaloids			Other							
Vegetable	Beta-carotene	Alpha-carotene	Lutein/zeaxanthin	Beta-cryptoxanthin	Lycopene	Saponins	Monoterpenes	Triterpenoids	Phytosterols	Sequiterpene lactones	Anthocyanins	Flavonols	Flavones	Flavanones	Flavan-3-ols	Isoflavones	Proanthocyanidins	Phenolic acids	Lignans	Coumarins	Coumestans	Glucosinolates/isothiocyanates	Allium Sulphur Comounds	Betalains	Glycoalkaloids	Capsaicinoids	Polyacetylenes	Lipoic acid	Chlorophyll	Glutathione	Glucaric acid	Fructans (FOS, inulin)	Capsaicin	Capsiate	
Group A																																		
Mint	0	0	0	0	0	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	
Oregano	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	
Basil	1	0	1	1	0	0	1	0	0	0	0	1	1	0	0	0	1	1	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	
Cabbage, white/green	1	0	1	0	0	0	0	0	0	0	0	1	1	0	0	0	0	1	1	0	0	1	0	0	0	0	0	0	0	0	1	1	0	
Carrots	1	1	1	0	0	0	0	0	0	0	0	1	1	0	0	0	0	1	1	1	0	0	0	0	0	0	1	1	0	1	0	0	0	
Cauliflower	0	0	0	0	0	0	0	0	1	0	0	1	1	0	0	0	0	1	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	
Parsnips	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	1	0	0	0	0	0	0	1	0	0	0	1	0	0	
Peas, green	1	0	1	0	0	1	0	0	1	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	1	1	0	1	1	0	
Pumpkin	1	1	0	1	0	0	0	1	0	0	0	1	1	0	0	0	0	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	
Silverbeet	1	1	1	0	0	0	0	0	0	0	0	1	0	0	1	0	0	0	0	0	0	0	0	1	0	0	0	0	1	0	0	0	0	
Spinach	1	0	1	0	0	1	0	0	0	1	0	1	1	0	0	0	0	1	0	0	1	0	0	0	0	0	0	1	1	1	0	0	0	
Sweetcorn	0	0	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	
Sweet potato	1	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	
Watercress	1	0	1	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	1	0	0	0	0	
Zucchini, green	1	0	1	0	0	0	0	1	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	
Total	**10**	**3**	**9**	**3**	**0**	**3**	**3**	**2**	**2**	**1**	**0**	**10**	**5**	**0**	**0**	**0**	**1**	**6**	**3**	**2**	**2**	**3**	**0**	**1**	**0**	**0**	**2**	**3**	**6**	**2**	**2**	**4**	**0**	**0**	**77**
Group B																																		
Beans, green	1	1	1	0	0	1	0	0	0	0	0	1	0	0	0	1	0	0	1	0	0	0	0	0	0	0	0	0	1	0	1	1	0	0	
Bok Choy	1	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	1	0	0	0	1	0	0	0	0	0	0	1	0	0	0	0	
Broccoli	1	0	1	0	0	0	0	0	1	0	0	1	0	0	0	0	0	1	1	0	0	1	0	0	0	0	0	1	1	1	1	0	0	
Cabbage, Chinese	1	0	0	0	0	0	0	0	0	0	0	1	1	0	0	0	0	1	1	0	0	1	0	0	0	0	0	0	1	0	1	1	0	
Cabbage red	1	0	1	0	0	0	0	0	0	0	1	1	1	0	0	0	0	1	1	0	0	1	0	0	0	0	0	0	0	0	1	1	0	
Cucumber, common	1	0	0	0	0	0	0	1	0	0	0	1	0	0	0	0	0	1	1	0	0	0	0	0	0	0	0	0	1	0	0	0	0	
Garlic	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	1	0	1	0	
Leeks	1	0	1	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	1	0	0	1	0	
Lettuce, iceberg	1	0	1	0	0	0	0	0	1	1	0	1	1	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	1	0	1	0	0	
Mushrooms, white button	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	
Onions	0	0	0	0	0	0	0	0	0	0	0	1	1	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	1	0	
Parsley, continental	1	0	1	0	0	0	1	0	0	0	0	1	1	0	0	0	0	0	0	1	0	0	0	0	0	0	1	0	1	0	0	0	0	
Sage	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	
Rosemary	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	
Rocket	1	0	1	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	1	0	0	0	0	
Spring Onions	1	0	1	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	1	0	1	0	0	
Tomatoes, standard red	1	1	1	0	1	1	0	0	0	0	0	1	0	1	0	0	0	1	0	0	0	0	0	0	1	0	0	0	0	1	1	0	0	
Total	**11**	**1**	**8**	**0**	**1**	**1**	**3**	**1**	**2**	**1**	**1**	**13**	**5**	**1**	**0**	**0**	**0**	**8**	**4**	**1**	**0**	**5**	**4**	**0**	**1**	**0**	**1**	**1**	**8**	**4**	**5**	**6**	**0**	**0**	**97**

0 Absent
1 Present

2.10 Recommendations for fruit and vegetable intake

Increased consumption of fruit and vegetables has been linked with multiple health benefits for an ageing population including reduced bone loss and fracture prevention (New et al., 2000; Ashwell et al., 2008), preservation of lean muscle tissue (Dawson-Hughes, Harris, & Ceglia, 2008), reduction in cardiovascular disease (He et al., 2006), improvement in cognitive decline (Shukitt-Hale, Lau, & Joseph, 2008; Son et al., 2008) and decreased cancer incidence (Kudlich, Gostner, Holub et al., 2007). The epidemiological evidence for increased consumption of fruit and vegetables to reduce disease is very substantial. This has led to policies encouraging increased consumption of fruit and vegetables.

Many national and international organizations conclude increased consumption of fruit and vegetables is to be encouraged with recommendations ranging up to 10 servings/day (Lock et al., 2005; USDA, 2005; Tobias et al., 2006; National Heart Foundation NZ, 2009; Whitney et al., 2009) and public health goals of 600 grams/day (7.5 servings/day) as a population minimum target, not including starchy vegetables and fruit (World Cancer Research Fund / American Institute for Cancer Research, 2007).

The New Zealand Ministry of Health's current guidelines for fruit and vegetables are one of the lowest in the western world as its recommendation "To eat at least 5 servings a day of fruit and vegetables (three servings of vegetables and two servings of fruits) is inclusive of starchy vegetables (Ministry of Health (NZ), 2003, 2008; University of Otago et al., 2011). The UK fruit and vegetable recommendations don't include starchy vegetables and fruit in their recommended 5 servings/day (Bond, 2013) and Australia's recommendations, which are for a minimum of 5 vegetables with fruit additional (Australian Government et al., 2013). Therefore, by including starchy vegetables such as potatoes, New Zealand's fruit and vegetable recommendations for number of servings/day are comparatively lesser than other western countries.

2.11 Conclusions

Bone loss during ageing is a result of many factors. Studies investigating dietary factors to enhance bone health have centred on adequate micronutrient and protein intake, anti-inflammatory phytochemicals and reducing the chronic metabolic acidosis associated with ageing and the western diet, with the latter still controversial (Ashwell et al., 2008; Fenton et al., 2008; Pizzorno et al., 2010; Moseley et al., 2013). How fruit and vegetables protect bone health is still not completely understood; however evidence across different age groups and genders, demonstrate greater consumption is linked to higher bone mineral density (Ashwell et al., 2008) and this may be because of the non-nutrient factors such as specific phytochemicals or the contribution to acid-base balance during ageing.

Lack of intervention studies that aim to significantly reduce dietary net endogenous acid production via increases in fruit and vegetables have hindered understanding how reductions in dietary acid load by increased alkaline provision from the metabolism of fruit and vegetables may affect bone loss in middle aged women. However, reducing renal acid load and subsequent calcium excretion through increased provision of buffer precursors, whether from potassium citrate supplementation of fruit and vegetables, remains a compelling part of the equation to ameliorate midlife bone loss (Pizzorno et al., 2010; Adeva et al., 2011; Shi et al., 2012; Moseley et al., 2013). Differing phytochemicals from common vegetables and fruit affect bone loss in midlife via direct effects on inflammation and cell signalling pathways and indirectly via antioxidant effects (Visioli et al., 2011). Plant phytochemicals suppress the activation and up-regulation of redox sensitive transcription factors NF-κB and AP-1 therefore modulating inflammation and bone loss. Some phytochemicals modulate the signalling proteins in the MAPK signalling family which may result in less ROS formation due to increased antioxidant defence (Weaver et al., 2013). Phytochemicals can also activate transcription factor Nrf2 that binds to the antioxidant response element in the promoter region of genes thereby indirectly affecting antioxidant enzyme levels. A cumulative effect of all these mechanisms may account for a reduction in inflammation and resulting inflammatory bone loss. Understanding how

phytochemicals influence these mechanisms as well as how a reduction in dietary renal acid load may reduce bone loss at midlife may help explain some of the beneficial effects on bone health seen in populations with increased consumption of fruits and vegetables. The findings may also provide impetus for dietary change that will be beneficial for maintenance of bone mass during the critical midlife period.

CHAPTER 3. INCREASING FRUIT AND VEGETABLES IN MIDLIFE WOMEN: A FEASIBILITY STUDY

In order to determine whether an increased intake of vegetables/herbs and fruit to nine servings/day is feasible in a group of community dwelling midlife women we designed the following feasibility study. The study would assess the women's ability to comply with the dietary increase, strategies the women participants employed to achieve the dietary change and also whether this strategy may increase urine pH by 0.5 pH units, a level determined to result in reduction in markers of bone of resorption. Urine pH was self-monitored by participants via daily urine pH dipstick testing. The morning's urine pH was then to be recorded in a diary, along with the daily intake of fruit and vegetables. An increase in vegetables (6 servings/day) was a specific strategy employed to maximise polyphenol intake and lower dietary acidity.

Data published in Nutrition Research (Refer to Appendix)

Gunn C.A., Weber J.L., Coad J, Kruger M.C. (2013). Increasing fruit and vegetables in midlife women: A feasibility study.

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3.1 Abstract

The positive link between bone health and fruit/vegetable consumption has been attributed to the lower renal acid load of a diet high in alkaline forming fruit/ vegetables. Other important dietary determinants of bone health include micronutrients and bioactives found in fruit/vegetables. We hypothesised that increased intake of fruit/vegetables to ≥9 servings/day would significantly lower net endogenous acid production (NEAP) (~20mEq/day) and increase urine pH (0.5 pH units). This 8 week feasibility study investigated if 21 midlife women (40-65 years), currently consuming ≤5 servings/day of fruit/vegetables could increase their intake to ≥9 servings/day to substantially lower net endogenous acid production (NEAP) and include specific vegetables daily. Three day diet diaries were completed at baseline and end of study and assessed for NEAP (estimated) and number of servings from all food groups. Urine pH dipsticks were provided for the participants to assess and record their fasting urine pH daily (second void). Seventy six percent of women increased their intake of fruit/vegetables to ≥9 servings of fruit/vegetables, on at least 5 days per week. There was a reduction in the number of bread/cereal servings. NEAP (estimated) was reduced significantly with a mean urine pH increase of 0.68 pH units (95% CI 0.46-1.14), however, daily urine pH measures showed high variability. This study demonstrated a group of midlife women can change their diet over 8 weeks by significantly increasing fruit/vegetable servings and include specific "bone friendly" vegetables daily resulting in a significant decrease in estimated dietary NEAP and an increase in urine pH.

3.2 Introduction

Bone health and fruit and vegetable intake are positively linked with population and intervention studies, highlighting superior bone status in those groups consuming higher amounts of fruit and vegetables (Tucker et al., 1999; New et al., 2000; New et al., 2004; Lanham-New, 2006; Prynne et al., 2006; Welch, Bingham, Reeve et al., 2007; Gannon et al., 2008; Busque et al., 2009; Wynn, Krieg, Lanham-New et al., 2009). While diet is commonly known to exert a positive influence on bone through the provision of protein, vitamins and minerals such as calcium, magnesium and potassium (New et al., 2000; Heaney, 2009; Kerstetter, 2009), other important determinants may include phytochemicals and bioactive compounds found in a range of fruit and vegetables (Mühlbauer et al., 1999; Lister et al., 2007; Trzeciakiewicz et al., 2009), and the lower renal acid load from a diet containing large amounts of fruit and vegetables.

Increasing fruit and vegetable consumption to reduce the risk of chronic disease associated with ageing (Lock et al., 2005; Tobias et al., 2006), including osteoporosis (New et al., 2000; Lanham-New, 2006; Berkemeyer et al., 2008), is a recurrent theme in the literature. National and international health organization recommendations of 5-10 servings/day (Lock et al., 2005; Tobias et al., 2006; National Heart Foundation NZ, 2009; Whitney et al., 2009; USDA et al., 2010) with proposed public health goals of 600 grams (7.5 servings) as a population minimum target, not including starchy vegetables and fruit (World Cancer Research Fund / American Institute for Cancer Research, 2007).

Only two thirds of the New Zealand population reach the Ministry of Health daily recommendations of at least 2 servings of fruit and 3 servings of vegetables/day and this recommendation is inclusive of starchy fruit and vegetables (Ministry of Health (NZ), 2003, 2008; University of Otago et al., 2011).

The specific dietary factors associated with fruit and vegetables, which are thought to have beneficial influences on bone include: high mineral content (potassium, magnesium and calcium) contributing to lower net endogenous acid production (NEAP), vitamin K and bioactive compounds.

Alkaline buffer precursors are only supplied in fruit and vegetables and this forms one of the tenets of the link between high fruit and vegetable diets, lower NEAP and higher bone mineral density (BMD) (Macdonald, New, Fraser et al., 2005; Lanham-New, 2008). Whole food sources of vitamin K (green leafy vegetables) may be important for ageing populations (Yan, Zhou, Greenberg et al., 2004; Booth, 2007), as low vitamin K intake is associated with increased risk of hip fracture and reduced bone mineral density (BMD) in women (Szulc, Chapuy, Meunier et al., 1996; Booth, Tucker, Chen et al., 2000; Booth, Broe, Gagnon et al., 2003; Cockayne, Adamson, Lanham-New et al., 2006; Cheung, Tile, Lee et al., 2008).

A recent study by Bullo and colleagues (2011), demonstrated superior bone attributes (reduced porosity/increased elasticity) in elderly men and women with increased intake of phylloquinone (Vitamin K1) mainly sourced from green leafy vegetables (85%) in their diet. This offers a possible explanation for lower fracture rates seen with increased vitamin K intake in previous studies (Booth et al., 2000; Cockayne et al., 2006). Bone turnover has also been shown to be positively influenced by a range of bioactive compounds present in various whole vegetables and fruit, in particular allium, cruciferous and green leafy vegetables (Mühlbauer et al., 1999; Arjmandi et al., 2002; Franklin et al., 2006; Huang, Muhlbauer, Tang et al., 2008; Trzeciakiewicz et al., 2009; Bullo et al., 2011).

Despite recommendations in the literature (Ashwell et al., 2008; Berkemeyer et al., 2008), no intervention study has specifically recommended increased vegetable consumption (≥ 6 servings/day) or specified daily inclusion of green leafy, cruciferous and allium vegetables (Chapman et al., 2012).

A few studies have used a whole food approach to increase fruit and vegetable intake to reduce dietary acid load (Macdonald et al., 2008; Nowson, Wattanapenpaiboon, & Pachett, 2009), but not emphasised significant increases in vegetable intake over fruit, or prescribed the range of selected vegetables associated with bone health.

While NEAP has been reduced (7.1mEq/day) using "a low sodium base producing diet" (Nowson, Wattanapenpaiboon, et al., 2009) and mineral water consumption shown to increase urine pH and positively affect bone markers (Maurer, Riesen, Muser et al., 2003; Wynn, Krieg, Lanham-New, et al., 2009), it is unknown if a significant reduction in NEAP (~20mEq/day) (Gannon et al., 2008), achieved by increasing fruit and vegetables alone can affect urine pH and if so, whether this positively impacts bone health.

Increasing fruit and vegetable intake to the level expected to be required (9 servings/day) to appreciably affect NEAP requires a significant dietary change (Dawson-Hughes et al., 2009) which can be difficult to achieve in a free living population (Mhurchu, Margetts, & Speller, 1998; Steptoe, Perkins-Porras, McKay et al., 2003; Resnicow, Davis, Zhang et al., 2008).

Additionally, it is possible that a substantial increase in vegetable intake may displace other foods from the diet, resulting in a nutrient imbalance. However increasingly, evidence is being provided that the nutrients supplied in vegetables and some fruit may contribute to bone health in synergy, and in yet to be elucidated ways, therefore using a whole food approach provides a better matrix than supplementation with individual nutrients (Mühlbauer et al., 1999; Lister et al., 2007).

As the number of New Zealanders over 50 years of age increases steadily, the cost of treating fractures and secondary illnesses related to osteoporosis is expected to rise rapidly (P. Brown et al., 2011). Determining if dietary changes can forestall bone loss and prevent fragility

fractures becomes imperative as costs to the public health system increase with an ageing population.

This feasibility study used a human model comprised of 21 midlife women (40-65 years) who represented a group at increased risk of bone loss, to investigate the following objectives: whether an increase in fruit and vegetable intake to ≥9 servings/day (including daily consumption of allium, cruciferous and green leafy vegetables) is achievable, how this dietary change affects estimated dietary NEAP, overall nutrient intake and intake from other food groups, as well as urine pH.

This last objective was based on the strong relationship purported to exist between diet and urine pH (Remer, 2000; Michaud et al., 2003; Welch et al., 2008) and a previous study which demonstrated an increase of 0.5 units in urinary pH achieved with consumption of alkaline mineral water, reduced levels of bone resorption marker serum C-terminal telopeptide of type 1 collagen (CTX) (Wynn, Krieg, Aeschlimann, et al., 2009).

We hypothesised that an increased intake of fruit and vegetables to ≥9 servings/day would lower NEAP significantly by approximately 20mEq/day and increase urine pH by approximately 0.5 pH units. The results of this feasibility study would provide the rationale for further investigation of the effects of ≥9 servings/day fruit and vegetables (including specific vegetables) on bone and inflammatory markers in a randomised controlled trial.

3.3 Methods and materials

3.3.1 Recruitment

This feasibility study was approved as "low risk" by the Massey University Human Ethics Committee with all subjects giving written informed consent prior to commencing. The women were recruited by fliers at Massey University (refer Appendix A) and word-of-mouth in the community. Study inclusion criteria were: female, age (40 to 65 years), healthy (no current significant health issue) and not currently meeting the New Zealand guidelines of consumption of at least five servings/day of fruit and vegetables (two fruit and three vegetables). Women were still included if they were taking some medications e.g. antihypertensive and thyroid medication.

3.3.2 Screening and introductory session

There were 33 inquiries about the study with 28 women undergoing an initial screening interview to confirm that inclusion criteria were met. Participants were asked about their health status, age and fruit and vegetable intake over the last 3 days (average < 5 servings/day) with five women excluded due to high fruit and vegetable intakes (≥5 servings/day) and one excluded on health grounds. The study requirements were explained, including how this was a feasibility study (rather than a randomised controlled trial) to determine if this dietary strategy was achievable in midlife women.

Following the interview, a group introductory session outlined the study dietary rationale and addressed practical considerations with a food demonstration with plates, cups and common serving utensils to give participants the opportunity to visually inspect, weigh and handle single serving measures of common vegetables and fruit in both raw and cooked forms. Participants were also shown how to read a urine dipstick (Phion Diagnostic Test Strips, Apex Wellness Group, UC, Scottsdale, AZ85260, USA) with 0.25 pH unit graduations. Participants dipped the dipsticks in different pH solutions and were monitored as to how they read the colours against

the chart. The diaries for recording daily urine pH and fruit and vegetables intake were distributed with detailed instructions on how to complete.

To aid compliance, the daily diaries required the participants to record not only their urine pH (fasted, second void) but also total numbers of servings/day separately of fruit and vegetables and servings from the allium, cruciferous or other green leafy vegetable families (refer Appendix A).

3.3.3 Dietary strategy

The women were asked to increase their intake of fruit and vegetables to 9 or more servings per day. A maximum of 3 servings of fruit was allowed, however both the number (≥6 servings/day) and type of vegetables (≥4 servings from allium, cruciferous and other green leafy vegetable families) was prescribed. These instructions were given both verbally and in written format in the daily diary along with tips for achieving an intake of this amount (refer Appendix A).

Nine servings were chosen being the number of servings recommended by the USDA for healthy, fit midlife women (USDA, 2005), and also the number recommended in the DASH Trial (P. Lin et al., 2003) and by the New Zealand (NZ) National Heart Foundation (National Heart Foundation NZ, 2009). One serving of vegetables was defined as 0.5 cup of cooked vegetables (50-80 grams) or 1 cup raw leafy salad greens or 1 medium potato or other root vegetable (135 grams); fruit servings were 0.5 cup cooked or 1 medium size raw fruit (Ministry of Health (NZ), 2003).

The dietary change was staged with 2 “ramp-up” weeks involving 1-2 serving increases in fruit and vegetables per week to allow physiological adjustment to the increased dietary fibre intake (Frassetto, Schloetter, Mietus-Synder et al., 2009). The expectation, as explained during the group introductory session and also written in the diaries, was that by the end of the first 2 week period, participants were to be consuming ≥ 9 servings of fruit and vegetables per day. The 9

servings would include 4/6 servings/day from the prescribed vegetable families (one serving from both the allium and cruciferous family and two servings of other green leafy vegetables daily). The fruit servings were to be no more than 3 per day to avoid fruit intake increasing at the expense of vegetables.

No other specific directions for other food group intake (increase or decrease) were given and the women were instructed that weight loss was not a goal of the study. The women recorded their daily fruit and vegetable intake in the diary provided including the number of servings of vegetables they consumed each day from the following three groups: allium, cruciferous and green leafy families. Three Day Diet Diaries (3DDDs) were completed at baseline and at the end of the study (week 8). Participants received written (refer Appendix A) and verbal instruction at the introductory session and email reminders on how to complete the 3DDD's. The women were asked to record all food and beverages consumed over 2 week-days and 1 weekend day, including types, brands and amounts (cups, tablespoons, etc.) of foods, as well as recipes for homemade dishes. Participants 3DDD's were checked for accuracy and completeness by a NZ registered nutritionist.

The data were first imputed into Foodworks (Xyris, NZ, 2007) by a M.Sc (nutrition) graduate, checked by a N.Z. registered nutritionist and then transferred to SPSS (Version 15) via Microsoft Access (2007) and Excel (2007). New Zealand dietary reference values were used to assess all nutrient intakes (Ministry of Health (NZ), 2006). Estimated average requirement (EAR) was used to assess nutrient adequacy and Adequate Intake (AI) if no EAR value was available. Likewise, Suggested Dietary Target (SDT) was used instead of Recommended Daily Intake (RDI), if the RDI was unavailable (sodium, fibre and potassium) (Table 7).

3.3.4 Potential renal acid load (PRAL) and net endogenous acid production (NEAP)

To confirm that the prescribed dietary change was theoretically sufficient to produce the required change in NEAP, a sample diet (Ministry of Health (NZ), 2003)(Table 5) was used to calculate likely baseline intakes from food groups and probable PRAL and NEAP values. The sample diet was then varied to the proposed daily diet, including 9 servings of fruit and vegetables. Sample PRAL/ NEAP values were recalculated and used for comparison with participants' actual baseline and ending 3DDD food records. Net Endogenous Acid Production (NEAP) was calculated according to the algorithm from Remer and Manz (Remer et al., 1995; Remer, Dimitriou, & Manz, 2003; Frassetto, Lanham-New, et al., 2007).

Net Endogenous Acid Production (NEAP) was determined from 2 components,

- NEAP = PRAL + OA
- PRAL represents the average intestinal absorption rates of ingested protein and additional minerals. OA is an anthropometry-based estimate for organic acid excretion.

The two components of NEAP are given as follows:

- PRAL (mEq/d) =0.49 protein (g/d) + 0.037 x phosphorus (mg/d) - 0.021 x potassium (mg/d) – 0.026 x magnesium (mg/day) - 0.013 x calcium (mg/day)
- OAest (mEq/day) = individual body surface area x 41/1.73

Body surface area was calculated according to the method of du Bois and du Bois (Dubois et al., 1916) and is available on the website.

http:/www-users.med.cornell.edu/~spon/picu/calc/bsacalc.htm

- BSA = $(W^{0.425} \times H^{0.725}) \times 0.007184$

NEAP and PRAL are expressed in milliequivalents (mEq) as ions in solution interact according to their charge. Milliequivalents are obtained by converting milligrams (mg) to millimoles (mmol) and multiplying by valences to give charge quantities (Frassetto, Lanham-New, et al., 2007).

Table 5 Food Group servings in a reference New Zealand adult diet and recommendations for feasibility study

Servings	Reference diet[1]	Predicted Feasibility study diet
Vegetables [2]	**3**	**≥ 6**
Fruit [2]	**2**	**≤ 3**
Cereals and bread	**6**	**≤ 6**
Dairy	**2**	**2**
Protein [3]	**1**	**1**
PRAL (mEq) [1, 4]	**0.7**	**- 2.4**
NEAP (mEq) [1, 5]	**41**	**17**

[1] Sample diet and number of serving based on New Zealand Ministry of Health guidelines
[2] Vegetables: 1 serving = 0.5 cup cooked or 1 cup raw vegetables (salad greens) (50-80 g), or 1 medium starchy vegetable e.g. potato/kumara (~ 135 g). Fruit: 1 serving = 0.5 cooked (50-80 g) or 1 medium size fruit.
[3] Protein includes meat, fish, eggs, nuts, seeds and beans.
[4] Estimated potential renal acid load (PRAL) expressed in milliequivalents per day (mEq/d) = 0.49 protein (gms/day) + 0.037 phosphorus (mg/day) - 0.021potassium (mg/day) – 0.026 magnesium (mg/day) -0.013calcium (mg/day).
[5] Estimated net endogenous acid production (NEAP) expressed in milliequivalents per day (mEq/day) = PRAL (mEq/day) + organic acid excretion (mEq/day).

3.3.5 Anthropometric data

Anthropometric data (height and weight) was collected in the middle of the day (12-2pm), both at baseline and end of study. Body weight was measured to the nearest 0.5 kg (light clothing without shoes) using digital scales (UWE Gilbarco, NZ) and height measured without shoes to the nearest 0.5 cm using a stadiometer (SECA 213). BMI was calculated as weight (kg)/height (m^2) (Whitney et al., 2009).

3.3.6 Physical activity

Physical activity levels were determined by a physical activity questionnaire (NZ PAQ-SF) designed by the NZ Ministry of Health and SPARC. The questionnaire was used to assess frequency, duration and intensity of exercise and is available on the SPARC website http://www.sparc.org.nz/research-policy/research/nzspas-97-01/nzpaq.

3.3.7 Urine pH

Daily pH recordings of urine were taken by the women on their second voided urine using dipsticks. The dipsticks had been validated as within ± 0.2 pH units of the reading with a pH meter. Validation was achieved by asking 9 people not involved in the study, to read and compare the pH of 3 dipsticks briefly wetted in different solutions, against a colour chart on the dipstick container. The solution's pH was measured with a pH meter (ISFET/ IQ125 Mini Lab). An average difference of 0.2 pH units from the 27 readings was calculated compared to the reading from the pH meter. Each study participant recorded their urine pH reading in a daily diary after passing the dipstick through a midstream urine flow (fasted, second void) and matching the colour panel on the dipstick against the container colour chart. Urine pH tested in the morning will typically be the lowest of the day. After an overnight fast, the kidneys have made the necessary adjustments with excretion of the non-volatile (fixed) acids produced by metabolism of sulphur containing proteins consumed the day before (Tietz, 1970).

3.3.8 Monitoring

Participants' engagement with the requirements of the study was monitored via emails (weekly) and telephone calls (fortnightly). The telephone calls were to aid compliance by answering participants queries personally and promptly (participants could also email the study coordinator for immediate response). The group emails served as an avenue for sharing helpful tips and recipes from other study participants.

3.3.9 Statistical analyses

Data analysis was performed using SPSS (version 17) and R statistical package. To assess normality of data, a one sample Kolmogorov-Smirnov test was used, and as test distribution was normal, parametric tests were done. Within subjects student paired t- tests were performed to assess changes from baseline for all variables. Values are presented as means and standard errors (SEM). Change in urine pH was also assessed using repeated measures ANOVA and regression analysis. The 56 urine pH values for each participant were plotted using a Lowess

Smoother where each smoothed value is given by a weighted linear least squares regression over the span. The relationship between daily fruit and vegetable intake and the following day's urine pH was examined by regression analysis. This was done to determine if a relationship existed between daily serving numbers of fruit and vegetables and the following day's urine pH. Predictor variables used were the number of fruit servings, vegetable servings or combined fruit and vegetable servings. Regression analysis was performed on the group as a whole and on an individual basis (n = 21) due to urine pH, vegetable and fruit intake being highly variable between participants. Informal examination of the data with histograms and scatter plots was used to determine any threat to underlying assumptions of the residuals. (Note: when visual inspection of histogram was doubtful, residuals were checked with Kolmogorov-Smirnov and Shapiro-Wilk Test).

3.4 Results

Twenty-two women began the study with 1 woman withdrawing on the first day. The remaining 21 completed the 8 week study. Three quarters of the participants were 45-55 years of age (16/21); two were younger (40-45 years) and three older (60-65 years). Over half the women (11/21) were taking some form of medication. The women had an average BMI of 30 (±1.3), with no significant change over the course of the study. The women were advised to maintain their normal activity levels throughout the study; however, a few women increased activity levels, which they said was to aid weight loss. There was a small, non-significant decrease in mean weight (0.3kg ± 1.34) as well as a slightly decreased energy intake (590 kJ/day ± 523) with most participants' weight varying by 1-2 kilos over the 8 week intervention period.

3.4.1 Changes in number of servings from food groups

In line with the aims of the study there was a highly significant increase in dietary intake from vegetables and fruit food groups. Reported fruit intake increased from 1.3 servings/day in baseline 3DDD's to 2.7 servings/day (p <0.000) in the final 3DDD, and vegetable intake increased from 2.7 servings/day to 5.0 servings/day (p <0.000) (Table 6). Protein and dairy

servings remained unchanged but cereal/bread servings reduced significantly from 4.1 ± 0.3 to 2.7± 0.3 servings/day (p <0.002).

Compliance with both number and type of fruit and vegetables servings/day was assessed from daily fruit and vegetables and urine pH diaries the women kept throughout the 8 weeks. Most participants increased to 9 servings of fruit and vegetable within the first week however, compliance was measured after the two ramp-up weeks.

Seventy six percent (16/21) of the women showed written adherence to the aim of ≥ 6 servings of vegetables (including requisite 4/6 servings from specified families) and 3 servings of fruit on at least 5 days each week with compliance reducing on weekend days to 48%. Twenty four percent (5/21) participants either didn't achieve ≥9 servings on at least 5 days/week or didn't include the requisite 4/6 servings from specified vegetable families (allium, cruciferous and green leafy) on at least 5 days/week.

Table 6 Participants food group servings, estimated potential renal acid load and net endogenous acid production at baseline and end of study (eight weeks)

Dietary intake	Baseline	8 weeks	Change	P baseline vs end [3]
Servings/day [1,2]				
Vegetables [4]	**2.7 ± 0.3**	**5.0 ± 0.3**	**2.3 ± 0.4**	**<0.001**
Fruit [4]	**1.3 ± 0.2**	**2.7 ± 0.2**	**1.4 ± 0.3**	**<0.001**
Cereal/bread	**4.1 ± 0.4**	**2.7 ± 0.3**	**-1.5 ± 0.4**	**<0.002**
Protein foods	**2.2 ± 0.2**	**2.0 ± 0.2**	**-0.14 ± 0.3**	**0.63**
Dairy	**1.1 ± 0.2**	**1.0 ± 0.2**	**-0.2 ± 0.2**	**0.34**
PRAL(mEq) [5]	**5.1 ± 4**	**-16.5 ± 3**	**-21.6 ± 5**	**<0.001**
NEAP (mEq) [6]	**50.2 ± 5**	**28.5 ± 4**	**-21.7 ± 5**	**<0.001**
Urine pH [7]	**5.46 ± 0.16**	**6.14 ± 0.2**	**0.68 ± 0.16**	**<0.001**

[1] Serving numbers have been calculated from three day diet diaries completed at beginning and end of study.
[2] Values are means ± SEM (standard error of mean).
[3] P value for the difference between baseline and end of study were determined using paired student t tests.
[4] Vegetables: 1 serving = 0.5 cup cooked or 1 cup raw vegetables (salad greens) (50-80 g), or 1 medium starchy vegetable e.g. potato/kumara (~ 135 g). Fruit: 1 serving = 0.5 cooked (50-80 g) or 1 medium size fruit.
[5] Estimated potential renal acid load (PRAL) expressed in milliequivalents per day (mEq/d) = 0.49 protein (gms/day) + 0.037phosphorus (mg/day) - 0.021potassium (mg/day) – 0.026 magnesium (mg/day) -0.013calcium (mg/day).
[6] Estimated net endogenous acid production (NEAP) expressed in milliequivalents per day (mEq/day) = PRAL (mEq/day) + Organic acid excretion (mEq/day).
[7] Urine pH is self-reported, second void, fasted, mid-stream, using pH dipstick (Phion Diagnostic Test Strips, Apex Wellness Group, UC, Scottsdale, AZ85260, USA) at beginning and end of study.

3.4.2 Nutrient intake at baseline and end of study (week 8)

Table 7 shows participants' average daily intake of nutrients and how they compared to New Zealand dietary reference values (Ministry of Health (NZ), 2006). Significant increases were seen in baseline to end of study values for a range of nutrients. Dietary fibre increased from 20 ± 2 grams/day to 25 ± 1.5 grams/day ($p < 0.015$) (SDT 28 grams/day), Vitamin C increased from 220 ± 39 mg/day to 519 ± 105 mg/day ($p < 0.016$) (SDT 190 grams/day), Vitamin A increased from 2268 ± 329 µg/day to 3514 ± 366 µg /day ($p < 0.013$) (SDT 1220 µg/day), potassium increased from 3143 ± 172 mg/day to 3797 ± 168 mg/day ($p < 0.003$) (SDT 4700 µg /day) and magnesium increased from 296 ± 18 mg/day to 313 ± 18 mg/day ($p < 0.05$) (RDI 320 mg/day). A significant decrease was seen with sodium intake reducing from 2583 ± 475mg/day at baseline to 1817 ± 144 mg/day by end of study ($p < 0.015$) (SDT 1600 mg/day). There was a trend for an increase in folate from 739 ± 52 µg/day to 922 ± 101 µg /day ($p < 0.06$) (SDT 600 µg/day) and a trend for a decrease in fat intake from 63 ± 5 µg/day to 51 ± 4 µg /day ($p < 0.07$) and selenium intake from 131 ± 17 µg/day to 99 ± 14 µg /day ($p < 0.07$) (EAR 60 µg/day).

Table 7 Participants' nutrient intake at baseline and end of study (eight weeks)

	Baseline (n=21)	8 weeks (n=21)	Reference values		$P_{baseline\ vs\ end}$ [1]
Dietary Intake [2]			EAR[3]/AI[4]	RDI[5]/SDT[6]	
Energy (kilojoules/day)	**6934 ± 499**	**6345 ± 341**	**7900**[7]		**0.27**
Protein (g/d)	**80 ± 6**	**73 ± 5**	**37**[3]	**46**[5]	**0.29**
Fat (g/d)	**63 ± 5**	**51 ± 4**	**N/A**		**0.07↓**
Carbohydrate (g/d)	**200 ± 26**	**183 ± 10**	**N/A**		**0.53**
Fibre (g/d)	**20 ± 2**	**25 ± 2**	**25**[4]	**28**[6]	**0.02↑**
Energy from protein (%)	**20 ± 1**	**20 ± 1**	**15-25%**		**0.73**
Energy from Fat (%)	**32 ± 1**	**29 ± 1**	**20-35%**		**0.11**
Energy from carbohydrate (%)	**44 ± 2**	**49 ± 2**	**45-65%**		**0.02↑**
Vitamin C (mg/day)	**220 ± 39**	**519 ± 105**	**30**	**45**[5]**/190**[6]	**0.02↑**
Vitamin E (mg/day)	**31 ± 11**	**33 ± 9**	**N/A**	**7**[5]**/14**[6]	**0.90**
Vitamin A (ùg/day)	**2268 ± 329**	**3514 ± 366**	**500**	**700**[5]**/1220**[6]	**0.01↑**
Thiamine (mg/day)	**8 ± 2**	**7 ± 4**	**0.9**	**1.1**	**0.9**
Riboflavin (mg/day)	**12 ± 6**	**5 ± 0.3**	**0.9**	**1.1**	**0.2**
Niacin Equivalents (mg/day)	**42 ± 4**	**44 ± 5**	**1.1**	**14**	**0.76**
Vitamin B6 mg/day	**3 ± 2**	**4 ± 2**	**1.3**	**1.5**	**0.76**
Vitamin B12 (mg/day)	**19 ± 7**	**16 ± 6**	**2.0**	**2.4**	**0.71**
Folate (µg/day)	**739 ± 52**	**922 ± 101**	**320**[3]	**400**[5]**/600**[6]	**0.06↑**
Calcium (mg/day)	**680 ± 97**	**729 ± 66**	**1100**[3]	**1300**[5]	**0.47**
Magnesium (mg/day)	**296 ± 18**	**313 ± 18**	**265**[3]	**320**[5]	**0.05**
Potassium (mg/day)	**3143 ± 172**	**3797 ± 168**	**2800**[4]	**4700**[6]	**0.00**
Sodium (mg/day)	**2583.4 ± 475**	**1817 ± 144**		**1600**[6]	**0.02↓**
Zinc (mg/day)	**11 ± 0.8**	**10 ± 0.9**	**6.5**[3]	**8**[5]	**0.34**
Phosphorus (mg/day)	**1312.3 ± 103**	**1216 ± 73**	**580**[3]	**1000**[5]	**0.38**
Iron (mg/day)	**13 ± 1**	**13 ± 2**	**5**[3]	**8**[5]	**0.96**
Manganese (mg/day)	**3838 ± 462**	**3850 ± 326**	**5**[4]		**0.98**
Selenium (µg/day)	**131 ± 17**	**99 ± 14**	**50**[3]	**60**[5]	**0.07↓**

[1] P values for testing the difference between baseline and end of study were determined using paired student t tests.
[2] Values are means ± standard error of the mean (SEM) with percentages for energy contribution from fat, protein and carbohydrate.
[3] Values are New Zealand Ministry of Health nutrient reference values for estimated average requirements (EAR) except for those with superscript [4] being adequate intake (AI).
[5] Values are recommended daily intake (RDI) except for those with superscript [6] being suggested dietary target (SDT).
[7] Estimated energy requirements (EER) female 51-70 years with physical activity level (PAL 1.6) = light activity. Foodworks has not yet been updated to include Vitamin K values for foods.

3.4.3 Change in net endogenous acid production (NEAP)

There was a 44 % reduction in mean estimated net endogenous acid production (NEAP) from 50 ± 5 mEq/day at baseline to 28.5 ± 4 mEq/day by end of study (Figure 14) and PRAL declined similarly, -22 ± 5.0 mEq/day. Both results were highly significant (p <0.001). The effect size measured by *Cohen's d* was 0.93, a value corresponding to a large effect.

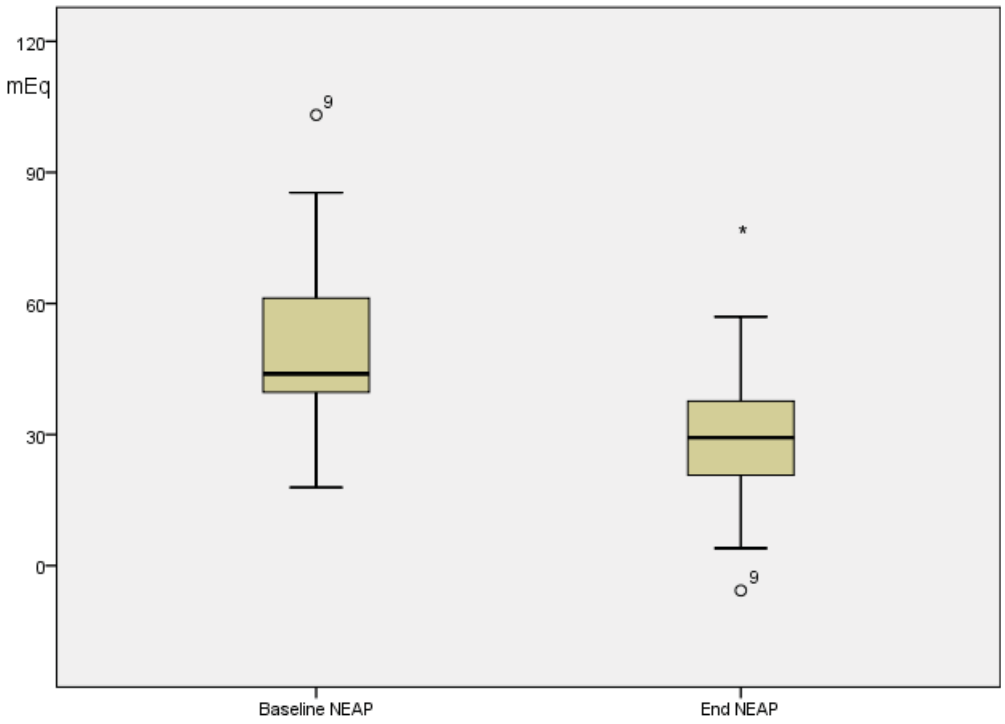

Figure 14 Dietary estimated net endogenous acid production (NEAP)

Dietary estimated net endogenous acid production (NEAP) in milliequivalents (mEq) based on 3 day diet diaries at baseline and end of study for participants (N=21) consuming a diet high in fruit and vegetables. NEAP is expressed in the stem and leaf plots as the median (44) and interquartile range (41-60) at baseline reducing to 29 (21-36) milliequivalents by end of study with one outlier (No.9). * Asterix change in NEAP from baseline to end of study P < 0.001.

3.4.4 Effect of change in diet on urine pH

Results of the repeated measures Anova performed on daily urine pH values showed an upward trend (refer Figure 15). The within subjects t-Test (Table 6) showed a pH increase of 0.68 ± 0.16 pH units from baseline (p<0.000) (95% CI 0.46-1.1).

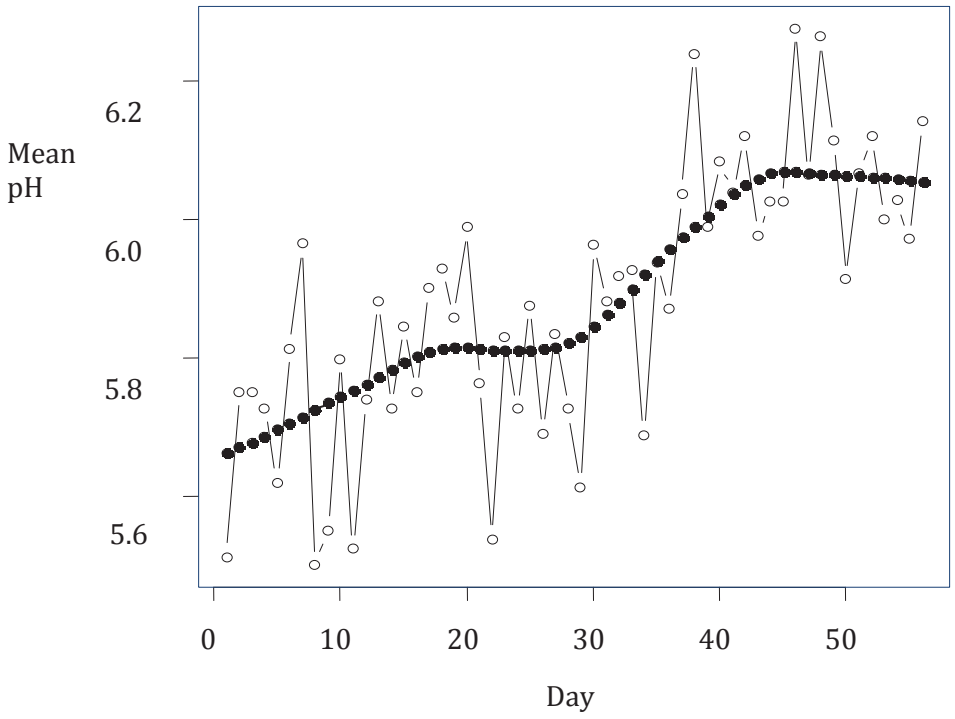

Figure 15 Urine pH changes

Readings were self-monitored every day by the participants (n=21) over 56 days during the feasibility study. A Lowess smoother demonstrates the upward trend of urine pH values. Urine pH was measured fasted, in the second void daily sample with a urine pH dipstick with 0.25 pH unit graduations (Phion Diagnostic Test Strips, Apex Wellness Group, UC, Scottsdale, AZ85260, USA).

3.4.5 Regression analysis

No strong linear relationship was found between the previous day's fruit or vegetable intake for the group as a whole or on an individual basis, however there were moderate significant linear relationships seen for combined vegetables and fruit intake with the following day's urine pH for 6/21 participants ($r = 0.36$, $p < 0.004$; $r = 0.34$, $p < 0.006$; $r = 0.27$, $p < 0.02$; $r = 0.27$, $p < 0.03$; $r = 0.25$, $p < 0.03$; $r = 0.25$, $p < 0.03$). These six participants had similar lifestyle profiles for nutrient intake, BMI (20-25), exercise levels (1 hour/day) and alcohol intake (<8 units/wk). For a sample of typical urine pH readings and the changes over the 8 weeks refer Appendix A.

3.5 Discussion

Recommendations to increase fruit and vegetable intake to reduce rates of chronic disease including osteoporosis have become more important with worldwide ageing populations coupled with a global economic downturn. Strategies for implementing increased intake from the fruit and vegetable food group successfully within smaller groups of people are imperative to determine how to achieve an increased intake in wider populations.

This study demonstrated that it is possible for a free-living population of midlife women to substantially increase their intake of fruit and vegetables on a daily basis. Although the increase to 9 servings per day was expected to pose considerable challenges for some participants the daily fruit and vegetable diaries showed 16/21 (76%) reported consuming ≥9 servings/day (3 fruit and 6 vegetable servings) as well as including at least 4 of the 6 vegetable servings from the allium, cruciferous or other green leafy vegetable families on at least 5 out of 7 days each week and 5/21 (24%) met the requirements less regularly. Weekends were a time when compliance with the study vegetable intake requirements reduced due to changes in routine.

Strategies used by some women who successfully increased their vegetable intake included taking vegetable salads or dinner vegetable leftovers for lunch, increasing intake at dinner or for snacks and adapting commonly used recipes to include requisite vegetable families. Another study with a similar aged population of midlife women demonstrated a small change in vegetable intake (39 grams/day), indicating when given a choice, many participants choose fruit over vegetables to increase intake from this food group (Macdonald et al., 2008). The increase in vegetables to a mean of 5 servings/day and the regular inclusion of specific vegetable families was noteworthy as participants had to source and purchase their own fruit and vegetable supplies.

The significant increase in fruit and vegetables was seen in conjunction with a reduction in servings of breads/cereals. This indicates fruit and vegetables may have replaced some servings

from breads/cereals food group, whereas the number of servings from other food groups (dairy and protein based foods) remained unchanged. Dietary fibre increased closer to the New Zealand (NZ) dietary recommended SDT of 28 grams/day of fibre to reduce risk of chronic disease (Ministry of Health (NZ), 2006). Significant micronutrient increases were seen with Vitamin C and A to above their SDT's, and while potassium intake significantly increased, it was still 900 mg/day below the SDT of 4700mg/day.

Significantly lower intake of sodium was noted and this reduction most likely reflected reduced intake of breads/cereals which are major contributors of sodium in the NZ diet (Ministry of Agriculture and Fisheries, 2009), although average intake was still in excess of the suggested dietary target (SDT) for sodium of 1600 mg/day. The change in the sodium: potassium ratio from ~2600:3100 mg/day to ~1800:3800 mg/day is considered favourable as it is closer to recommended intakes (Lanham-New, 2008) and differs considerably from reported sodium: potassium ratios in the United States, United Kingdom and Australia of ~4000:2500 mg/day (Lanham-New, 2008). Overall, changes made during the study reflect closer alignment with NZ dietary recommendations for micronutrient intakes.

This study aimed to significantly reduce the mean NEAP from an estimated figure derived from a standard Ministry of Health diet (Ministry of Health (NZ), 2003) of 41.4 mEq/day by approximately 20 mEq/day. An average reduction of -21.7 ± 5.0 mEq/day was achieved from a mean value of 50.2 ± 5 mEq/day at baseline to 28.5 ± 4 mEq/day by the end of study. This is a significant reduction ($p \leq 0.001$) and more than twice that achieved in a three month study of similar aged women (45-75 years) (Nowson, Patchett, et al., 2009), where the effects of a low sodium base producing diet and one with a higher acid load on bone markers were assessed. The lower acid/DASH type diet achieved an estimated NEAP reduction of -7.1mEq/day (Nowson, Patchett, et al., 2009).

Urine pH is an indirect measure of net acid excretion (Remer, 2000; Michaud et al., 2003; Welch et al., 2008) and was expected to show an increase over time due to the decreased dietary

NEAP (Remer et al., 1995). An important finding from this study was the significant increase achieved in urine pH. Some women reported daily use of the pH dipsticks to self-monitor their urine pH helped motivate them to increase fruit and vegetable intake. Some participants reported the pH dipstick provided personal daily feedback reflecting the dietary changes they made and this affected their food choices the following day as they wanted their urine pH to increase (Welch, 2008) while others disliked the urine pH monitoring. A significant increase in mean urine pH of 0.68 ± 0.16 pH units was achieved, however, there was a high variability in daily urine pH and this study shows a single measure may not be reflective of the acid load of an individual's diet due to the high daily variability (Fenton, Eliasziw, et al., 2009). Different types of food intake will cause urine pH to vary (Wachman et al., 1968; Michaud et al., 2003) and 71% of the variability in urine pH has been attributed to the amount of potassium and protein in the diet (Frassetto et al., 1998). Kidney function can also affect renal excretion of acid (Frassetto et al., 1996; Scialla & Anderson, 2013) but glomerular function was undetermined nor whether any participants had undiagnosed renal disorders which may have influenced renal clearance of acid. Also urinary tract infections can influence urine pH but none were reported. Besides diet and age-induced decline in renal function (Remer, 2000; Berkemeyer et al., 2008; Welch et al., 2008), urine pH can vary with hypertension, medication (Welch et al., 2008), exercise status (Michaud et al., 2003) and race (Michaud et al., 2003; Taylor et al., 2007). Participants were all non-smoking but some were on medications which may have influenced their urine pH e.g. blood pressure medications, and age, exercise levels and BMI varied. Women recorded in their diaries any significant change in exercise level, stress or medications (Remer, 2000; Michaud et al., 2003; Welch et al., 2008) and these environmental factors were reported to have remained stable over the 21 days for most participants. Representative samples of individual participant's urine pH charts demonstrating the daily changes over the 8 weeks are provided in Appendix A.

The increase in urine pH (5.46 ± 0.16 to 6.14 ± 0.2) is higher than that reported previously by increasing vegetables and fruit intake (Macdonald et al., 2008; Nowson, Patchett, et al., 2009),

and most likely is a reflection of the increased dietary intake of vegetables and their higher alkaline provision. This magnitude of change has been achieved however, with mineral water (Wynn, Krieg, Aeschlimann, et al., 2009), supplements (Maurer et al., 2003) or a combination of both mineral water and supplements (Buclin et al., 2001). This level of urine pH change has been associated with a decrease in bone resorption marker C-terminal telopeptide of type 1 collagen (CTX) (Wynn, Krieg, Aeschlimann, et al., 2009).

Despite compliance with dietary aims, a few participants' urine pH values showed little change over the 8 week trial period. A second void fasting spot urine was used rather than a 24 hour collection due to less burden of time on those involved. Previously, similar correlations between acid load determined from diet and urine pH were found, whether pH was measured from a 24 hour urine sample or a part day sample (Welch et al., 2008). Urine pH was self-monitored, so there may have been some errors in reporting urine pH. However with the daily readings, if the colour bars were misread and reported higher or lower than actual values, there would be consistency in this misreporting and as we were looking for change in pH this would have minimised reporting error.

The purpose of the regression analysis was to see if a model could be developed of fruit and vegetable daily intake and urine pH daily change, however, only a few participants' (6/21) previous day's intake of fruit and vegetables had a predictive effect on the following morning's urine pH. Other dietary factors, possibly the contribution to PRAL from meat and/or bread/cereals intake may have a more significant influence on urine pH, as could health (medications, infection) or environmental factors (stress, exercise) (Michaud et al., 2003; Taylor et al., 2007; Welch et al., 2008). Daily urine pH measurements allowed assessment of correlation with change in diet over 8 weeks rather than relying solely on paired *t* tests of urine pH values at baseline and end of study. Results from this study have shown the high daily variability in this measure.

This study trialed a different nutritional approach to maintenance of bone health at midlife by providing detailed directions to consume specific vegetables and minimum numbers of vegetable servings. It demonstrated that a daily increase in number and type of vegetables is feasible and achievable in a community setting and urine pH is increased, therefore we accept the research hypothesis that a substantial change in dietary NEAP was achieved by increasing fruit and vegetable intake to ≥9 servings/day and this dietary change positively influenced urine pH. The limitations with this study included its design being a pilot study rather than a randomised trial and therefore lacked a control group, and its reliance on personal report of dietary intake and serving size, as no urine or blood samples were taken to check fruit and vegetable biomarkers such as serum vitamin C, A, potassium or beta carotene. A weakness is the measurement of urine using pH dipsticks, which is known to be less reliable than laboratory based methods such as pH meters but afforded ease of use (Kwong, Robinson, Spencer et al., 2013). Another limitation of this feasibility study was the increase in women's urine pH over the 8 weeks of the study provided an incentive for some of them to maintain the dietary changes required, however several women were disappointed when their urine pH did not change over the 8 weeks of the study despite significant reported dietary changes. In consideration of this I decided not to advise the women in the next trial that their urine pH would likely increase, to avoid disappointment and undue concern there was something wrong with them if no change was observed. Several women reported that daily urine recordings were quite inconvenient (particularly when travelling) and time consuming. In light of this feedback, the reporting requirements of urine pH for the next trial were changed to any two days of the week at the women's convenience, thereby reducing the burden of reporting this study variable. There appears to be no disadvantage of using only 2 measurements per week over the study time period to assess changes in urine pH. Another limitation of this feasibility study was that some women expected a weight loss with the "healthier diet" of increased fruit and vegetables. The women were not advised that weight loss would occur but some expected it and on average half the participants lost 1-2 kilos and half gained 1-2 kilos. For the next trial I was more specific

with instructions that weight loss should not be expected nor did we want this to occur as it would affect bone markers.

This study demonstrated a group of midlife women can significantly increase intake of vegetable servings (including specific vegetables). This dietary change resulted in a reduction in the number of servings from the bread /cereal food group and sodium intake. The dietary changes had a large, reliable effect on estimated NEAP, as well as an increase in alkalinity of mean urine pH. Daily urine pH measurement was shown to be highly variable, thus a single measurement may not be a useful indicator of renal acid load.

3.6 Acknowledgments

This study was supported by a grant from the Institute of Food, Nutrition and Human Health, Massey University, NZ. The study participants are gratefully acknowledged.

CHAPTER 4. MIDLIFE WOMEN, BONE HEALTH, VEGETABLES, HERBS AND FRUIT STUDY. THE SCARBOROUGH FAIR STUDY PROTOCOL

This study protocol outlines the practical aspects of study 2, a human dietary intervention study which involved 143 post-menopausal women in three locations in the North Island of New Zealand: Hawke's Bay, Manawatu and Auckland. The protocol details specific aspects of the dietary regime and its implementation. This study protocol includes all the scientific methodologies for collection and laboratory analysis of plasma blood markers of bone, inflammation and metabolism and 24 hour urine samples for mineral urinary excretion. In addition, data collection methods are included for anthropometric measurements, dietary intake and DXA bone mineral density assessment.

Data published in BMC Public Health (Refer to Appendix)

Gunn, C., Weber, J., & Kruger, M. (2013). Midlife women, bone health, vegetables, herbs and fruit study. The Scarborough Fair Study protocol. BMC Public Health, 13(23).

4.1 Abstract

Bone loss is accelerated in middle aged women but increased fruit/vegetable intake positively affects bone health by provision of micronutrients essential for bone formation, buffer precursors which reduce acid load and phytochemicals affecting inflammation and oxidative stress. This study aimed to increase fruit/vegetable intake in post-menopausal women to 9 servings/day using a food specific approach to significantly reduce dietary acid load and include specific vegetables, fruit and herbs with bone resorbing inhibiting properties (animal studies) to assess effect on bone turnover, metabolic and inflammatory markers. The Scarborough Fair Study is a randomised active comparator, controlled, multi-centre trial. It aimed to increase fruit and vegetable intake in 100 post-menopausal women from ≤5 servings/day to ≥9 servings/day for 3 months. The women in the dietary intervention were randomly assigned to Group A or B. Both groups consumed ≥9 servings/day of fruit/vegetables and selected herbs but the diet of each group emphasised different fruit/vegetables/herbs with one group (B) selecting from a range of vegetables, fruit and culinary herbs with bone resorbing inhibiting properties. 43 women formed a third group, who were to be the negative control group and consumed their usual diet (Group C). Primary outcome variables were plasma bone markers assessed at baseline, 6 weeks and 12 weeks. Secondary outcome variables were plasma inflammation and metabolic markers and urinary electrolytes (calcium, magnesium, potassium and sodium) assessed at baseline and 12 weeks. Dietary intake and urine pH change were also outcome variables. The dietary change was calculated with 3 day diet diaries and a 24 hour diet recall. Intervention participants kept a twice weekly record of fruit, vegetable and herb intake and urine pH. This study will provide information on midlife women's bone health and how a dietary intervention increasing fruit and vegetable/herb intake affects bone, inflammatory and metabolic markers and urinary electrolyte excretion. It assessed changes in nutrient intake, estimated dietary acid load and sodium: potassium ratios. The study also explores whether specific fruit/vegetables and herbs with bone resorbing properties has an effect on bone markers.

Trial registration: ACTRN12611000763943

4.2 Introduction

Osteoporosis meaning “porous bone” is the term for inadequate bone mass. It is a global problem seen most often in the elderly and in women (80%) (National Osteoporosis Foundation, 2010) and is considered one of the ten most important diseases affecting the world’s population (Räth et al., 2008). Osteoporosis is particularly prevalent in developed countries with ageing populations and longer life spans (P. Brown et al., 2011). Bone loss is accelerated at early menopause resulting in increasingly fragile bones prone to breakage. Inflammation also increases with age and exacerbates bone loss (Miranda et al., 2001; Ginaldi et al., 2005; De Martinis et al., 2007).

Osteoporosis poses a significant health and economic burden for New Zealand families and the public health system. The number of older (>50 years) New Zealanders is increasing steadily and the cost of treating fractures and secondary illnesses related to osteoporosis is expected to rise from $330 million in 2007 to $458 million by 2020 (P. Brown et al., 2011).

Fruit and vegetables (F/V) are positively associated with bone status. The beneficial effect is thought to be through provision of micronutrients potassium, magnesium, calcium, vitamins A, C, E and K, and potentially a lower dietary acid load conferred by the fruit and vegetables food group (Lanham-New, 2008; Macdonald et al., 2008; Welch et al., 2008). Typical western diets are acidic because predominantly acid (hydrogen ions) rather than base (bicarbonate) is created during the metabolism of the daily food intake. Acid forming grains and high protein food derived from animal origin (meat, fish and eggs) contain sulphur based amino acids, methionine and cysteine which create acid when metabolised. Alkaline forming foods contain potassium salts, which can be broken down to make alkaline buffers (Remer, 2001). Vegetables and fruit are considered alkaline because of their high mineral content in the form of salts of organic acids. The salts, predominantly potassium based but also calcium and magnesium, generate bicarbonate to balance the acid produced from the rest of the diet.

Western diets are low in F/V and high in grains and animal protein compared to the typical diet of early man. The change from plant based diets to modern, western diets characterised by foods that are acid rather than alkaline forming results in a low grade systemic metabolic acidosis (Frassetto et al., 1998; Sebastian et al., 2002; Frassetto et al., 2008). The level of acidity created can be estimated from the dietary intake.

A significant change in estimated net endogenous acid production (est.NEAP) is said to have occurred from pre-agricultural times (−88mEq/d) to today (+ 48 mEq/d) (Sebastian et al., 2002). The chronic, low grade metabolic acidosis induced by the modern, western diet is exacerbated during ageing when renal function begins to decline (Jehle, Zanetti, Muser et al., 2006; Lanham-New, 2006), requiring the body's skeletal reserves to be called upon to relinquish bicarbonate to produce alkaline buffers needed to continuously balance the acid load. This results in bone mass that is worn away gradually and indefinitely after the age of 30 years, accelerating at menopause, to lower bone strength and mineral density (New et al., 2003; Jehle et al., 2006; Prynne et al., 2006; Berkemeyer et al., 2008). The influence of F/V on acid–base balance is crucial as they are the sole dietary source of alkaline precursor constituents and for this reason alone, an important reason to recommend increased consumption during ageing to forestall bone loss (Frassetto & Sebastian, 2005; Pizzorno et al., 2010).

Additional benefits on bone metabolism ensue from bioactive constituents found predominantly in vegetables but also some herbs and fruit. Phytochemicals, antioxidants and other bioactive compounds influence bone metabolism through a variety of mechanisms (Basu et al., 2001; Mühlbauer, Lozano, Reinli, et al., 2003; Tobias et al., 2006; Lister et al., 2007), particularly by reducing inflammation and oxidative stress (Hunter et al., 2008; Trzeciakiewicz et al., 2009).

This pharmacological effect of F/V on bone resorption was first observed a decade ago by Mühlbauer (Mühlbauer et al., 2002; Wetli et al., 2005) who, in precise and controlled conditions with animals, demonstrated specific vegetables, herbs and fruit positively affected bone resorption quite apart from their effects on diet acid load. Mühlbauer (Mühlbauer et al.,

2002; Mühlbauer, Lozano, Reinli, et al., 2003) determined the effect was additive, therefore, the more of this specific range of vegetables, herbs and fruit consumed, the more bone resorption reduced. This effect has previously been shown only in the animal model not in humans.

Intervention studies with midlife women assessing acid load and bone health have been limited to modest increases in self-selected fruit and vegetables (Macdonald et al., 2008; Nowson, Patchett, et al., 2009), use of supplements (L. Rao et al., 2007; Macdonald et al., 2008) or use of alkaline water (Dawson-Hughes et al., 2001; Burckhardt, 2008), mimicking F/V alkali forming effect. No study has increased F/V intake significantly to reduce NEAP by approximately 20 mEq/day or specified daily intake of this specific range of vegetables, herbs and fruit shown to have bone resorption inhibiting properties in the animal model. A diet high in F/V and including some from the specific range of vegetables, herbs and fruit with bone resorption inhibiting properties could be a useful dietary strategy to ameliorate bone loss, particularly at critical times such as menopause.

Despite the numerous reports in the literature attributing health benefits with increased consumption of F/V and improvement in chronic disease risk factors (Tobias et al., 2006), most New Zealanders don't reach the Ministry of Health target of 2 servings of fruit and 3 servings of vegetables every day (Ministry of Health (NZ), 2003; University of Otago et al., 2011).

It is hypothesised that an increase in vegetable and fruit consumption to ≥ 9 servings/day will reduce the estimated Net Endogenous Acid Production (NEAP) by approximately 20 mEq/day. The reduction in NEAP will result in a reduction in bone markers of resorption C-Terminal Telopeptide of Type I Collagen (CTX) and bone formation marker Procollagen 1 N Propeptide (P1NP) in post-menopausal women. Women who include 4–5 servings of vegetables, herbs and fruit with bone resorption inhibitory properties (BRIPs) as half of the 9 servings/day will reduce resorption marker CTX by a greater amount.

It is also hypothesised that an increase in fruit and vegetable intake will significantly reduce inflammatory markers including: C- reactive protein (CRP), interleukin 6 (IL-6), interleukin 10 (IL-10) and tumour necrosis factor (TNF) and positively modulate metabolic markers adiponectin, triglycerides, cholesterol, fibrinogen and plasminogen activator inhibitor-1 (PAI-1). This study therefore aims to investigate the effect of increased fruit, vegetables and herbs on bone, metabolic and inflammatory markers and whether including specific fruit, vegetables and herbs with BRIPs (Mühlbauer et al., 2002) as part of an increased fruit/vegetable intake has any additional effect.

4.3 Methods/Design

Table 8 outlines the study design. This study is a randomised active comparator controlled intervention (as bone studies cannot be crossover design) to increase fruit and vegetable intake in healthy post-menopausal women over a 3 month period (refer Appendix B for flier).

Table 8 Scarborough Fair Study design

Study phase	Activities
Recruitment phase	Advertisements/fliers in local newspapers/magazine and workplaces for women (≥5 yrs postmenopausal) to participate in Fruit/vegetable dietary intervention Phone/email inquiry from potential participants/ Information sheet emailed/posted. Phone response from women -clarification of queries Screening questionnaire administered 150 participants eligible for study emailed/posted consent forms and 3 Day Diet Diary information and instructions for first visit Phone response from women -clarification of queries Screening questionnaire administered 150 participants eligible for study emailed/posted consent forms and 3 Day Diet Diary information and instructions for first visit
Week 1 of study	1st visit to Human Nutrition Research Unit for both intervention and control groups Randomisation of intervention group into group A and B Double check consent form signed and any queries answered Fasted blood sample taken between 0700 and 1000hrs (Light breakfast provided) Questionnaire regarding usual diet, lifestyle and nutritional knowledge Dietary assessment (3 Day Diet Diary) reviewed with nutritionist (food portion size atlas) Study dietary requirements reviewed with participants in intervention with demonstration of serving sizes and how to fill in weekly diary. Anthropometric tests: weight, height, blood pressure, spot urine pH. DXA scan performed (first or second visit) Participants willing to provide a 24 hour urine collection are given container with instructions (verbal and written). Pickup of 24 hour urine specimen

Study phase	Activities
Week 6 of study	Participants in the intervention arms of the study attend the clinic again for a fasted blood sample (0700 and 1000hrs) and for a 24 hour dietary recall with nutritionist
	Researcher emailed participants fortnightly with general answers to any queries, tips, recipes etc. appropriate to each group in the intervention arms of the study. Participants could email the researcher with a query and receive a prompt response (within 24 hours).
Week 11 of study	All participants contacted to complete second 3 Day diet diary (3DDD) and to bring 3DDD to their clinic visit the following week. Those who volunteered a first 24 hour urine collection reminded to commence another collection 24 hours prior to attending final clinic visit
Week 12/13 of study	Final visit to Human Nutrition Research Unit for blood sample (fasted), 24 hr urine collection (Light breakfast provided). Anthropometric tests: weight, blood pressure, spot urine (pH). 3 DDD reviewed with nutritionist and final questionnaire for all participants

4.3.1 Sample size

The number of subjects required to demonstrate a reduction in resorption marker C-Terminal Telopeptide of Type I Collagen (CTX) was calculated to be 32 (minimum) in each group and this was determined using a power calculation based on demonstrating a difference of ~8% in the primary outcome variable (CTX) with 80% power and alpha of 0.05 (2 sided test) and accepting 0.4µg/ml as mean CTX of this population (26). To detect any differences between the 2 diets and allowing for withdrawals, non-compliance or maintenance (~25%) approximately 50 women were needed in each group. Since there were 2 diets emphasising different vegetables and fruit and a control group who consumed their usual diet (≤5 servings F/V/day), three groups of 50 participants were required.

4.3.2 Inclusion/Exclusion criteria

The target population were healthy, post-menopausal (≥ 5yrs) women aged between 50–70 years. Women were included if they were taking some medications e.g. hypertensive tablets, thyroxine (if thyroid function stable) and diuretics other than potassium sparing but excluded if on medication for diabetes, heart disease, osteoporosis (including hormone replacement therapy) or medication that could affect bone or calcium metabolism (oral corticosteroids, warfarin, dilantin, potassium sparing diuretics and regular use of proton pump inhibitors). Regular use of NSAIDs (non-steroidal anti-inflammatory drug) including aspirin was not permitted as these

NSAIDs (non-steroidal anti-inflammatory drug) including aspirin was not permitted as these could interfere with anti-inflammatory markers. Participants who had stopped use of an NSAID 1 month prior to the study commencing were included. Women were also excluded if they had any of the following conditions: osteoporosis previously diagnosed, both hips replaced, previous fractures of the lower vertebra or hip, severe osteoarthritis* of the lower spine or hips, gastrointestinal, liver or renal disease and any severe* disease including treatment for cancer within the last 3 years. Women who smoked, drank more than 20 standard alcoholic drinks/week or were already consuming > 6 servings fruit and vegetables every day, or were taking calcium supplements and unwilling to stop one month prior to the study, or for the duration of the study were excluded. Any participant who developed an illness during the study that required treatment with steroids or medication that affected bone, inflammatory and other metabolic markers was also excluded. The intervention group participants had to be willing to increase their intake of fruit and vegetables to 9 servings/day and the negative control group willing to continue their normal diet.

*Severe defined as requiring daily pain relief.

4.3.3 Setting and recruitment

This was a multi-centre trial, with 50 participants at each trial site in Hawke's Bay, Palmerston North and Auckland (NZ). The study was conducted at Massey University's clinical nutrition research units in Palmerston North and in Albany, Auckland. Hawke Bay participants attended Choices Medical Centre in Hastings. Participants were recruited using 2 different fliers. One flier recruited 100 women to form the intervention group and be randomised to one of two groups (A or B) to increase intake of fruit and vegetables to 9 servings/day. The other flier recruited 50 women (Group C) who were willing to have their bone, inflammatory and metabolic markers tested on two occasions 3 months apart (baseline and end of study) and who would continue eating their usual diet. This control group was called the Diet and Metabolic Markers group (DMM) and referred to in this protocol as Group C. Because of the motivation

and commitment involved, it was considered preferable to recruit a control group of women separately rather than randomising women to a control group when they were attracted to the study because of a conscious decision to participate in the dietary change. The same exclusion and inclusion criteria applied to the control group apart from the requirement for dietary change.

The study was advertised in local newspapers in the 3 centres, in two workplaces and in a small advertisement on the health page of The Listener (a popular national magazine) over July/August 2011.This advertising and word of mouth returned a good response rate (>350 enquiries) with enquiries mainly to participate in the dietary intervention rather than the negative control study (Group C). Recruitment was completed within 6 weeks of first advertising (for study flier refer Appendix B)

4.3.4 Screening

Prospective participants who phoned or emailed expressing an interest in the study were initially sent out the appropriate detailed information sheet for perusal. If they replied (email/ phone) and were willing to participate, a screening questionnaire was completed over the phone. This questionnaire included demographics, health status (including medications) and biographic information. Over 300 women were screened, with over half being declined due to a significant health issue or their being on medication deemed incompatible with the study e.g. regular use of proton pump inhibitors.

4.3.5 Randomisation and blinding for intervention groups

Participants were stratified according to the 3 cities and randomly allocated to either group (A or B) using block randomisation to reduce variability in groups from different regions. The study period was over the Spring months for all groups to minimise differences in sunshine exposure and availability of some fruits vegetables and herbs. The random allocation sequence was generated by administrative personnel (not the researcher who recruited participants) and intervention group participants were assigned to group A or B as they arrived for their first

appointment. A crossover design is not considered appropriate for bone studies due to the effects on bone markers.

As intervention participants were required to source, store, prepare and consume specific vegetables, fruits and herbs they were not blinded to which diet (A, or B) they were on once randomisation had occurred, but were blinded to which group contained the fruit, vegetables and herbs with BRIPs. The randomisation codes were maintained by an alpha numeric added to the participant's unique identifier and laboratory staff involved in the analysis of blood or urine samples were blinded to participants' group.

4.3.6 The intervention rationale

Women in the intervention groups A and B were asked to consume a minimum of 9 servings a day of fruit and vegetables with herbs additional. 9 servings per day was chosen as the target number of fruit and vegetable servings as it allows for a significant decrease in estimated net endogenous acid production (NEAP) of the diet (~20 mEq/day)(Gunn, Weber, Coad et al., 2013) and was the number of servings recommended by the USDA for healthy, fit 50 year old women (USDA, 2005), as well the number recommended in the DASH Trial (P. Lin et al., 2003). This increase in fruit and vegetables could then be compared to a group of 50 women consuming their normal diet (≤5 servings/day) (Group C).

In order to assess the impact of BRIPs, both groups were asked to emphasise a different selection of vegetables, herbs and fruit and to avoid others. Group B included 4–5 servings of specific vegetables, fruit and herbs with bone resorption inhibitory properties (BRIPs), while Group A avoided those specific vegetables, herbs and fruit and emphasised other specific fruit, vegetables and herbs. For specific dietary advice given to Groups A and B refer Appendix B. To allow for change in bone turnover markers particularly P1NP, the minimum study period was determined to be 3 months (P. Lin et al., 2003).

Bone resorption inhibiting properties have been ascribed to a limited range of common fruit, vegetables and herbs. These include the herbs dill, sage, garlic, parsley, thyme and rosemary. Vegetables and fruit with BRIPs include onion, tomatoes, green beans, cucumber, broccoli, lettuce, prunes and oranges (Mühlbauer et al., 2002), with the effect on bone resorption of the foods with BRIPs said to be additive (Mühlbauer et al., 1999). The minimum effective dosage of fruit/vegetables and herbs (F/V/H) with BRIPs calculated in the animal model is 170 mg/day. This corresponds with 6.2 grams of fresh F/V/H per kilogram human body weight. This amount is equivalent to 3–5 servings /day (Ministry of Health and the University of Auckland, 2003) of any F/V with BRIPs for a 60–70 kilo woman. Culinary herb servings were to be additional and usual culinary measures in meals were advised to all intervention participants (2–3 cloves garlic, 0.5-1 teaspoon for dried/fresh culinary herbs and up to 0.25 cup of parsley (Group B-BRIPS) and basil (Group A- non-BRIPS). Vitamin K is also known to affect bone health and fracture risk through its action on cytokines and the gamma carboxylation of the bone protein osteocalcin (Booth, Broe, Peterson et al., 2004; Cockayne et al., 2006; Shea, Dallal, Dawson-Hughes et al., 2008). To control for vitamin K intake all participants in the intervention were asked to include one serving of a leafy green vegetable in their diet every day. They were also asked to have at least 2 servings of dairy or calcium enriched soy milk or other alternative to control for calcium intake (Ministry of Health (NZ), 2003). Twice a week the intervention group women recorded their total vegetable, fruit and herb intake as well as their urine pH (fasting, second void) to the nearest 0.25 pH unit with dipsticks provided (PHion Diagnostic Test Strips, Apex Wellness Group, UC, Scottsdale, AZ85260, USA). The urine pH was checked as the second voided urine after an overnight fast. This method is used to standardise the timing as testing of early morning urine will most likely give the lowest pH reading of the day due to the kidneys having processed the acid load of the previous day and made the readjustments necessary, particularly the excretion of the non-volatile (fixed) acids produced by metabolic processes (Tietz, 1970). Urine pH will also be influenced by renal function and age related decline in kidney function(Scialla et al., 2013) but these were undetermined in our study participants.

Group A ≥ 9 servings of fruit and vegetables (≤3 servings fruit and ≥6 servings of vegetables) Specific fruit/ vegetables/herbs (all non BRIPs) specified for over half the servings and avoiding F/V/H with BRIPs.

Group B ≥ 9 servings of fruit and vegetables (≤3 servings fruit and ≥6 servings of vegetables) Specific fruit/ vegetables/herbs (all BRIPs) specified for over half the servings and avoiding some of Group A's specified fruit/vegetables/herbs.

Group C Control group who were to consume their usual diet

4.3.7 Blood and urine sampling

Procedures for taking blood, urine and anthropometric measurements were standardised to reduce errors and variability. Bone markers show circadian variability (Qvist, Christgau, Pedersen et al., 2002) therefore all fasted blood samples were taken between 0700 and 1000hr, with blood drawn by certified phlebotomists. After their first appointment women were advised to make their subsequent appointments as close as possible to the exact time of day as their first, and most were able to comply with this. The bone markers chosen were serum CTX and serum P1NP as these two bone markers are recommended by the IOF (Vasikaran et al., 2011). Serum CTX has several advantages over urinary markers of type I collagen. Some of the main advantages include: measurement of urinary creatinine is not required, its versatility either as a manual ELISA or as an immunochemiluminescent assay on fully automated platforms which allow faster and improved analytical performance, normative data from large and well-documented populations of healthy women at all age ranges are available and finally, a low within subject variability exists for this bone marker, half the variability of urinary markers (Vasikaran et al., 2011; Biver et al., 2012).

Plasma was used for analysis of bone, inflammatory and metabolic markers and drawn into vacutainers containing EDTA, citrate or heparin. After centrifugation at 3000 rpm (1560 RCF) for 15 minutes (4°C), the plasma was dispensed in aliquots and frozen at −80°C. Participants

collected a 24 hour urine sample (information sheet) the day prior to attending their clinic visits and kept the sample refrigerated. At the clinic the sample quantity was measured and 3 aliquots of 100 ml were frozen immediately at −20°C. All blood and urine samples were analysed at the end of the 3 month study period. To reduce inter-assay variability both baseline and end of study samples were analysed in the same assay run. Canterbury Endocrine laboratory, accredited with IANZ to ISO 15189, determined all serum bone and metabolic markers using a fully automated platform which reduces analytical error. Laboratory bone markers average coefficient of variation (CV_A) was 6.5% for CTX and 5.1% for P1NP (further laboratory details are included in appendix). Cytokine analysis was conducted at Plant and Food Research Laboratory using flow cytometry methods (Table 9). Unfortunately CVs were unavailable due to staff changes. Mineral analysis of 24 hour urine samples were done in duplicate at EIT Research Laboratory (no IANZ accreditation). Creatinine was measured at Massey University Laboratory, Palmerton North (IANZ accreditation 17026). Laboratory creatinine coefficient of variation (CV) was 2.3% with samples measured singly and in duplicate every 10th sample. The Gold standard test for each bone marker was used as well as for urine analysis of minerals (refer Table 9).

4.3.8 Biochemical analysis

Refer Table 9 for details of biochemical analysis done

Table 9 Outcome variables and analysis method

Outcome Variables	Start	Mid 6 wks	End (3mths)	Method
CTX bone resorption marker	I & C	I	I & C	Roche Elecsys 2010. Roche Diagnostics, Canterbury Health Endocrine laboratory (CHL). Accredited with IANZ to the ISO 15189
P1NP bone formation marker	I & C	I	I & C	Roche Elecsys 2010.Roche Diagnostics, Canterbury Health Endocrine laboratory.
Cholesterol	I & C		I & C	CHL Enzymatic Cholesterol Oxidase Abbott c8000 analyser, Abbott reagents.
Triglycerides	I & C		I & C	CHL Enzymatic hydrolysis of Triglycerides. Abbott c8000/c16000 analyser, Abbott reagents.
LDL	I & C		I & C	CHL calculated as follows: LDL Chol = Total Chol - HDL Chol -(Trigs/2.22) mmol/L.
HDL	I & C		I & C	CHL Enzymatic assay. Abbott c8000 analyser. Abbott reagents
Fibrinogen	I & C		I & C	Fibrinolysis reference plasma (Cat. No. T6010) (Kordia, Leiden, The Netherlands).
PA1	I & C		I & C	Plasma PAI-1_{act} bio-immunoassay, TriniLIZE PAI-1 activity assay kit (Cat. No. T6004)
CRP	I & C		I & C	Plant and Food Research Auckland FlowCytomix kits (Bender Medsystems, Austria) FC500 MPL flow cytometer (Beckman Coulter, Miami, FL)
IL-6	I & C		I & C	Plant and Food Research Auckland FlowCytomix kits (Bender Medsystems, Austria) FC500 MPL flow cytometer (Beckman Coulter, Miami, FL)
IL-10	I & C		I & C	Plant and Food Research Auckland FlowCytomix kits (Bender Medsystems, Austria) FC500 MPL flow cytometer (Beckman Coulter, Miami, FL)
TNF	I & C		I & C	Plant and Food Research Auckland FlowCytomix kits (Bender Medsystems, Austria) FC500 MPL flow cytometer (Beckman Coulter, Miami, FL)
OPG	I & C		I & C	Plant and Food Research Auckland FlowCytomix kits (Bender Medsystems, Austria) FC500 MPL flow cytometer (Beckman Coulter, Miami, FL)
Adiponectin	I & C		I & C	Plant and Food Research Auckland FlowCytomix kits (Bender Medsystems, Austria) FC500 MPL flow cytometer (Beckman Coulter, Miami, FL)
24hr Calcium	I & C		I & C	E.I.T Research Laboratory - Shimadzu AA-6200 Atomic Absorption Flame Emission Spectrophotometer with air/acetylene flame.(Method adapted from Gimblet El)
24hr Magnesium	I & C		I & C	AAS as above (Method adapted from Gimblet El)
24hr Sodium	I & C		I & C	AAS as above (Method adapted from Castenmillar JM)
24hr Potassium	I & C		I & C	AAS as above (Method adapted from Castenmillar JM)
24hr Creatinine	I & C		I & C	Massey University Nutrition laboratory. Jaffe Method Flexor E, Vital Scientific NV, 6956 AV Spankeren/Dieren, The Netherlands
Iodine	I & C		I & C	Hills Laboratory - inductively-coupled plasma mass spectrometry.
Selenium	I & C		I & C	Hills Laboratory - inductively-coupled plasma mass spectrometry.
Dietary assessment				

Outcome Variables	Start	Mid 6 wks	End (3mths)	Method
3DDD macro and micronutrient intake	I & C		I & C	3 day food intake (2 week days and 1 weekend) Recipes/packaging. Foodworks NZ,2009 (Xyris software)
24 hour dietary recall		I		Previous day 24 hour recall second clinic visit nutritionist (NZ reg.)
Estimated NEAP and PRAL	I & C		I & C	Derived from algorithms and using Food works (Xyris, NZ) data from 3DDDs
No. serves each food groups	I & C		I & C	Derived from 3DDD nutritionist (NZ reg.) converted quantities in grams/mls/cups etc. to servings
F/V/Herb Diary	Intervention group only. Twice weekly- self reported intake			
Urine pH (2/wk)	Intervention group Twice weekly self-reported urine pH (second void /fasting) Phion Diagnostic Test Strips (0.25 graduations), Apex Wellness Group, UC, Scottsdale, AZ85260, USA			
Weight/height BMI	I & C		I & C	Digital scales UWE Gilbarco, NZ / Stadiometer/ BMI =weight/height^2
Blood pressure	I & C		I & C	Omron Digital HEM-907 automatic blood pressure monitor (duplicates) high values repeated manually
Waist/hip measurements	I & C		I & C	Anthropometry methods performed by trained nutrition staff using standardised procedures and equipment. Tape measure, Douglas Pharmaceuticals Limited, Auckland.
Body composition and BMD	I & C	DXA Hologic Discovery QDR 4500A densitometer, Hologic Inc. Bedford, Massachusetts		

Intervention (I) and control group (C).

4.3.9 Dietary assessment

3 Day Diet Diaries (3DDDs) were done at baseline and end of study (week 12). Participants received written and verbal instruction on how to complete the 3DDD's via email and a phone call prior to attending their first clinic appointment. They were asked to record all food and beverages consumed over 2 weekdays and 1 weekend day, including types, brands and amounts (cups, tablespoons, etc.) of foods as well as recipes for homemade dishes. Participants supplied nutritional information panels from processed food packets (for 3DDD refer Appendix B). All 3DDD's were checked for accuracy and completeness by a NZ registered nutritionist at the first visit. Prompting methods were used for incomplete quantities or to ascertain specific food types e.g. milk (skim or full fat).

Dietary data were entered into Foodworks (version 2009, NZ, Xyris software) by a M.Sc (nutrition) graduate, checked for accuracy and completeness by a N.Z. registered nutritionist and then transferred to SPSS (20) via Microsoft Access (2007) and Excel (2007). Dietary vitamin K intake was not assessed as reference data was unavailable in Foodworks (version 2009, NZ, Xyris software) at the time of analysis. Serum Vitamin K analysis was not conducted due to costs.

New Zealand dietary reference values were used to assess all nutrient intakes (Ministry of Health (NZ), 2003). Net endogenous acid production (NEAP) was calculated according to the algorithm from Remer and Manz (Remer et al., 2003; Frassetto, Lanham-New, et al., 2007).

NEAP = Potential Renal Acid Load (PRAL) + Organic Acid (OA)

PRAL represents the average intestinal absorption rates of ingested protein and additional minerals. OA is an anthropometry-based estimate for organic acid excretion.

The two components of NEAP are as follows:

- PRAL (mEq/d) = 0.49 protein (g/d) + 0.037 × phosphorus (mg/d) - 0.021 × potassium (mg/d) – 0.026 × magnesium (mg/day) - 0.013 × calcium (mg/day)
- OAest (mEq/day) = individual body surface area × 41/1.73

Body surface area was calculated according to the method of du Bois and du Bois

- BSA = ($W^{0.425} \times H^{0.725}$) × 0.007184 (Website http:/www-users.med.cornell.edu/~spon/picu/calc/bsacalc.htm)

NEAP and PRAL are expressed in milliequivalents (mEq) as ions in solution interact according to their charge. Milliequivalents are obtained by converting milligrams (mg) to millimoles (mmol) and multiplying by valences to give charge quantities (Frassetto, Lanham-New, et al., 2007).

4.3.10 Questionnaire

At the first clinic visit, a baseline questionnaire was administered with the intent of focusing participants on their motivations for this dietary behaviour change, and how they would overcome common barriers to dietary change (Appleton, McGill, Neville et al., 2009) and how they would incorporate the vegetables, fruit and herb increase required into their daily dietary intake.

4.3.11 Compliance diaries

Intervention participants were provided with a weekly diary in which they recorded their intake of fruit, vegetables, herbs (type and quantity) and urine pH (fasted second void) on 2 days each week for the duration of the study (refer Appendix B for study diaries). This diary was to aid compliance with the dietary requirements and also to see if urine pH increased with increased amounts of fruit and vegetables (Remer et al., 1994; Michaud et al., 2003). An increase of 0.68 pH units was previously demonstrated (Gunn, Weber, Coad, et al., 2013) and the change in urine pH was said by some participants to be a motivator.

4.4 Adverse event reporting

Participants were advised to report any side effects from the increase in vegetables and fruit intake. They also recorded in their diaries any incidence of adverse side effects (e.g. stomach upset), which was reviewed at each clinic visit. The study's clinical collaborator was available to provide guidance on medical issues. Participants were advised to report immediately their concerns with any serious adverse side effect from this dietary regimen e.g. chronic diarrhoea (> 48 hours). Any participant recording serious adverse effects due to the diet would have been immediately advised to consult their medical practitioner for assessment if they had not already done so, and withdrawn from the study.

Follow-up: The researcher/clinical collaborator would follow-up with any participant that had a medical issue, that they had received medical treatment if necessary and the issue was resolved satisfactorily. In the unlikely event of a serious adverse side effect from participation in this intervention study, the individual after consultation with their medical practitioner would have been advised to contact ACC (Accident Compensation Corporation). No significant adverse side effects however, were reported.

4.5 Statistical methods

SPSS 20 was used for statistical testing. Repeated measures and one way analysis of variance (ANOVA) was done on serum bone markers (baseline, 6 and 12 weeks) to determine change over the study period, multiple regression and Pearson's correlation was also used for daily F/V intakes and bone markers. Analysis of nutrient intake before and during the intervention was by paired *t*-tests and repeated measures, main outcome variables were checked for normal distribution (Shapiro-Wilk test) and any data not normally distributed was log transformed before comparisons were made. A level of significance of 0.05 was used. Due to multiple comparisons being made, the Bonferroni correction was applied for all comparisons between groups and the Sidak post hoc test was used for investigating interaction effects.

The primary analysis was the comparison of change in bone markers between the intervention groups and negative control group following the "intention to treat" principle at 6 weeks and 3 months. Secondary analyses involved any intervention treatment effects on inflammatory and metabolic markers. Per protocol analyses was performed and compared with intention to treat to assess bias.

4.6 Participant's results

All participants received individualised dietary assessment analysis and their DXA body composition results, but not individual blood biochemistry or urinary test results (requirement of MUHEC).

4.6.1 Dietary analyses

Following input of 3DDD's into Foodworks (2009), all participants in group C (negative control group) received an overview of their baseline three day diet diary analysis. This provided detailed information on macronutrient and micronutrient intake. Due to this study involving dietary change, all intervention participants received both beginning and ending 3DDD results to assess micronutrient changes associated with their increased vegetable and fruit intake. Participants with queries about their nutrient analysis were invited to email the nutritionist (NZ registered) and received immediate feedback.

4.6.2 DXA analysis

All participants received a letter informing them of their DXA bone scan result. This was reported as normal (including mild osteopenia), osteopenia or having a T score ≥2.5 SDs below normal. Participants with DXA results not within normal parameters for their age group (latter 2 groups) also received the radiologists report on their bone scan with their DXA t and z scores details and a letter advising them to discuss the results with their doctor.

4.7 Funding and ethics

Funding for this study was provided from the following organisations: Hawke's Bay Medical Research Foundation, The National Heart Foundation, Massey University Research Fund (MURF),

AMGEN funding (Osteoporosis Australia, Glaxo Smith Kline, Australian and NZ Bone Mineral Society).

This study was approved by Massey University Human Ethics Committee (MUHEC) (Southern A) Reference 11/11 in June 2011. It was conducted in compliance with the protocol approved by the Massey University Ethics Committee (MUHEC) and will be reported according to CONSORT guidelines. No deviation from the protocol was implemented without the prior review and approval of MUHEC. Participants were informed they could withdraw from the study at any point. All subjects for this study were provided with a detailed information sheet describing the study and providing sufficient information to make an informed decision about participation before they gave signed consent.

4.8 Discussion

This study "The Scarborough Fair Study" will provide detailed information about the influence of increased fruit and vegetable intake on the bone health of post-menopausal New Zealand women (50-70 years). The dietary intervention involves a significant dietary behaviour change with increased intake of fruit, vegetables and herbs which may have an effect on markers of bone and inflammation as well as metabolic markers.

This study differs from other fruit and vegetable dietary interventions, in that it includes: a combination of prescribed fruit, vegetables and herbs previously shown to affect bone resorption in the animal model but not previously trialed in humans (Mühlbauer et al., 1999), its primary emphasis on vegetables (≥6 servings) rather than fruit (≤3 servings) (Ashwell et al., 2008) and the inclusion of herbs (Mühlbauer, Lozano, Palacio, et al., 2003) and its use of urine pH monitoring to assess change in dietary acid load (Wynn, Krieg, Aeschlimann, et al., 2009). This differs from previous intervention studies on bone health using the fruit and vegetable food group (Macdonald et al., 2008; Nowson, Patchett, et al., 2009) where smaller increases in fruit and vegetable consumption were achieved.

Limitations with the study include the element of selection bias introduced as a result of stringent inclusion and exclusion criteria. In some ethnic groups, health status is likely to be already compromised by midlife due to chronic illness (diabetes and heart disease) and these groups were under represented. Predominantly European women formed the study population.

A high element of commitment to a behaviour/lifestyle change was required with the need to purchase, store and prepare all fruit and vegetables and herbs consumed. There were no financial incentives and participants received a petrol voucher ($10/per visit) at the conclusion of the study.

Results from this study may provide further evidence to encourage increased consumption of fruit, vegetables and herbs with emphasis on those with bone resorption inhibiting properties.

4.9 Acknowledgements

The study was financed by grants from the Hawke's Bay Medical Research Foundation, the National Heart Foundation, Massey University Research Fund (MURF) and AMGEN funding (Osteoporosis Australia, Glaxo Smith Kline, Australian and NZ Bone Mineral Society). Dr Anne-Thea McGill, Auckland University, acted as clinical advisor for the study.

CHAPTER 5 DIET, WEIGHT, CYTOKINES AND BONE HEALTH IN POST-MENOPAUSAL WOMEN

This study details the baseline data obtained from the Scarborough Fair Trial. Dietary intake data from the baseline three day diet diaries provided by the women was analysed and compared to data with their fasting plasma cytokines, twenty four hour urinary mineral excretion and their DXA bone mineral density scan data. Primarily, correlations between dietary intake data and bone mineral density measures were investigated, to determine if a diet higher in plant food was associated with favourable bone mineral density at middle age. Participant's weight and other anthropometric measures was correlated with cytokines and bone mineral density to assess associations between fat mass and inflammation and a multiple regression was performed with relevant factors.

Data published in Journal of Nutrition, Health and Aging, (Refer to Appendix)

Gunn, C. A., Weber, J. L., & Kruger, M. C. (2014). Diet, weight, cytokines and bone health in postmenopausal women. *The Journal of Nutrition, Health & Aging, 18*(5), 479-486

5. 1 Abstract

The aim of the study was to investigate diet and nutrition-related factors associated with bone loss in a group of post-menopausal (PM) women. Nutritional intake, inflammatory markers and body composition (weight, body mass index, fat/lean mass) were analysed for associations with bone mineral density (BMD). A cross sectional study examining correlations between BMD (Duel-energy X ray absorptiometry; (DXA) and dietary intake (3-day diaries), body composition and plasma bone and inflammatory markers: C-terminal telopeptide of type I collagen (CTX) and procollagen type I N propeptide (P1NP), C- reactive protein (CRP), interleukin 6 and 10 (IL-6, IL-10), tumour necrosis factor (TNF) and osteoprotegerin (OPG). Setting: Community dwelling women from Auckland, Hawke's Bay and Manawatu regions in New Zealand. Participants: 143 healthy postmenopausal women aged 50-70 years. OPG (per kilogram fat mass) was increased in women with osteoporosis ($p<0.001$) compared to groups classified with normal BMD and osteopenia. Protein, vitamin B_{12}, zinc, potassium and dairy intake were all positively correlated with higher BMD, while dairy and potassium intakes also inversely correlated with CTX. Body composition (weight, BMI and fat/lean mass) had strong positive associations with BMD. Multiple regression analysis showed body weight, potassium and dairy intake were predictors of increased BMD in PM women and explained 39% ($r^2=0.39$, $p< 0.003$) of variance. BMD was negatively correlated with OPG and positively with body weight, dairy and potassium intake. This study highlights the importance of maintaining adequate body weight and emphasising dairy and potassium predominantly sourced from fruit/vegetables to reduce bone loss at midlife.

5.2 Introduction

Osteoporosis has emerged as a leading public health issue in New Zealand (P. Brown et al., 2011) and the rest of the world health (Woolf & Pfleger, 2005; Dawson-Hughes & National Osteoporosis Foundation Committee, 2008). Current estimates in 2013, are for over 100,000 osteoporosis related fractures in NZ per year, with females over 50 years of age accounting for two thirds (P. Brown et al., 2007). Bone loss accelerates at menopause therefore determining modifiable influences is imperative.

Nutrition has a direct influence on bone health (Cashman, 2007; Ashwell et al., 2008; Anderson, 2012), also the immune system and levels of inflammation (Cashman, 2008; Dinkova-Kostova et al., 2008; Bakker et al., 2010) and the protective influence of weight including fat mass on bone density is central to bone health in PM women (Reid, 2008; Breuil, Ticchoni, Rous et al., 2010; Reid, 2010).

Diet and exercise may account for up to 25% of variation in bone mineral density (BMD) (Anderson et al., 2012b) with important nutritional contributions attributed to adequate protein (Jesudason & Clifton, 2011; Calvez et al., 2012; Filion, Barbat-Artigas, Dupontgand et al., 2013) calcium and dairy intake (Heaney, 2009; Pedrera-Zamorano, Calderon- Garcia, Roncero-Martin et al., 2012). Long chain polyunsaturated fatty acids contribute to increased bone mass and reduced resorptive activity (Kruger, Coetzee, et al., 2010; Hutchins-Weise, Kleppinger, Annis et al., 2013) while fruit/vegetable's contribution to bone health is due to their micronutrients (Nieves, 2005; Cashman, 2007), phytochemicals (Lister et al., 2007; Habauzit et al., 2008; Hunter et al., 2008; Salminen et al., 2012) and bicarbonate precursors which lower the renal acid load (Pizzorno et al., 2010; Shi et al., 2012).

Osteoporosis and low bone mineral density (BMD) have traditionally been referred to as a disease of low body mass index (BMI) and body fat composition (Reid, 2008; Breuil et al., 2010) and in older adults, low body weight is an established risk factor (Bhupathiraju, Dawson-Hughes, Hannan et al., 2011). There is also, however, agreement that increasing body fat levels contribute to rising levels of adipokines and other inflammatory cytokines (Reid, 2010). Bone metabolism and immune regulation is now viewed as intertwined, due to the increased numbers of immune cells (macrophages, T and B cells) seen with obesity and therefore increased levels if inflammation and cytokines influencing bone (Galic, Oakhill, & Steinberg, 2009). Increased levels of inflammatory cytokines emanating from immune cells in fat tissue contribute to inflammatory bone loss, therefore, obesity may also be a negative determinant of bone health (Cao, 2011; Halade et al., 2011) with abdominal (Bhupathiraju et al., 2011) or visceral fat particularly implicated (Ziccardi, Nappo, Giugliano et al., 2002; Gilsanz, Chalfant, Mo et al., 2009; Bredella, Torriani, Ghomi et al., 2010; Bhupathiraju et al., 2011).

While older New Zealand women (51-70 years) are known for consuming a better diet (more fruit/vegetables) than younger age groups or men (Ministry of Health (NZ), 2008; University of Otago et al., 2011), there has been no study assessing the combined effects of dietary composition and renal acid load, body weight (BMI, fat and lean mass) and levels of inflammatory cytokines with bone status in this group. The aim of the present study was therefore to explore the relationship between these factors and bone health in a group of healthy, post-menopausal (PM) New Zealand women.

5.3 Methods

5.3.1 Study population

Ethical approval was obtained from Massey University Human Research Ethics Committee (Southern A) Reference number 11/11. All participants were fully informed of the study requirements and gave written informed consent. The trial was registered with the Australian and New Zealand Clinical Trials Registry (ANZCTR) http://www.ANZCTR.org.au. **Trial Registration**: ACTRN 12611000763943.

The study commenced in August 2011, with 142 women between 50-70 years, who were at least 5 years post-menopausal and who had volunteered for a dietary intervention (The Scarborough Fair Study). The study was conducted by Massey University, New Zealand and women were recruited from 3 regions: Auckland, Palmerston North and Hawke's Bay by advertisement in local papers. Exclusion criteria included any known significant health condition or regular use of medication which could affect bone or inflammation including HRT, NSAID's and proton pump inhibitors. Results presented here represent the baseline data obtained on bone turnover and inflammatory markers, dietary intake and bone mineral status determined by dual-energy X ray absorptiometry (DXA).

5.3.2 Plasma markers

The plasma markers included: bone markers of resorption, C-terminal telopeptide of type I collagen (CTX) and formation, procollagen type I N propeptide (P1NP) and inflammatory markers: C- reactive protein (CRP), interleukin 6 and 10 (IL-6, IL-10), tumour necrosis factor (TNF) and osteoprotegerin

(OPG). In short, overnight fasted blood samples were taken between 8-10am, immediately centrifuged at 3000 rpm (1560 RCF), separated and stored at -80ºC until bone markers were analysed at Canterbury Health Endocrine Laboratory (Roche Elecsys 2010.Roche Diagnostics) and inflammatory markers at Plant and Food Research Auckland (FlowCytomix kits (Bender Medsystems, Austria). Inflammatory markers are expressed per kilogram fat mass as production is correlated with increasing fat mass (Reid, 2010).

5.3.3 Dual-energy X ray absorptiometry (DXA)

DXA of lumbar spine (L1-L4) and hip (total and femoral neck) was performed using a Hologic QDR-Discovery A densitometer (Hologic Inc, Bedford, Mass., USA) giving measures of bone mineral content (BMC)(grams), BMD (grams/cm^2), T and Z scores, body fat and lean measures (android and gynoid). The machines were calibrated daily with in-vivo reproducibility of coefficient of variation 0.45-0.54% for all measured sites.

5.3.4 DXA classification

The four groups (1-4) DXA classification was based on the W.H.O classification (World Health Organisation, 2004; Kanis, McCloskey, Johansson et al., 2008), however, in this study, the osteopenic group was further divided into mild or significant osteopenia according to T-score as below.

1) Normal: A value for BMD that is higher than 1 SD below the young adult female reference mean (*T*-score greater than or equal to − 1 SD).
2) Mild osteopenia: A value for BMD 1 SD or more below the young female adult mean (*T*-score < − 1 and > − 1.5 SD).
3) Significant osteopenia: A value for BMD 1.5 SD or more below the young female adult mean (*T*-score < − 1.5 and > − 2.5 SD).
4) Osteoporosis: A value for BMD 2.5 SD or more below the young female adult mean (*T*-score less than or equal to − 2.5 SD).

The division into mild and significant osteopenia in women in this age range (50-70 years) was based on the specialist radiologist's recommendations for referral. Women with normal BMD or mild osteopenia were not referred for follow-up, whereas those with significant osteopenia and osteoporosis were.

5.3.5 Diet

Dietary intake data was assessed from 3 Day Diet Diaries (3DDDs). These were considered more reliable than a FFQ for monitoring short term change in dietary habits as well as assessing potential renal acid load (PRAL). Participants received verbal and written instructions on how to complete the diaries and examples of standard portion sizes were listed. All food and beverages consumed over 2 weekdays and 1 weekend day were recorded along with information on brands and amounts, along with recipes for homemade dishes. A registered nutritionist reviewed the diary with each participant, using prompting for specific food types and a food display demonstrating portion sizes of both raw and cooked food to ascertain quantities and servings. Data was entered into Foodworks (version 9, Xyris NZ) and compared with New Zealand dietary reference values (Ministry of Health (NZ), 2006). A random subset (random sampling method in EXCEL) of 20 diaries were analysed to determine the main food sources of potassium. PRAL and net endogenous acid production (NEAP) was determined according to Remer and Manz (Frassetto, Lanham-New, et al., 2007).

- PRAL (mEq/d) = 0.49 protein (g/d) + 0.037 × phosphorus (mg/d) - 0.021 × potassium (mg/d) – 0.026 × magnesium (mg/day) - 0.013 × calcium (mg/day).
- NEAP = Potential Renal Acid Load (PRAL) + Organic Acid (OA)

5.3.6 Anthropometric data

Body weight was measured to the nearest 0.5 kg with subjects in light clothing without shoes using digital scales (UWE Gilbarco, NZ). Body height was measured without shoes to the nearest 0.5 cm using a stadiometer (SECA 213). BMI was calculated as weight (kg)/height (m^2). Hip and waist

circumference were measured by trained nutrition staff using standardised procedures and equipment (Tape measure, Douglas Pharmaceuticals Limited, Auckland).

5.3.7 Statistical analyses

The Statistical Package for Social Sciences version 20.0 (SPSS Inc. Chicago, IL, USA) was used for all analysis with a significance level of 0.05 (2 tailed). Data was checked for distribution and if normally distributed was expressed as means and standard deviations (SD), or if not normally distributed, as both means (SD) and medians (interquartile range) for comparison purposes. Log transformations of non-normally distributed data were used for correlations and models. Pearson and partial correlations were used to determine associations between nutritional indices (servings, macro and micronutrients and PRAL/NEAP), weight, BMI and soft tissue indices, inflammatory and bone markers and BMD. Potential differences among groups were evaluated with one way analysis of variance. Regression analyses were used to determine the influence of single predictor variables (nutrients, inflammatory markers and body composition variables (fat mass, lean mass, body fat percentage, android and gynoid fat percentages) on BMD (spine, hip and femoral neck of hip).

A multiple regression model was used to assess the simultaneous effects of soft tissue indices, inflammatory markers and nutrients shown in the Pearson's correlation to have a significant effect on bone mineral density. Coefficients of variation were used to check the validity of the regression model. Nutrients were energy adjusted and multicollinearity avoided by not including variables such as weight and fat/lean mass together or nutrients protein and vitamin B12 in the model. Variance inflation factors with values above 2 were used to indicate problems with the model.

5.4 Results

The majority of the 143 women recruited were European (98%) with 2% Maori or Pacific Islander. This is not representative of the population (68% European, 15% Maori, 7% Pacific Islander), but reflects the difficulty in recruiting older indigenous women without significant health issues. Mean BMI was 25.7, age 60.4 years and 11 years since menopause (YSM). Nearly half the women had

normal BMD (<1 SD below normal) (36%) or were mildly osteopenic (1.0-1.49 SD below normal) (15%) while the other half were either significantly osteopenic (1.5-2.49 SD below normal) (37%) or osteoporotic (≥2.5 SD below normal) (12%). The older women in this study had higher bone resorption and lower bone formation markers but no significant differences were seen in bone density or body composition between the two age groups 50-60 years and 61-70 years. 50% of women had normal BMI, 27% were overweight and 23% were classified as obese (Table 10). Bone turnover markers were lowest in the group with normal BMD and increased across each group with decreasing BMD.

Table 10 Bone markers and bone mineral density of post-menopausal study women (n=142)

Groups based on BMD	1 Normal BMD (n=51)	2 Mild Osteopenia (n=21)	3 Significant Osteopenia (n=53)	4 Osteoporotic (n=17)	p <
Age (yrs)	60 (5)	63(4)	60(4)	61(4)	0.04
CTX base µg/L	0.34(0.11)	0.39(0.23)	0.40(0.15)	0.51(0.16)	0.001
P1NP base µg/L	41.7(14)	46.9(24)	48.4(17)	54.6(16)	0.046
BMI	27.9(5.6)	27.7(3.4)	24.1(4.1)	22.0(3.1)	0.001
BMI (18.5-24.9)	17(24%)	4(6%)	33(48%)	15(22%)	-
BMI (25-29.9)	15(36%)	11(26%)	15(36%)	1(2%)	-
BMI (30-34.9)	14(58%)	5(21%)	4(17%)	1(4%)	-
BMI >35	5(71%)	1(14%)	1(14%)	0(0%)	-
YSM	11(5)	13(4)	9(4)	12(5)	-
Spine BMC(g/cm)	60(11)	54(15)	51(9)	45(11)	0.007
Spine BMD(g/cm^2)	1.1(1)	1.0(0.1)	0.9(0.1)	0.8(0.07)	0.001
Spine T-score	0.25(0.85)	-0.23(0.9)	-1.4(1.1)	-2.55(0.6)	0.001
Hip BMD (g/cm^2)	0.96(0.1)	0.88(0.1)	0.84(0.1)	0.72(0.06)	0.001
Hip BMC (g/cm)	33(4)	30(4)	28(5)	25(3)	0.001
Hip T-score	0.11(.67)	-0.48(.67)	-0.85(1.0)	-1.87(.5)	0.001
FN Hip BMD(g/cm^2)	0.85(1.)	0.74(.1)	0.69(.1)	0.59(.1)	0.001
FN Hip BMC(g/cm)	4.2(.6)	3.7(.4)	3.4(.6)	2.96(.5)	0.001
FN Hip T-score	-0.05(.8)	-1.0(.6)	-1.4(.7)	-2.3(.6)	0.001

Values are means and standard deviations (SD) except for BMI which also are number of participants in each BMI category and percentage (%) of women within each BMI classification in each category of BMD.P values determined from ANOVA.CTX= C terminal telopeptide of type 1 collagen, P1NP= procollagen type 1N propeptide. BMI= body mass index (weight (kg)/height m^2), YSM=years since menopause, FN= Femoral neck of hip, BMD=bone mineral density, BMC=bone mineral content. DXA groups 1-4 are based on W.H.O classifications of T scores (World Health Organisation, 2004):Normal T score <1.0 SD below normal, osteopenia T score 1.0-2.49 SD below normal and osteoporosis T score ≤-2.5 SD below normal. Osteopenia is separated into mild (T-score ≥1-1.49 SD below normal and significant osteopenia (T score ≥1.5 ≤ 2.49 SD below normal).

Table 11 Dietary intake data of post-menopausal study women (n=142)

	Total	Group 1 Normal BMD	Group 2 Mild Osteopenia	Group 3 Significant Osteopenia	Group 4 Osteoporosis	NZ reference values	
	(n=142)	(n=51)	(n=21)	(n=53)	(n=17)	EAR/AI	RDI/SDT
Alcohol(units/week)	7(8)	6(9)	8(10)	7(7)	7(9)	≤14units/week	
Energy (kj)	7595(1730)	7550 (2099)	8220(1380)	7389(1642)	7602(1631)	N/A	
Protein (g)	79(20)	79(21)	83(18)	77(22)	76(21)	37	46
Fat (g)	70(23)	71(27)	78(22)	66(23)	74(21)	N/A	
Fibre (g)	26(9)	24(8)	30(9)	26(8)	26(12)	/25	/28
Vitamin C (mg)	195(199)	207(247)	203(156)	185(175)	196(173)	30	45/190
Vitamin E (mg)	12(11)	11(5)	12(5)	14(16)	13(12)	N/A	7/14
Vitamin B12 (ug)	5(6)	5(3)	4(4)	5(6)	5(6)	2.0	2.4
Folate (ug)	360(212)	314(134)	442(209)	347(148)	459(437)	320	400/600
Vitamin A equiv	1350(1397)	1328(713)	1081(575)	1395(1746)	1809(2295)	500	700/1220
Carotene equiv [1]	4835(3049)	5704(3501)	4324(2914)	4103(2533)	6107(3645)		
Sodium (mg)	2398(954)	2319(908)	2891(1054)	2271(916)	2488(860)		/1600
Potassium (mg)	3483(978)	3444(860)	3741(1077)	3390(896)	3427(1493)	/2800	/4700
Magnesium (mg)	350(118)	345(116)	389(104)	340(102)	354(102)	265	320
Calcium (mg)	829(342)	814(281)	763(228)	839(377)	891(504)	1100	1300
Iron (ug)	14(6)	13.5(5)	16(5)	13(6)	14(7)	5	8
Zinc (mg)	11(4)	10(3)	12(4)	10(4)	10(4)	6.5	8
Servings/day*							
Fruit	1.99	2.0	2.1	2.2	1.7	≥2	
Vegetable	3.46	3.5	3.3	3.4	3.8	≥3	
Bread/cereals	4.4	4.1	4.9	4.5	4.6	≥6	
Dairy [2]	1.58	1.9	1.1	1.5	1.4	≥2	
Protein	1.9	1.8	1.7	2.0	2.0	1	
PRAL mEq/day†	-1.5	-2	-2	0	-6	0.74mEq/day	
NEAP mEq/day†	39.4	39.5	38.5	39.1	41.2	41mEq/day	

[1, 2] Anova values are significantly different at p<0 .02 for[1] and p<0 .005 for [2]

** All serving sizes: according to NZ Ministry of Health guidelines: fruit/vegetables = 50-80 grams, or 0.5 cup cooked or 1 cup raw (salad greens) or 1 medium fruit, starchy vegetables (135grams), protein includes meat, fish, eggs, nuts/seeds and beans.*

† Estimated PRAL (mEq/d) = 0.49 protein (gms/day) + 0.037 phosphorus (mg/day) - 0.021potassium (mg/day) – 0.026 magnesium mg/day -0.013calcium mg/day, Estimated NEAP (mEq/day) = PRAL (mEq/day) + Organic acid excretion (mEq/day).PRAL and NEAP values determined from a sample diet Ministry of Health, NZ (Ministry of Health (NZ), 2003).NZ reference values are: estimated average requirement (EAR) (50% population requirements) when EAR not available adequate intake (AI) is used instead. Recommended daily intake (RDI) (98% of population requirements) and "suggested dietary target" (SDT) if available.

Reported dietary intake was similar between all groups of women (Table 11). The women's diets met or exceeded New Zealand estimated average requirement (EAR) or adequate intake (AI) for most nutrients except calcium. Sodium intake exceeded the suggested dietary target (SDT) while potassium, folate and fibre intake didn't reach the SDT. All groups met the 5-plus/day recommendations servings of fruit/vegetables and had adequate meat/protein intake (1.9 servings/day), however intakes of breads/cereals (4.4 servings) and dairy (1.6 servings) were lower than recommended (Ministry of Health (NZ), 2003).

A significant difference was noted between the four BMD groups for dairy consumption with group 1 (normal BMD) having the highest consumption (1.9 serves). No significant differences were seen in estimated dietary PRAL and NEAP in the 4 BMD groups. Partial correlations (correcting for age and years since menopause) showed inverse relationships between bone resorption marker CTX and energy adjusted potassium intake and dairy consumption (Table 12). Positive associations (r=0.18 - 0.23) were also found between areal BMD and intakes of dairy products, protein, potassium, zinc and B vitamins thiamine, niacin and cobalamin, however, when these nutrients were combined in multiple regression models the significance only persisted for potassium and dairy intake. Analysis of a random subset of diaries for potassium demonstrated more than two thirds of potassium intake derived from fruit/vegetables.

Table 12 Correlations between selected nutrients, bone mineral density and bone turnover markers in post-menopausal study women (n=142)

	Cobalamin	Diary	Protein	K	Zn	Mg	Thiamine	Niacin
FN Hip BMD(g/cm^2)	.19*	.19*	.23*	.24*	.14*	.16*	.2	.24*
FN Hip T-score	.20*	.19*	.17*	.18*	.20*	.11	.14	.15
CTX	-.17*	-.2*	-.18*	-.18*	-.21*	-.09	-.12	-.13
P1NP	.12	-.19*	-.23*	-.24*	-.24*	-.20*	-.19*	-.2*

K= potassium, Zn = zinc, Mg = magnesium, Dairy = servings/day, FN = Femoral Neck, BMD = bone mineral density, CTX = C-terminal telopeptide of type 1 collagen, P1NP = Procollagen type I N propeptide, *Correlations r = ± .17 and above are significant at the p<0.05. Nutrients are energy adjusted values.
Pearson's correlation showed the women's areal bone mineral density was related to soft tissue compartments of fat and lean mass, weight and BMI. Body weight had the most consistently strong positive relationship with BMD at all 3 sites measured (Reid, 2008) (see Fig 16)

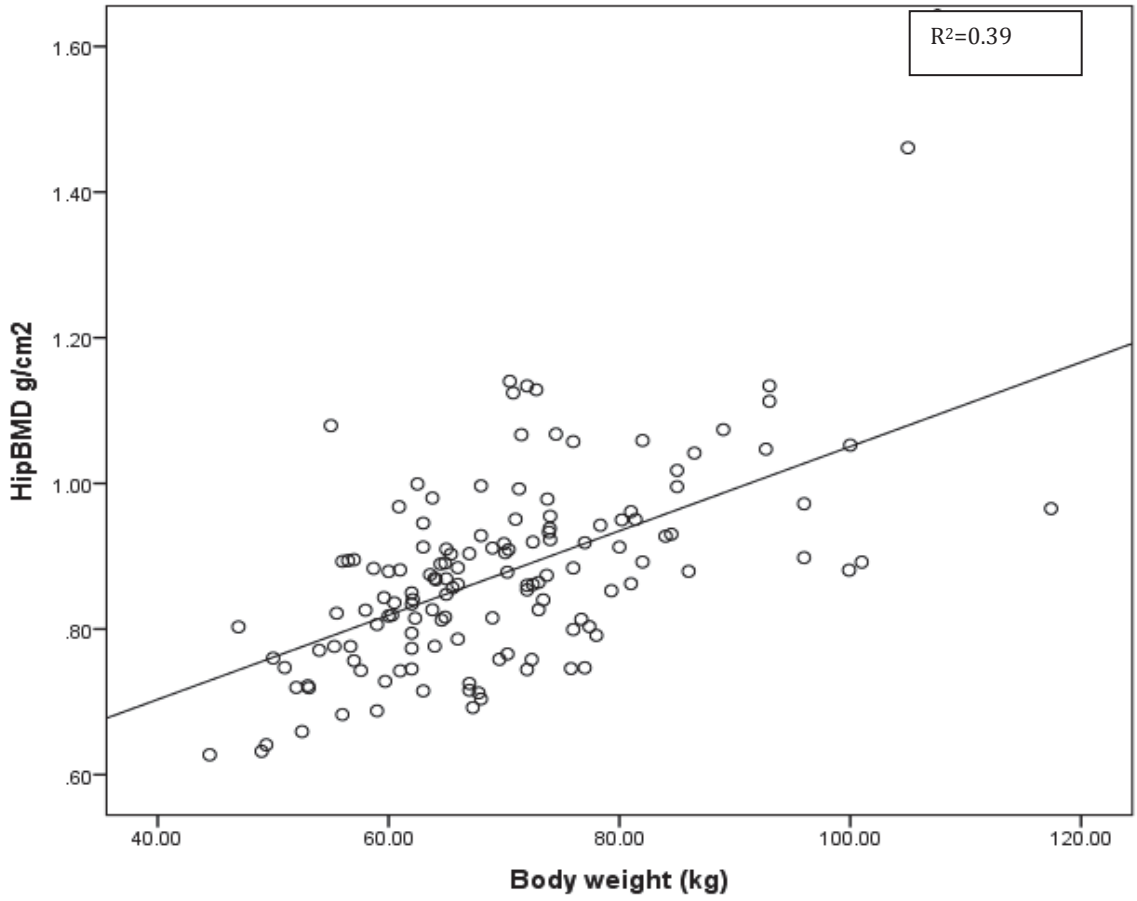

Figure 16 Relationship between body weight and hip bone mineral density in post-menopausal study women (n=142)

Regression relationship between weight in kilograms and hip bone mineral density (g/cm^2) in 142 postmenopausal women from three New Zealand regions. "r" is the Pearson correlation coefficient.

Bone turnover markers (CTX and P1NP) (Table 10) showed significant inverse correlations with BMD at all three measured sites spine, hip and neck of hip.

Increased levels of pro-inflammatory marker CRP (produced in the liver) were found in group 2 with highest percentage android (AFM) and total body fat, however, inflammatory cytokines (expressed per kg fat mass) associated with bone: OPG, IL-6, IL-10 and TNF were all higher in group 4 (osteoporosis) with OPG significantly higher than other groups. The median value for group 4 (Osteoporosis) being 0.17 ng/l (kg FM) (range-1.3, 0.08), $p< 0.007$. (Table 13, Figure 17).

Table 13 Comparison of cytokine production and body composition in post-menopausal study women based on bone density classification

	1 Normal BMD (51)	2 Mild Osteopenia (21)	3 Significant Osteopenia(53)	4 Osteoporosis(17)	p <
Weight (kg)	76(13)	72(9)	67(10)	61(9)	0.001
BMI (kg/m^2)	28(6)	28(3)	24(4)	22(3)	0.001
Lean mass (kg)	45(5.4).	43(5.3)	42(4.2)	39(5.1)	0.001
Fat mass (kg)	31(9.8)	29(9.1)	25(7.2)	22(8.2)	0.001
Body fat %	40(7)	41(7)	37(6)	37(7)	0.01
Android fat %	37(9)	41(8)	34(9)	32(7)	0.002
Gynoid fat %	42(6)	44(6)	41(6)	41(6)	0.25
CRP mg/l(kg FM) †	0.9 (0.9) (0.3-1.5,0.5)	1.3(1.3) (0.3-20,1.0)	0.77(0.88) (0.20-0.93,0.49)	0.91(1.2) (0.17-1.3,0.42)	0.73
OPG ng/l(kg FM) †	0.07(0.06) (0.03-0.08,0.05)	0.07(0.04) (0.04-0.08,0.06,)	0.07(0.04) (0.05-0.93,0.06)	0.13(0.12) (0.17-1.3,0.08)	0.007
IL-6 pg/ml(kg FM)†	0.06(0.05) (0.03-.08,0.05)	0.05(0.03) (0.04-0.08,0.05)	0.07(0.07) (0.02-0.08,0.05)	0.08(0.07) (0.04-0.09,0.07)	0.56
IL-10 pg/ml(kgFM)†	0.11(0.11) (0.05-.15,0.08)	0.11(0.07) (0.05-0.16,0.09)	0.10(0.09) (0.05-0.12,0.08)	0.20(0.3) (0.07-0.19,0.1).	0.53
TNF pg/ml(kg FM) †	0.31(0.28) (0.12-0.43,0.24)	0.29(0.19) (0.14-0.37,0.23)	0.29(0.25) (0.12-0.37,0.25)	0.30(0.16) (0.16-0.42,0.30)	0.74

Values are means (standard deviation) except inflammatory markers which given as both means (SD) and (range, median). † C - reactive protein (CRP), interleukin 6 and 10 (IL-6, IL-10), tumour necrosis factor (TNF) and osteoprotegerin (OPG) expressed per kilogram of fat mass (kg FM).
P values derived from ANOVA. P values <0.05 are considered statistically significant. Android fat= central/abdominal fat, gynoid fat =fat in gluteofemoral regions.

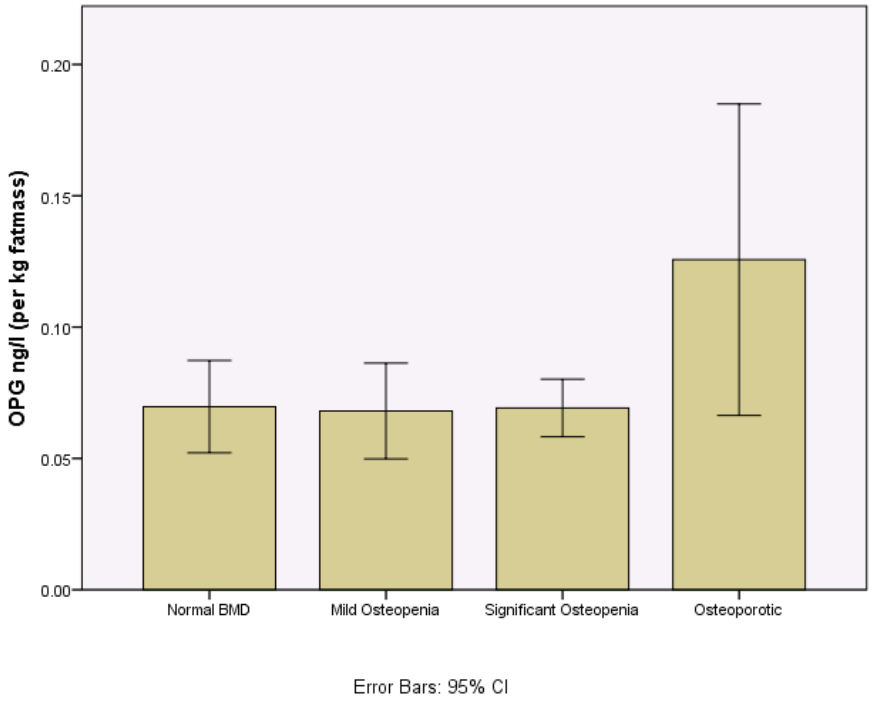

Figure 17 Osteoprotegerin (OPG) and bone density classification (DXA)

Osteoprotegerin is expressed per kilogram of fat mass. DXA groups are based on W.H.O classifications of T scores (World Health Organisation, 2004).

Normal T-score <1.0 SD below normal, osteopenia T -score 1.0-2.49 SD below normal and osteoporosis T- score ≥2.5 SD below normal. This study divided the osteopenic group into mild and significant osteopenia with mild osteopenia those participants with a T score ≥1-1.49 SD below normal and significant osteopenia participants with a T score ≥1.5 - ≤2.49 SD below normal.

Multiple regression analysis was used to assess the combined effects of OPG, weight, CTX, dairy and potassium intake on BMD. The equation that predicts hip BMD from the independent variables was found to be:

Hip BMD= -.221 +.026(dairy) +0.006(weight) -0.001(OPG) +.18(K) -0.003(CTX)

The value of R^2 (for Hip BMD) was 0.39 (adjusted R^2 was 0.36) a value that was highly significant (p<0.00) (Figure 16). F (5,119) = 15.14, $MS_{residual}$ was 1.2, p< 0.00. The standard error of the estimate was 0.10. Although each independent variable alone correlated significantly with hip BMD, only

body weight, dairy intake and potassium accounted for a significant amount of unique variance of hip BMD.

This multiple regression shows when body weight, dairy and potassium intake are used to predict a woman's hip BMD, 39% of the variance in BMD is removed. The coefficient of variation for the hip BMD model was 10.7% indicating a fair to good fit (a good fit being 10% or less). Semi partial r values and values of Beta (standardised coefficients) for all the independent variables are shown in Table 14 together with results of the significance tests.

Table 14 Regression coefficients β for independent predictors of BMD

	Hip BMD R^2=0.39				Femoral Neck of Hip BMD R^2=0.27				Lumbar spine BMD R^2=0.28			
	Semi partial r	β	T (124)	*P* value	Semi partial r	β	T (124)	*P* value	Semi partial r	β	T (124)	*P* value
OPG	.001	.001	.015	.99	-.02	-.03	-.3	.77	.01	-.02	.18	.86
Weight	.450	.60	6.3	.00	.31	.40	4.0	.00	.35	.45	4.6	.00
Potassium †	.166	.17	2.30	.02	.21	.22	2.7	.01	.14	.15	1.8	.07
Dairy	.171	.18	2.40	.02	.16	.17	-.76	.05	.15	.16	1.9	.06
CTX	-.003	-0.004	-.049	.96	-.06	-.07	-1.03	.30	-.09	-.02	.18	.86

β coefficient = standardised regression coefficient.
† Energy adjusted value.
Coefficient of variation for Hip BMD 10.7%, Neck of hip BMD 12.2% and Spine BMD 13.7%.
BMD bone mineral density, OPG osteoprotegerin, CTX C terminal telopeptide of type 1 collagen.

5.5 Discussion

The women in this study were predominantly European (98%) and 12% were classified with osteoporosis (T score ≤-2.5 SD below normal). As prevalence of osteoporosis varies with ethnicity (Kanis, McCloskey, et al., 2008) this is higher than reported population estimates for white PM women in Canada (8%) but less than the U.S (17%), U.K and Sweden (21%) (Tenenhouse. A, Joseph. L, Kreiger. N et al., 2000; Kanis, McCloskey, et al., 2008).

There were no significant differences between the four BMD groups, for reported nutrient intake or dietary acid load (Table 10 and 11) except for dairy intake with the group with normal BMD having the highest intake. Dairy products provide calcium, protein and potassium (Heaney, 2009) and positive associations with BMD were found for protein, potassium, vitamin B12 and zinc, all linked to improved bone status and reflective of higher meat/dairy and fruit/vegetable intake (Tucker et al., 1999; New et al., 2003; Anderson, 2012).

Reported measures of dietary acid load (PRAL and NEAP) (Table 11) were similar to other studies (Macdonald et al., 2008; Nowson, Patchett, et al., 2009) and no significant association with BMD was found, however, an inverse relationship existed between levels of bone resorption marker CTX and both dairy servings and potassium intakes (Table 12) reflecting the positive correlation of fruit/vegetables and dairy on bone health, which has previously been noted (Tucker et al., 1999; Demigne et al., 2004; Doyle et al., 2004; Rafferty & Heaney, 2008; Tylavsky, Spence, Harkness et al., 2008; Heaney, 2009; Whiting, 2011).

Increased levels of obesity in post-menopausal women are now seen as a risk factor for osteoporosis (Halade et al., 2011). While half (49%) of the women in this study had a BMI within the normal range, the other half were either overweight (30%) or obese (21%) (Table 10). Body weight, BMI and soft tissue compartments of fat and lean mass were investigated for their relationships with BMD with body weight being the most positively associated with BMD.

Body weight is made up of both lean mass and fat mass, with lean mass said to have more significant influence on BMD in pre-menopausal women while fat mass is more significant in post-menopausal women (Reid, 2002, 2010). In this study, women's fat mass was more highly correlated with hip BMD than lean mass. While lean mass is positively associated with BMD, the effect of fat mass on BMD is less conclusive, with both positive effects attributed to skeletal loading and negative effects attributed to inflammation (Reid, 2008, 2010). We found lower levels of both fat and lean tissue were associated with increased bone loss as seen in group 3 and 4 (Table 13).

Fat is an endocrine organ therefore obesity and increased fat mass particularly visceral or android fat (Gilsanz et al., 2009; Kawai, de Paula, & Rosen, 2012) are now considered a potent source of cytokines (adipokines). Increased secretion of TNF and IL-6 and other inflammatory cytokines by macrophages which infiltrate fat tissue may induce a chronic low grade inflammatory state which contributes to inflammatory bone loss leading to osteoporosis (Galic et al., 2009; Zhao, Grimes, Li et al., 2012).

Increased production of pro-inflammatory markers such as TNF and IL-6 affect bone formation particularly osteoclastogenesis (Kawai et al., 2012), however, a moderate inverse relationship was found between BMI and osteoclastogenesis in this study with resorption marker CTX ($r = -0.3$, $P<0.00$) and formation marker P1NP ($r = -0.22$, $p<0.00$) (Revilla, Villa, Sanchez-Atrio et al., 1997), indicating increased body mass in this study was associated with reduced levels of bone turnover.

There was a significant difference seen between the BMD groups OPG production, with higher levels in the osteoporotic group OPG (Figure 17). Group 2 had the highest level of CRP which is produced in the liver and is a measure of systemic inflammation (Bhupathiraju et al., 2011). CRP production has been strongly linked to fat mass and in particular, abdominal fat mass (android) (Lapice, Maione, Patti et al., 2009; Bhupathiraju et al., 2011) and in this study Group 2 had a significantly higher percentage body and android fat. Higher CRP values may be a result of systemic inflammation

associated with higher abdominal adiposity and therefore represent an additional cardiometatabolic risk factor (Lapice et al., 2009).

OPG production reflects a variety of metabolic processes (Wagner & Fahrleitner-Pammer, 2010), and the increased levels seen in the osteoporotic group may be due to elevated bone turnover with a higher pool of osteoblasts and precursors releasing OPG (Wagner et al., 2010). This group also had significantly higher levels of resorption marker CTX reflecting increased osteoclastogenesis which may be due to less OPG attached to receptor activator of nuclear factor *kappa B* ligand (RANKL) therefore increasing osteoclastic activity (Reyes-Garcia, Munoz-Torres, Garcia et al., 2010; Wagner et al., 2010) which is central to bone loss. The significant positive relationship seen in this study between CTX and OPG and the inverse seen with both these markers and weight/BMI reinforces the protective relationship between fat and bone (Reid, 2010).

5.6 Conclusion

This study of post-menopausal women demonstrated significantly increased OPG (per kilogram of fat mass) and lower bodyweight were associated with osteoporosis indicating increased body weight was protective of bone loss. Higher CRP levels were seen with higher abdominal fat mass and increased dairy and potassium intake (fruit/vegetables) predicted lower levels of bone resorption marker CTX as well as higher BMD. Our findings highlight the importance of dietary intake of dairy and potassium sourced from fruit/vegetables to maintain bone health at midlife.

5.7 Acknowledgements

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CHAPTER 6 EFFECTS OF INCREASED VEGETABLES/HERBS/FRUIT ON BONE AND INFLAMMATORY MARKERS INCLUDING ADIPONECTIN

This study describes the changes that occurred in bone and inflammatory markers over the three month dietary intervention in three groups of post-menopausal women who consumed different diets high in potassium from fruit/vegetables. It also describes some of the changes in nutrient intake and the effect of the dietary change on calcium excretion. The third group who were a control group (C) is also described. This group was to make no changes from their usual diet but large increases in potassium urinary excretion were noted. This phenomenon has been described as the "Hawthorne effect " a well-known effect in human intervention studies in Psychology whereby by signing a consent form and being part of a study unconsciously leads participants to focus on the study topic and make associated changes regardless of which group they are assigned. However, despite the control group making dietary changes which were not expected, this afforded the opportunity to compare three groups of women with diets high in potassium.

Manuscript submitted to Nutrients for review

Caroline Ann Gunn, Janet Louise Weber, Anne-Thea McGill and Marlena Cathorina Kruger. Effects of increased intake of vegetables/herbs/fruit on bone and inflammatory markers.

6.1 Abstract

Consumption of vegetables/herbs/fruit may reduce bone loss in midlife women through provision of polyphenolic compounds, potassium and lower renal acid load, which reduce inflammation and urinary calcium excretion. We hypothesised that 9 servings/day of vegetables and fruit would reduce inflammatory and bone turnover markers and urinary calcium excretion, and the emphasis on vegetables/herbs/fruit containing bone friendly polyphenols may enhance the effect. Using a randomised controlled design, two intervention groups of healthy post-menopausal women (n=50) consumed ≥ 6 servings vegetables and ≤ 3 servings of fruit for 3 months. Intervention group A emphasised a generic range of vegetables, herbs and fruit whereas intervention group B (Scarborough Fair group) emphasised vegetables/herbs/fruit with bone resorbing inhibiting properties. A control group (n=43) was instructed to consume their usual diet. Plasma bone and inflammation markers, urinary electrolytes (24 hour), dietary intake (three day diaries) and estimated dietary potential renal acid load (PRAL) were assessed at baseline and 12 weeks. Bone marker PINP was reduced (-3.2µg/L, $p<0.01$) in the Scarborough Fair group CTX was reduced (-0.065µg/L, $p<0.01$) in women with osteopenia in this group. Inflammatory markers decreased in all groups and urinary potassium increased in control group reflecting dietary change ("Hawthorne effect"). Urinary calcium conservation occurred in all three groups but intervention groups A and B (SF) with decreased PRAL and increased urine pH had a significantly decreased percentage of urinary calcium loss. The Scarborough Fair group demonstrated positive changes in both turnover markers and calcium conservation.

6.2 Introduction

Loss of bone mass is a public health issue due to increased life expectancy paralleling rapid ageing of the world's population (Dawson-Hughes & National Osteoporosis Foundation Committee, 2008; Dawson-Hughes et al., 2009; P. Brown et al., 2011). Women are vulnerable to increased bone loss during and after menopause which may lead to bone fragility, fractures and disability in later years. Maintenance of bone health with ageing is attributed to genetics, sun exposure, exercise and diet.

Dietary choices based on unprocessed foods such as vegetables and fruit (Ashwell et al., 2008) are important for bone health through provision of raw nutrients: potassium (K. Zhu, Devine, & Prince, 2009), polyphenols (Weaver et al., 2012) and fibre (Wood & McDonald, 2013), and decreased sodium intake (Nordin, Need, Morris et al., 1993). Increased consumption of vegetables and fruit provides a favourable ratio of sodium/potassium, dietary acidity (New et al., 2004) which alleviates associated hypercalciuria (Shi et al., 2012). Increased phytochemical intake may counteract the pro-inflammatory milieu associated with ageing.

Emerging evidence of the interdependence of immune and skeletal systems demonstrates bone loss during ageing is exacerbated by increased inflammation (Schett, 2011). Metabolic inflammation (metaflammation), the same nutritional related disturbance seen in metabolic syndrome, cardiovascular and cancer risk (Hummasti & Hotamisligil, 2010), causes bone loss by uncoupling the balance between bone resorption and formation through effects on receptor activator of nuclear factor kappa B (RANK), its ligand (RANKL) and osteoprotegerin which are highly susceptible to circulating levels of inflammatory cytokines interleukin 6 (IL-6), interleukin 10 (IL-10) and tumour necrosis factor (TNF).

Plant compounds may suppress this inflammatory response (Son et al., 2008; Birringer, 2011; Salminen et al., 2012). Decreasing inflammation by dietary patterns which increase vegetables and fruit while reducing intake of less nutrient dense foods may offer another important element in reducing bone loss (Habauzit et al., 2008) and other diseases associated with ageing (Dinkova-Kostova et al., 2008; Trzeciakiewicz et al., 2009; WHO., 2012).

Paradoxically, adiponectin, an anti-inflammatory adipokine made in fat cells and positively associated with plant-based diets (Cassidy et al., 2008), is negatively associated with bone mass and higher levels predict increased fracture risk (Biver et al., 2011; Barbour et al., 2012). Intervention studies have shown increased adiponectin levels with lower glycaemic diets (Neuhouser et al., 2012), lower fat or lower carbohydrate diets, and supplements of fish oil, omega 3 fatty acids and fibre and

an increase in this cytokine is purported to be beneficial (Silva et al., 2011). The effect of an intervention with increased intake of vegetables and fruit on adiponectin levels has not been investigated.

While it has been acknowledged for over a decade that plant phytochemicals modulate bone metabolism (Huang et al., 2008; Weaver et al., 2012), greater understanding has emerged of mechanisms at the molecular level. The seminal work of Mühlbauer (2002) determined that bone resorption was reduced in animals fed particular vegetables/herbs/fruit due to pharmacologically active phytochemicals rather than their base excess (Mühlbauer et al., 1999; Mühlbauer, 2006; Huang et al., 2008). Since then, the effect of phytochemicals in dried plums (Franklin et al., 2006; Hooshmand & Arjmandi, 2009) and onions (Horcajada-Molteni et al., 2000; Huang et al., 2008) on osteoclast inhibition, reduced bone loss and increased BMD (Matheson, Mainous, & Carnemolla, 2009) has been affirmed.

Recently, research has focused on a wider array of phytochemicals in plants and impact on cell signalling pathways (Crozier et al., 2009) directing differentiation of osteoblasts and osteoclasts (Habauzit et al., 2008). The flavonoid hesperidin (citrus)(Trzeciakiewicz et al., 2009) regulate osteoblast differentiation, while quercetin (plums) and kaempferol (onions, broccoli) inhibit osteoclastic resorption with kaempferol particularly inhibitory. Although there are interwoven connections and multiple signalling cascades, inflammation with suppression of transcription factors nuclear factor kappa B (NFκB) and activator protein-1 (AP-1) is strongly associated with osteoclasts number and activity (Habauzit et al., 2008).

This study differs from previous studies investigating a dietary approach to reducing bone loss by emphasising an increase in vegetables (≥6 servings/day), rather than fruit (≤3 servings/day), inclusion of herbs and combining vegetables/herbs/fruit with known inhibitory effects on bone turnover (animal model) (Mühlbauer et al., 2002) in group B (The Scarborough Fair group) compared to another

intervention group (A) consuming vegetables/herbs/fruit (9 servings/day) with no known effect on bone turnover markers and a control group (C) (usual diet).

6.3 Subject and Methods

6.3.1 Study design

A three month randomised active comparator intervention compared effects of consuming ≥9 servings of vegetables and fruit/day plus selected culinary herbs on two groups of PM women emphasising differing vegetables/herbs/fruit and a control group (usual diet). Outcome measures included plasma bone turnover markers: C-terminal telopeptide of type I collagen (CTX) and procollagen type I N propeptide (P1NP), inflammatory markers: adiponectin (total molecular weight), IL-6, IL-10, TNF, OPG and C- reactive protein (CRP), urinary mineral excretion, urinary pH and dietary intake including estimated dietary potential renal acid load (PRAL).

6.3.2 Participants

We recruited 100 healthy women (non-smokers) aged between 50 and 70 years from three New Zealand regions and using block randomisation technique assigned them to group A and B. The inclusion criteria were: at least 5 years post-menopausal (PM), not on medication affecting bone and inflammatory markers e.g. hormone replacement therapy (HRT) within the last 2 years, proton pump inhibitors or non-steroidal anti-inflammatory medication, calcium or dietary supplements. We separately recruited 43 PM women using the same inclusion and exclusion criteria as a control group (Gunn, Weber, & Kruger, 2013). The 143 recruited women were predominantly white (98%). Ethical approval was obtained from Massey University Human Research Ethics Committee (Southern A) Reference number 11/11. All participants were fully informed of the study requirements and gave written consent. The trial was registered with the Australian and New Zealand Clinical Trials Registry (ANZCTR) http://www.ANZCTR.org.au. **Trial Registration**: ACTRN 12611000763943.

6.3.3 Study diets

To optimise reaching a physiological dose of bone modulating phytochemicals, we combined vegetables/herbs/fruit with proven bone modulating effects as dietary choices for one group of women (B) named the Scarborough Fair group (SF). At least half of total daily vegetable servings were to include prescribed vegetables such as: lettuce, onions, garlic, Chinese cabbage, broccoli and fruits: prunes and oranges with herbs additional (parsley, sage, rosemary and thyme). The other intervention group (A) also consumed 9 servings/day e.g. cabbage (non-Chinese), pumpkin, silverbeet, apples, bananas with herbs basil, mint and oregano. We asked both intervention groups to: consume ≥6 servings of vegetables, including ≥2 servings/day of green leafy vegetables to control for vitamin K intake, and dairy (or calcium enriched milk substitute) to control for calcium intake, and ≥1 culinary herb daily (dried/fresh) and refrain from consuming the alternate group's selection of vegetables/herbs/fruit.

Fruit intake was limited (≤3 servings/day) to avoid fruit rather than vegetable intake increasing (Dragsted et al., 2004; Macdonald et al., 2008) and intervention groups were blinded to which vegetables/herbs/fruit had proven bone modulating effects. The women were advised this intervention was not a weight loss strategy and to maintain their normal exercise levels. Significant changes in health/medication were recorded in a bi-weekly diary along with daily urine pH and vegetable/herbs/fruit intake (Gunn, Weber, & Kruger, 2013). Participants received fortnightly emails and could email queries to the study coordinator. A control group received instruction to maintain their normal diet but was aware their purpose was for comparison with two intervention groups increasing vegetables and fruit.

6.3.4 Dietary analysis

3 Day Diet Diaries (3DDDs) were completed at baseline and end of study (week 12). Participants recorded all food and beverages consumed over 2 weekdays and 1 weekend day, including types, brands and amounts (cups, tablespoons, etc.) of foods as well as recipes for homemade dishes. Participants supplied nutritional information panels from processed food packets. All 3DDD's were

checked for accuracy and completeness by a New Zealand (NZ) registered nutritionist at the first visit. Prompting methods were used for incomplete quantities or to ascertain specific food types.

Data were entered into Foodworks (version 2009, NZ, Xyris software) then transferred to SPSS (version 20) via Microsoft Access (2007) and Excel (2007). Estimated potential renal acid load (PRAL) expressed in milliequivalents per day (mEq/d) = 0.49 protein (gms/day) + 0.037 phosphorus(mg/day) - 0.021 potassium(mg/day) – 0.026 magnesium(mg/day) -0.013 calcium(mg/day) (Frassetto, Lanham-New, et al., 2007). Nutrients were energy adjusted using the nutrient energy model (Willett, Howe, & Kushi, 1997).

6.3.4.1 Specimen collection and analysis

Baseline and end of study plasma markers included: bone markers of resorption, C-terminal telopeptide of type I collagen (CTX) and formation, procollagen type I N propeptide (P1NP) and inflammatory markers: C- reactive protein (CRP), interleukin 6 and 10 (IL-6, IL-10), tumour necrosis factor (TNF), adiponectin (total molecular weight) and osteoprotegerin (OPG). In short, overnight fasted blood samples were taken between 8-10 am, centrifuged (3000 rpm,1560 RCF), separated and stored (-80ºC) until bone markers were analysed at Canterbury Health Endocrine Laboratory, Christchurch, NZ (Roche Elecsys 2010, Roche Diagnostics) and inflammatory markers at Plant and Food Research Auckland, NZ (FlowCytomix kits (Bender Medsystems, Austria)(Gunn, Weber, & Kruger, 2013). 24 hour urine samples were collected, measured and frozen until samples analysed by atomic absorption flame emission spectrophotometry (Gunn, Weber, & Kruger, 2013).

6.3.4.2 Bone densitometry

Dual X-ray absorptiometry (DXA) scans of lumbar spine (L1-L4) and hip (total and femoral neck) was performed using a Hologic QDR-Discovery A densitometer (Hologic Inc, Bedford, Mass., USA) giving measures of bone mineral content (BMC)(grams), BMD (grams/cm^2), T and Z scores, body fat and lean measures (android and gynoid). The machines were calibrated daily with in-vivo reproducibility of coefficient of variation 0.45-0.54% for all measured sites.

6.3.5 Statistical analyses

The Statistical Package for Social Sciences (SPSS Inc. Chicago, IL, USA) version 20.0 was used for all analysis with a significance level of $p< 0.05$ (2 tailed). Data was checked for distribution using the Kolmogorov–Smirnov statistical test and Levene's Test for equality of variance. Parametric statistical analysis was done for normally distributed data otherwise non-parametric tests (Kruskal-Wallis Test) were used (potassium urinary excretion) or logarithmic transformations were applied prior to one way analysis of variance analysis (ANOVA). Log transformed values were back transformed for tables. ANOVA was used to compare inflammatory, bone and urinary mineral excretion data. Post hoc tests (Scheffe and Tukey-B) were used to determine group difference as well as student t-tests for within group differences. Two way ANOVA was used to determine interaction effects between groups and BMD groups on bone markers using MANOVA. To avoid type 1 errors due to multiple comparisons being made with the pairwise comparison of the levels of each factor (BMD) within the levels of the other factor (group), the Sidak correction was applied. Repeated measures ANOVA were used to determine differences between intervention groups using baseline CTX as covariate. Most data are expressed as means and standard deviations (SD) or 95% confidence intervals. Pearson and partial correlations (adjusted for age and years since menopause) determined associations between inflammatory markers and BMD.

6.4 Results

6.4.1 Compliance

Bi-weekly diaries kept by intervention group participants A and B compliance with dietary counselling to increase consumption of vegetables/herbs/fruit. Changes in urinary potassium excretion demonstrated that the control group who received no dietary counselling to increase fruit/vegetables inadvertently increased their intake, possibly through the "Hawthorne Effect" whereby signing an informed consent and understanding they were to be compared with a group, increasing fruit and vegetable intake influenced them to also make this dietary change (Konstantinou, 2012).

6.4.2 Anthropometric and bone mineral density measurements

Baseline measurements showed groups were similar in anthropometric and BMD measures. Body mass index (BMI) however, was higher in group A than group C ($p<0.02$) (Table 15). The mean BMI was 25.7 and 50% of women had a normal BMI and 50% were either overweight (27%) or obese (23%). There was no change in weight or BMI during the study. The women had an average age of 61±4.4 years with a mean of 11 ± 4.7 years since menopause (YSM). BMD was determined at baseline and results were classified according to W.H.O classification (World Health Organization, 1994; World Health Organisation, 2004). Over a third of the women had normal BMD (36%), over half had osteopenia (52%) while 12% were classified as osteoporotic.

Table 15 Baseline anthropometric and bone mineral density (BMD) characteristics of study population.

	Group A N=49	Group B N=50	Group C N=43	P [1]
Age (years)[2]	60 ± 4 (53-68,60)[1]	60 ± 4 (51-71,61)	61 ± 5 (51-71,61)	0.66
YSM[3]	11± 5 (9-12)	11 ± 4 (9-12)	11 ± 5 (9-12)	0.97
Weight (kg) (baseline)	72 ± 13 (68-76)	70 ± 13 (66-73)	66 ± 11 (63-70)	0.09
Weight (kg) (end)	72 ± 12.5 (68-74)	70 ± 13 (66-73)	66 ± 11 (62-70)	0.08
Height (metres)	1.6 ± .5 (1.61-1.64)	1.65 ± 7 (1.62-1.167)	1.63 ± 7 (1.61-1.66)	0.10
BMI[4] (kg/m^2)	27 ± 5 (26-29)	25 ± 5 (24-27)	24 ± 5 (23-26)	0.01
Body fat %	40 ± 6.6 (38-42)	38 ± 7 (36-40)	37 ±6 (35-39)	0.14
BP[5] systolic (mmHg)base	131 ± 16 (126-135)	132 ± 18 (127-137)	129 ±18 (124-135)	0.77
BP systolic (mmHg)end	131 ± 15 (126-135)	129 ± 17 (125-134)	127 ±18 (122-133)	0.62
BP diastolic(mmHg)base	80 ± 10 (77-83)	79 ± 10 (76-81)	78 ±9 (75-81)	0.51
BP diastolic (mmHg) end	79 ±10 (76-82)	79 ± 10 (76-82)	78 ± 9 (75-80)	0.76
BMD[6] (n=142)				
Normal BMD	20	16	15	
Osteopenia	26	26	22	
Osteoporotic	4	9	4	
Spine BMC(g/cm)	53 ±13 (49-57)	55 ± 10 (52-58)	55 ±13 (50-59)	0.77
Spine BMD(g/cm^2)	0.95 ±15 (0.9-0.99)	0.94 ± 14 (0.9-0.98)	0.97 ±.2 (0.9-1.0)	0.75
Spine T-score	ˉ0.81 ±1.2 (ˉ1.1- ˉ0.47)	ˉ.92 ± 1.3 (ˉ1.3-ˉ0.56)	ˉ.56 ±1.6 (-1.1-ˉ0.06)	0.44
HipBMC(g/cm)	30 ± 5 (29-32)	29 ± 5 (28-31)	30 ±5 (29-32)	0.56
HipBMD(g/cm^2)	0.88 ±11 (0.85-0.91)	0.86 ± .14 (0.82-0.89)	0.88 ±. 013 (0.84-0.92)	0.47
HipT-score	ˉ0.50 ± 0.9 (ˉ0.75-ˉ0.25)	ˉ0.69 ± 1.1 (ˉ1.0-ˉ0.25)	ˉ0.48 ±1 (ˉ0.8-ˉ0.15)	0.52
NeckHip BMC(g/cm)	3.7 ±.7 (3.5-3.9)	3.5 ± .82 (3.3-3.75)	3.80 ±.6 (3.6-3.99)	0.15
NeckHip BMD(g/cm^2)	0.76 ± 0.12 (0.72-0.79)	0.72 ± 0.11 (0.69-0.75)	0.75 ± 0.12 (0.72-0.79)	0.16
NeckHip T-score	ˉ0.84 ±1 (-1.2-ˉ0.54)	ˉ1.2 ± 0.95 (ˉ1.5-ˉ0.9)	ˉ0.86 ±1.1 (ˉ1.2-ˉ0.5)	0.17

[1] One way ANOVA p<0.05 considered significant between groups.
[2] Values are Mean ± SD(95%CI) except for age which is mean ± SD, (range, median).*
[3] YSM=years since menopause.
[4] BMI= body mass index (weight (kg)/height.
[5] BP=blood pressure.
[6] Normal: BMD higher than 1 SD below young adult female reference mean (T-score ≥− 1 SD).
Osteopenia: BMD 1 SD or more below the young female adult mean (T-score < − 1 and > − 2.5 SD).
Osteoporosis: BMD 2.5 SD or more below the young female adult mean (T-score ≤ − 2.5 SD).

6.4.3 Changes in food group servings

The main changes reported in serving numbers were in vegetables and fruit food group where intervention groups differed significantly from control group (Table 16). Fruit servings/day increased by 0.9 (Group A), 1.3 (Group B), and -0.06 (Group C) ($p<0.001$). Vegetable servings/day increased by 2 (Group A), 2.8 (Group B) and 0.2 (Group C) ($p<0.001$). Intake of dairy servings/day decreased in Groups A (-0.2, $p<0.03$) and C (-0.4, $p<0.003$) while the meat/protein food group was unchanged in all groups. Breads/cereals servings/day decreased in intervention groups by 1.4 (Group A) and 1.2 (Group B) compared to control group reduction of only 0.2 servings/day (Group C), ($p<0.03$). The increased servings in fruit/vegetable and decrease in breads/cereals significantly reduced PRAL (Frassetto, Morris, & Sebastian, 2006) in the intervention groups (-17,-22mEq/day) compared to the control group (-1.5mEq/day, $p<0.001$).

Table 16 Changes in food group servings and PRAL/NEAP from baseline to end of dietary intervention

Servings[1] /day	Group A N=47	Group B (SF)[2] N=50	Group C (control) N=41	p[3]
Fruit (baseline)[4]	2.0 ±0.9(0- 3.8)	1.8 ±0 .9(0.3-3.8)	2.2 ±1.2(0-4.7)	0.15
Fruit (final)	2.9 ±1.4(0.50-7.0)	3.0 ±1.0 (1.3-5.3)	2.2 ±1.4(0-6.3)	0.003
change	0.9±1.5 (¯2.3-4.8) p [5]<0.001	1.3 ±1.1(¯0.8-4.0) p <0.001	¯0.06 ±1.0(¯2.0-2.0) p<0.51	0.001
Vegetables(baseline)	3.4 ± 1.2(1.3-6.0)	3.3 ± 1.3(1.0-7.8)	3.7 ± 1.3(1.2-7.0)	0.21
Vegetables(end)	5. 5 ±1.9(1.7-10)	6.1 ± 1.9(2.7-12.2)	4.0 ± 1.2(0.7-6.3)	0.001
Change[5]	2.0 ± 2.2(¯3.7-7.3) p <0.001	2.8 ± 2.1(¯2.5-8.2) p <0.001	0.2 ± 1.5(¯3.7-2.8) p <0.49	0.001
Bread/cereals (baseline)	4.5 ±1.60 (1.0-8.3)	4.4 ±1.4(0.3-7.5)	4.2 ±1.4(1.7-7.5)	0.54
Bread/cereals (end)	3.4 ±1.52(0.83-7.8)	3.3 ±1.5(0-7.0)	4.1 ±0.7(0.5-8.0)	0.03
change[5]	¯1.4 ± 1.9(¯5.7-2.3) p <0.001	¯1.2 ± 1.5(¯5.7-1.5) p <0.001	¯0.22 ±1.5(¯4.7-2.2) p <0.48	0.03
Dairy (baseline)	1.5 ±0.74(.3-2.8)	1.5 ±0.9(.2-4.2)	1.8 ±1(.3-5.3)	0.28
Dairy (end)	1.3 ±0.98(.0-5.0)	1.5 ±0.8 (0.2-3.5)	1.4 ± 0(.0-4.5)	0.77
change	¯0.2 ± 1.0(¯2.0-3.3) p <0.03	¯0.08 ±0.8(¯2.3-1.7) p <0.5	¯0.4 ± 0.8(¯2.0-1.5) p <0.003	0.24
Meat and protein(baseline)	1.9 ± 0.91(0.3-4.3)	1.8 ± .8 (0.3-5.0)	2.0 ± .8 (.50-4.3)	0.43
Meat and protein (end)	1.8 ± 0.97 (0.2-4.8)	1.7 ± .8 (0.3-4.0)	1.95 ± 1.0 (0.2-6.3)	0.34
change	¯0.11± .98 (¯2.7-2.5) p <0.46	¯0.11 ± 0.9 (¯2.0-2.0) p <0.40	¯0.09 ± 1.0 (¯2.2-3.8) p <0.69	0.99
PRAL (baseline) mEq/day[6]	¯0.1 ±16 (¯36-31)	¯1.2 ±15 ¯ (34-26)	¯1.8 ±13 (¯43-27)	0.76
PRAL (end) mEq/day	¯17 ±17 (¯45-27)	¯23 ±16 (¯65-10)	¯3 ±16 (¯36-40)	0.001
change[5]	¯17±17 (¯55-15.) p <0.001	¯22 ± 25. (¯65-10) p <0.001	¯1.6 ±18 (¯30-60) p <0.57	0.001

[1]All serving sizes according to NZ Ministry of Health guidelines: fruit/vegetables = 50-80 grams, or 0.5 cup cooked or 1 cup raw (salad greens) or 1 medium fruit, starchy vegetables (135grams), protein includes meat, fish, eggs, nuts/seeds and beans.
[2] Scarborough Fair Group (SF).
[3] One way ANOVA. P values <0.05 are considered statistically significant.
[4] Values are means ± SD (minimum and maximum).
[5] Paired t-test showing significant differences in changes within each group.
[6]PRAL=Estimated potential renal acid load (PRAL) expressed in milliequivalents per day (mEq/d) = 0.49 protein (gms/day) + 0.037 phosphorus (mg/day) - 0.021 potassium(mg/day) – 0.026 magnesium(mg/day) -0.013 calcium(mg/day).

6.4.4 Changes in dietary nutrient intake

Nutrient intake changes were assessed from baseline and end of study three day diet diaries. Nutrient intake was similar for all groups at baseline (Table 17) and by end of study calcium, potassium, magnesium, folate and fibre intake had increased in intervention groups. Energy intake was adjusted (8000 kJ) to more accurately reflect nutrient intake change in composition of the diet during the intervention (Willett et al., 1997).

Table 17 Nutrient intake for each group at baseline and changes at 3 months

	Group A Intervention N=47	Group B (SF)[1] Intervention N=50	Group C Control N=41	EAR[2]/AI[3] RDI[4]/SDT[5]	P[6]
Baseline					
Protein (g)[7]	86±17 (49-128)	82±18(46-128)	83.5±13.6 (56-117)	37[2] 46[4]	
change	2.2±19 (-67-35)	2.0± 17(-34-45)	0.10± 16 (-53-35)		
Fat (g)	72±16(35-98)	75±14(47-102)	74±17(42-112)	N/A	
change	2.30±15(-36-24)	2±19(-49-57)	4±20(-69-41)		
CHO(g)	220±43(150-350)	216±32(107-275)	220±41(132-304)	N/A	
change	19± 72(-115-195)	5.2± 52 (-148-133)	6± 65(-155-124)		
Fibre(g)	27±8(14-50)	27±8(12-56)	28±8(13-43)	25[3] 28[5]	
change	5.7±6.6(-22-7)	6.4±10.3(-28-18)	-0.5±.00(-24-24)		0.001
Folate (µg)	368±153(102-812)	380±225(151-1646)	381± 134(205-687)	400[4] 600[5]	
change	109±213- (865-447)	121±250(-561-1177)	-9±164- (237-548)		0.009
Sodium (mg)	2345±618(1261-4046)	2766±900(1242-4593)	2551±979(947-6405)	1600[5]	
change	-63±887(-2466-1566)	-376±943(-1561-2461)	24.5±1256(-2147-3230)		
Potassium (mg)	3695±766(2400-6261)	3643±1000(2013-6902)	3781±790(2410-6027)	2800[3] 4700[5]	
change	935±953(-3246-775)	1393±1375(-5401-2483)	64±933(-1758-2414)		0.001
Magnesium(mg)	365±930(232-694)	366±105(208-667)	385±112(231-941)	265[2] 320[4]	
change	73±134(-548-298)	54±106(-285-229)	-20±113(-143-532)		0.001
Calcium (mg)	850±257(375-1532)	872±347(404-2166)	905±270(396-1632)	1100[2] 1300[4]	
change	181±353(-935-635)	164 ±41(-974-1514)	5±293(-621-679)		0.03

[1]Scarborough Fair Group (SF).
NZ reference values are estimated average requirement (EAR)[2] (50% population requirements) and/or adequate intake (AI)[3]. Recommended daily intake (RDI[4] (98% of population requirements) and/or "suggested dietary target" [5](SDT). Fibre, sodium and potassium have SDT (no RDI).
[6]One way ANOVA between groups, P<0.05 was considered significant and only those values listed.
[7]Values are means ± SD (minimum and maximum) or percentages. Nutrients energy adjusted (8000kj).

6.4.5 Bi-weekly diaries: urinary pH and vegetable/herb/fruit intake

Bi-weekly diaries kept by intervention groups demonstrated a mean increase in urinary pH from 6.41 to 6.65 pH units (Figure 18) compared to the control group's baseline and ending pH (6.43, 6.44), ($p<0.001$). Intervention groups (A, B) reported mean servings/day of green leafy vegetables (2.7, 2.3), total vegetables (6.1, 6.3), fruit (2.9, 2.9) and herbs (1.3, 0.91, $p<0.001$).

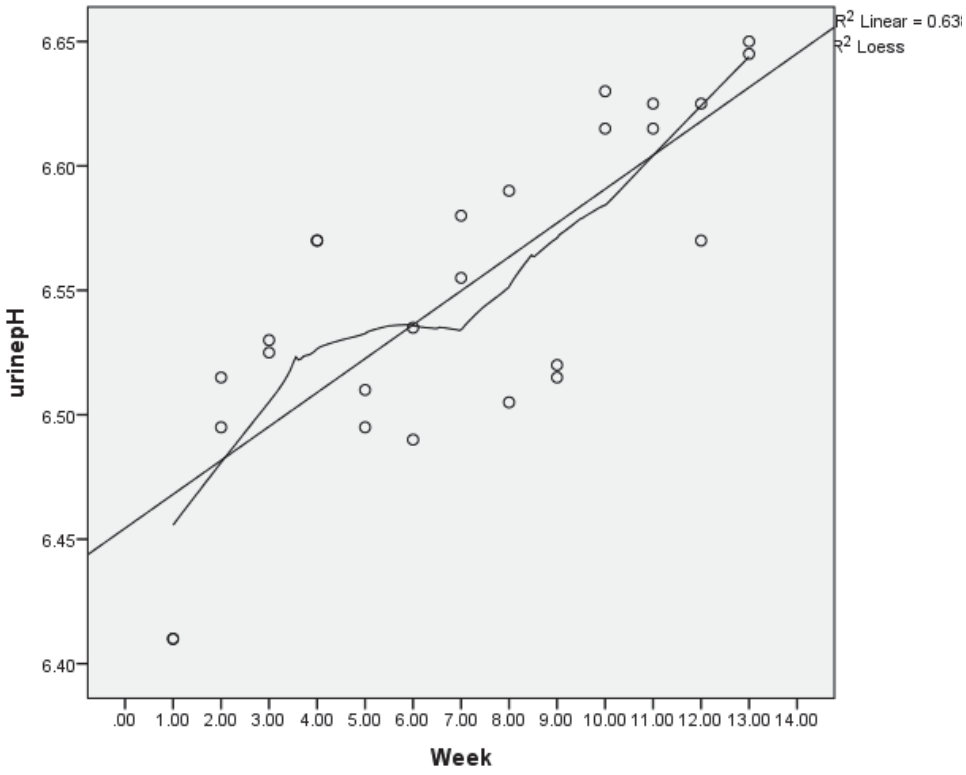

Figure 18 Change in urine pH in intervention groups A and B

Urine pH was second void, fasted and self-reported by intervention participants twice weekly using dipsticks (37).
Circles are weekly averages of groups (A and B).
Graph is fitted with a Lowess smoother line demonstrating increasingly alkaline pH.

6.4.5 Urinary mineral excretion

Subsets of each group A (n = 29), B (n = 36), C (n = 22) provided baseline/end-of-study 24 hour urine samples. No significant differences in mineral excretion were noted between groups at baseline. Urinary potassium excretion increased over the course of the study and change may confirm compliance of intervention groups with study aims to increase vegetables and fruit, thereby increasing urinary potassium output. Group A increased by 86 mmol/day (93%, $p<0.04$), Group B by 85 mmol/day (141%, $p<0.07$), however Group C (normal diet) increased by 104 mmol/day (93%, $p<0.10$) with no significant difference between intervention and control groups ($p<0.96$) (Table 18). A significant change in percentage calcium excretion was seen in the intervention groups compared to control with calcium excretion decreased by 1.2mmol/day in Group A (26%) and 1.3 mmol/day in Group B (24%) compared to a decrease of 0.6mmol/day (2%) in Group C. Sodium excretion decreased in all three groups: Group A by 164 mmol/day (48%, $p<0.001$), Group B by 166 mmol/day (50%, $p<0.001$) and Group C by 166 mmol/day (49 %, $p<0.02$) with no difference between groups.

Table 18 Changes in urinary mineral excretion in the three study groups from baseline

Urinary mineral excretion mmol/24hours	No	Baseline	No	End	Change	% change	*P* change[1]
Calcium Group A intervention[2]	29	3.9±2.0	29	2.7±1.5	¯1.2(¯1.8-¯0.5)	¯26	.002
Calcium Group B intervention (SF)[3]	36	4.8±2.1	36	3.5±1.8	¯1.3(¯2.0-¯0.6)	¯24	.001
Calcium Group C control	22	4.2±1.6	22	3.6±1.6	¯0.61(¯1.6-¯0.2)	2	0.14
p change between groups					N/S	<0.05	
Potassium A	29	135 ±91	29	221±199	86(¯5-167)	93	0.04
Potassium B	36	174±189	36	260±217	85(¯9-178)	141	0.07
Potassium C	22	187±150	22	291±265	104(¯27-235)	93	0.10
p change between groups					NS	NS	
Sodium A	29	256±119	29	90±50	¯164(¯117-¯212)	¯48	0.001
Sodium B	36	274±114	36	110±64	¯166(131-¯202)	¯50	0.001
Sodium C	22	260±108	22	103±54	¯166(¯120-¯213)	¯49	0.02
p change between groups					NS	NS	
Magnesium A	29	4.0±1.9	29	3.9±1.4	¯0.04(¯0.8- 0.8)	¯28	0.9
Magnesium B	36	4.7±1.6	36	4.4±2.3	¯0.08(¯0.9-0.7)	¯5	0.9
Magnesium C	22	4.1±1.5	22	4.3±1.5	0.1(¯0.6-0.8)	10	0.8
P change between groups					NS	NS	
Creatinine A	29	8929±3191	29	9881±2999	952(¯659-2563)	N/A	0.2
Creatinine B	36	9017±2591	36	8344±3279	-481(¯1992-1029)	N/A	0.5
Creatinine C	22	9169±2602	22	10069±2768	899(¯717-2516)	N/A	0.3
P change between groups					NS		

[1]Significance of change between groups determined by ANOVA except potassium (Kruskal-Wallis).Within group's change determined by student t test.
[2]Baseline and end values are means ±SDs, all change values are means (95% CIs).
P values <0.05 are considered statistically significant.
[3]Scarborough Fair Group (SF).

6.4.6 Baseline correlation of inflammatory markers with bone mineral density

Partial correlations were performed for log transformed cytokines (expressed per kilogram of fat mass due to their association with adipose tissue). While baseline cytokine values for OPG, IL-6, IL-10 and TNF were positively and significantly correlated with each other (e.g. IL-6 and TNF r = 0.84, p<.000) only OPG was significantly correlated with adiponectin (0.5, p<.001). The relationship between OPG and adiponectin corresponds to a large effect size and the coefficient of determination (r^2 =0.25) means 25% of the variance in either cytokine is shared. Higher levels of adiponectin and OPG were both negatively associated with BMD (Table 19). Adiponectin levels at the end of the study correlated with both IL-6 (r = 0.3, p<.001) and IL-10 (r = 0.34, p<.0001) as well as OPG (r = 0.54, p<.001).

Table 19 Baseline partial correlation coefficients between bone mineral density (BMD), adiponectin and osteoprotegerin (OPG) (per kg fat mass) (n=127)

Per kg fat mass	OPG		Adiponectin	
	r	p	r	p
Spine 1-4 BMD	-.25	.005	-.22	.011
FN Hip BMD	-.24	.006	-.24	.006
Hip BMD	-.37	.000	-.35	.000

Partial correlations are adjusted for age and years since menopause. Cytokines and adiponectin were log transformed. FN=Femoral neck of Hip, BMD=bone mineral density, OPG= osteoprotegerin. Measures of cytokines are per kilogram fat mass, calculated by dividing cytokine values by fat mass (kilograms)(DXA).

6.4.7 Inflammatory markers-changes

Baseline levels showed no significant differences between groups for inflammatory markers (OPG, IL-6, IL-10, TNF and CRP). At the end of the study, inflammation markers associated with bone IL-6, IL-10 and TNF significantly reduced within all groups but there were no significant differences between groups (Table 20). OPG decreased in Group B and C but not A. No change was seen in CRP by the end of this intervention.

Table 20 Changes in inflammatory markers within each group

		Intervention Group A		Intervention Group B(SF) [1]		Non-Intervention Group C	P between groups [2]
	n		n		n		
Adiponectin baseline (µg/ml)[3]	48	9.0 (7.9-10.1)	50	8.5 (7.5-9.3)	43	8.0 (7.1-9.2)	0.5
Adiponectin final (µg/ml)	47	6.7(5.8-7.5)	47	7.1(6.0-8.0	39	6.3(5.4-7.7)	0.8
change Adiponectin		ˉ2.3(ˉ3.4- ˉ1.2)		ˉ1.4(ˉ2.4- ˉ0.4)		ˉ1.2(ˉ2.3- ˉ0.1)	0.3
P change[4]		*p*<0.000		*p*<0.003		*p*<0.007	
OPG baseline ng/l	48	1.6(1.4-1.7)	50	2.0(1.7-2.4)	43	1.9(1.7-2.4)	0.3
OPG final ng/l	47	1.5(1.2-1.8)	47	1.4(1.3-1.6)	39	1.3(1.1-1.5)	0.6
change OPG		ˉ0.2(ˉ0.1-ˉ0.5)		ˉ0.6(ˉ1.0- ˉ0.2)		ˉ0.7(ˉ1.2- ˉ0.14)	0.2
P change		*p*<0.3		*p*<0.006		*p*<0.01	
IL-6 baseline pg/ml	48	1.5(1.1-1.8)	50	1.7(1.5-1.9)	43	1.7(1.2-2.2)	0.5
IL-6 final pg/ml	47	0.7(0.6-0.9)	47	0.8(0.7-1.0)	39	0.9(0.7-1.2)	0.2
change IL-6		ˉ0.7(ˉ1.0-ˉ0.4)		ˉ0.8(ˉ1.1-ˉ0.6)		ˉ0.9(ˉ1.3-ˉ0.4)	0.8
P change		*p*<0.000		*p*<0.000		*p*<0.000	
IL-10 baseline pg/ml	48	2.3(1.8-2.7)	50	3.2(2.5-3.8)	43	2.9(2.2-3.7)	0.1
IL-10 final pg/ml	47	1.6(1.3-1.9)	47	1.8(1.3-2.3)	39	1.5(1.3-1.8)	0.6
change IL-10		ˉ0.7(ˉ1.2-ˉ0.5)		ˉ1.2(ˉ1.9-ˉ0.5)		ˉ1.5(ˉ2.5-ˉ0.7)	0.2
P change		*p*<0.03		*p*<0.002		*p*<0.001	
TNF baseline pg/ml	48	7.4(5.8-8.9)	50	8.3(6.8-9.8)	43	6.5(5.0-8.1)	0.3
TNF final pg/ml	47	2.9(2.4-3.4)	47	3.2(2.7-3.8)	39	3.5(2.8-4.2)	0.4
change TNF		ˉ4.6(ˉ6.2-ˉ2.8)		ˉ5.3(ˉ7.1-ˉ3.5)		ˉ3.3(ˉ5.1-ˉ 1.4)	0.3
P change		*p*<0.000		*p*<0.000		*p*<0.001	
CRP baseline mg/l	48	2.7(2.0-3.4)	50	2.4(1.6-3.1)	43	2.1(1.4-2.8)	0.5
CRP final mg/l	47	2.7(2.0-3.4)	47	2.2(1.5-3.0)	39	2.7(1.8-3.6)	0.6
CRP change		0.1(ˉ0.6-0.9)		-0.3(ˉ1.2-0.6)		0.5(ˉ0.4-1.4)	0.4
P change		p<0.7		p<0.6		p<0.3	

[1]Scarborough Fair Group (SF).
[2]P values for group comparisons are derived from ANOVA.
[3]Values are means (95% CI).
[4] P change by student t tests. P<0.05 is statistically significant. Cytokines were log transformed for ANOVA. C - reactive protein (CRP), interleukin 6/10 (IL-6, IL-10), tumour necrosis factor (TNF), osteoprotegerin (OPG), CTX = C terminal telopeptide of type 1 collagen, P1NP= procollagen type 1N propeptide.

6.4.8 Adiponectin

Adiponectin levels reduced significantly in each group during this intervention, however there was little difference between groups by the end of the study (Table 20). Stratifying intervention and control groups by BMD demonstrated the significant differences between osteoporotic groups compared with normal BMD groups at baseline ($p<0.007$). The lower the BMD at baseline, the greater the reduction in adiponectin during the intervention, with values in each BMD group aligned similarly by end of study (Table 21).

Table 21 Change in adiponectin (per kilogram fat mass) during dietary intervention in post-menopausal women (n=141) stratified by bone mineral density

	Normal BMD n=51	Osteopenia n=73	Osteoporosis n=17	P [2]
Adiponectin (µg/ml)/kg fat mass[1]				
Baseline	0.32 (0.26,0.38)	0.34 (0.30,0.39)	0.52 (0.39,0.65)	0.007
Final	0.28 (0.21,0.35)	0.28 (0.24,0.33)	0.30 (0.18,0.41)	0.72
Change	0.04 (0.01,0.08)	0.06 (0.03,0.09)	0.22 (0.12,0.33)	0.001

1 Values are means (95% CI).
2 P values derived from ANOVA using log transformed values. P<0.05 statistically significant

6.4.9 Bone turnover markers

Baseline bone markers of resorption (CTX) and formation (P1NP) were the same in all groups (Table 22). There was no significant main effect of group (A, B, C) or BMD on change in CTX. A 2 way ANOVA examined the interaction effect of group and baseline BMD (normal BMD and osteopenia) on change in CTX (due to low numbers no comparison was available for women with osteoporosis). A significant interaction effect ($p<0.039$) existed between the three groups (A, B, C) based on BMD (normal or osteopenia). Women with osteopenia (n=22) consuming the Scarborough Fair combination of vegetable/fruit/herbs had significantly decreased levels of resorption marker CTX, $F(1,109)=6.5$, $p<0.01$, compared to women with normal BMD (mean difference of -0.065 µg/L) and this effect was not observed in Group A (n=24), $F(1,109)=0.26$, $p<0.60$ and Group C (n=20), $F(1,109)=0.42$, $p<0.52$. Repeated measures ANOVA was also performed on markers at midway and end of study using baseline CTX as covariate. CTX decreased from 0.43 to 0.41µg/L ($p<0.35$) in the Scarborough

Fair group (B) while CTX of group A increased from 0.39 to 0.40µg/L (p< 0.15) but neither change was statistically significant. Moreover, the increase in CTX of 0.01 µg/L in Group A is not considered biologically significant (Martínez-Abraín, 2008). Although every effort was made to reduce within subject variability of all bone markers, CTX may also vary due to between subject variability and analytical variability of bone markers (Alvarez, Ricos, Peris et al., 2000; Biver et al., 2012), therefore, a slight increase of 0.01 µg/L between two sampling time points does not present biological significance.

Table 22 Changes in bone markers within each group

Bone markers		Intervention Group A		Intervention Group B(SF) [1]		Non Intervention Group C	P between groups [2]
	n		n		n		
CTX baseline µg/L[3]	48	0.37(0.34-0.41)	50	0.42(0.38-0.45)	43	0.39(0.33-0.45)	0.4
CTX mid µg/L	41	0.39(0.35-0.43)	47	0.43(0.39-0.47)			0.2
CTX final µg/L	47	0.40(0.36-0.44)	47	0.41(0.38-0.45)	39	0.40(0.35-0.46)	0.8
Change in CTX		0.03(0.01-0.03)		-0.01(-0.03-0.03)		0.01(-0.01-0.03)	0.4
P change		0.03		0.9		0.3	
P1NP µg/L	48	44.2(39.8-48.7)	50	49.7(45.1-54.2)	43	45.0(39.0-50.8)	0.2
P1NP mid µg/L	41	43.8(38.9-48.6)	47	46.7(42.5-50.9)			0.4
P1NP end µg/L	47	43.3(39.3-47.3)	47	45.9(42.4-49.5)	39	46.6(38.5-54.6)	0.6
Change in P1NP		-0.9(-2.4-4.3)		-3.2(-5.9-0.4)		1.8(-3.1-6.6)	0.2
P change		*p*<0.6		*p*<0.01		*p*<0.5	

[1]Scarborough Fair Group (SF).
[2]P values for group comparisons are derived from ANOVA.
[3]Values are means (95% CI).
[4] P change by student t tests. P<0.05 is statistically significant.
CTX= C terminal telopeptide of type 1 collagen, P1NP= procollagen type 1N propeptide.

Although there was a decrease in CTX within the Scarborough Fair group (B), the differences between intervention groups did not reach significance, but there was a significant reduction in CTX in Group B between those with normal bone mineral density and those with osteopenia (Figure 19). Bone formation marker P1NP decreased significantly in intervention group B from 49.7 to 45.9µg/L (p<0.03) and this level of decrease is biologically significant and reflects an overall lowering of bone turnover. Changes in P1NP in both groups A and C were not significant. A weak positive association was observed at baseline and end of study, between adiponectin and CTX (r = 0.26; p<0.002) and baseline only for P1NP (r = 0.21; p<.016) as well as between OPG and CTX (r= 0.24; p<0.005) but not OPG and P1NP.

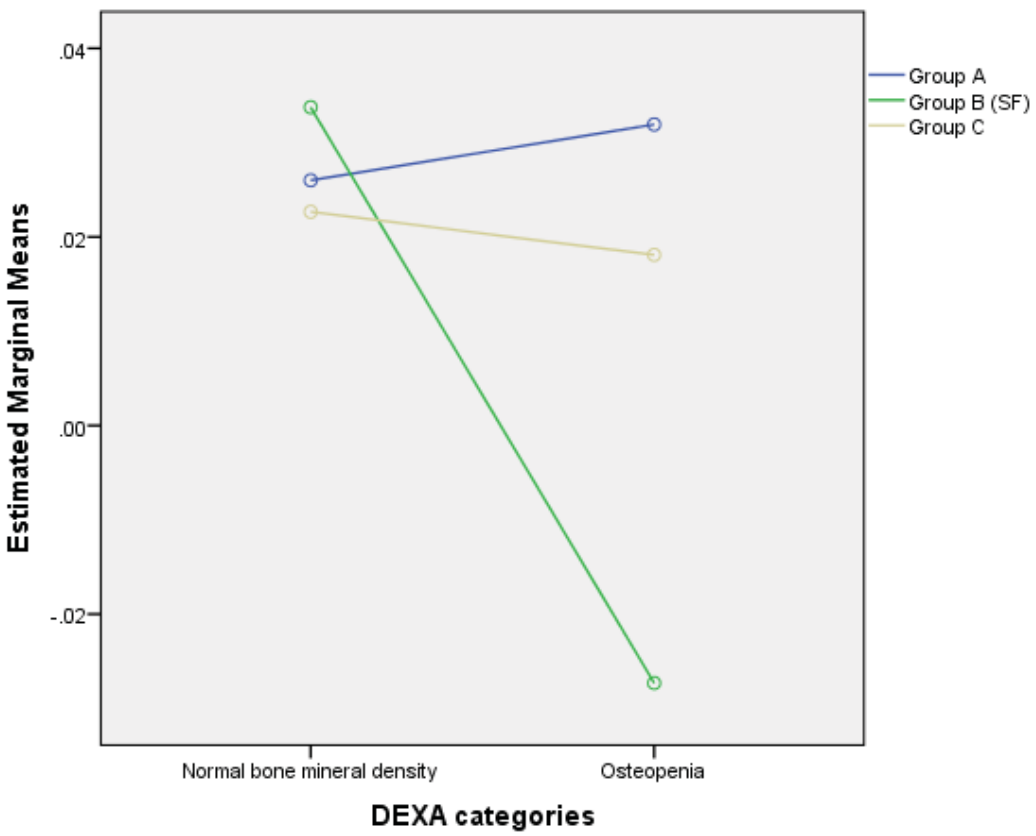

Figure 19 Change in CTX in women with normal bone mineral density or osteopenia

This figure demonstrates the interaction effect of group and bone mineral density status. C-Terminal Telopeptide of Type I Collagen (CTX) decreased significantly in the women with increased bone loss (osteopenia) in the Scarborough Fair group (B) compared to the other two groups of women (A, C) with osteopenia.

6.5 Discussion

The main finding of our study was decrease in bone turnover markers in women consuming the Scarborough Fair dietary mix of vegetables, fruit and herbs (group B). Resorptive activity (CTX) reduced in osteopenic women and formation activity (P1NP) reduced in all women in this group. The reduction in bone formation was considered biologically significant while the resorptive activity reduction was significantly different from the other two groups in the women with osteopenia. Downward modulation of bone turnover markers suggests the additive effect of mildly active pharmacological agents present in the SF selection of vegetables, herbs and fruit reduces bone turnover particularly in those with osteopenia. The SF vegetable/herbs/fruit decreased bone markers significantly compared with active comparator Group A, who also increased potassium/phytochemical

intakes, lowered sodium intake and estimated dietary PRAL, and a less defined control (Group C) that significantly increased urinary potassium excretion therefore potassium intake.

This indicates neither the increased potassium intake (both A and C), decreased dietary PRAL (A) or phytochemicals in Group A's selection of vegetables/herbs/fruit resulted in a significant effect on bone markers, whilst phytochemicals in the SF vegetables/herbs/fruit combination may have reduced bone turnover. The SF combination of vegetables/herbs/fruit has shown effects on resorption in the rat model (Mühlbauer, Lozano, Reinli, et al., 2003), but not previously in humans. The antiresorptive effect was however, evident only in women with increased bone loss (osteopenia), not women with normal BMD, therefore its usefulness as a dietary strategy may be limited to those with osteopenia.

Another finding was the considerable decrease of inflammatory cytokines IL-6 and TNF associated with osteoporosis (Schett, 2011), obesity and ageing (Schett, 2011; Sudhaa, Vishal, Shagun et al., 2014) in all groups. IL-6 decreased by -0.7 pg/ml (p<0.000) in Group A, -0.8 pg/ml (p<0.000) in Group B and -0.9 pg/ml (p<0.000) in Group C. TNF demonstrated similar sized reductions of -4.6 pg/ml (p<0.000) in Group A, -5.3 pg/ml (p<0.000) in Group B and -3.3 pg/ml (p<0.000) in Group C. Bone loss increases with raised levels of IL-6 and TNF which enhanced expression of receptor activator of nuclear factor kappa B ligand (RANKL) and osteoclast activity (Schett, 2011). A similar magnitude reduction in TNF has not previously been demonstrated with a dietary intervention (Ziccardi et al., 2002; Bakker et al., 2010). Dietary intake of vegetables/herbs/fruit (Group A and B) and/or potassium (Group C) increased in all groups and significant reductions in IL-6 and TNF were similar across the three groups as was IL-10, with IL-10 reducing in Group A , -0.7pg/ml (p<0.000); Group B,-1.2pg/ml (p<0.000); Group C-1.5pg/ml (p<0.000). IL-10 is a negative regulator of osteoclastogenesis (Zhao & Ivashkiv, 2011) suggesting the mechanism of action of the SF phytochemicals may not be limited to IL-6, TNF and IL-10 action.

A surprising finding in this study was a decrease in adiponectin (total molecular weight). Higher baseline levels and increased reductions in adiponectin were seen in study participants with lower

BMD at baseline. Higher adiponectin levels are found in slim older women with low grade inflammation/oxidative stress and reduction in inflammation may lower adiponectin (Sodi, Hazell, Durham et al., 2009). Adiponectin along with OPG, were negatively associated with all measures of BMD at baseline and adiponectin declined with increased vegetable/fruit intake and/or increased urinary potassium excretion. This reduction is in contrast to observational and animal studies linking higher adiponectin levels with diets higher in: plant food (Cassidy et al., 2008), fruit (Yannakoulia, Yiannakouris, Melistas et al., 2008), fibre (Silva et al., 2011), antioxidants (Detopoulou et al., 2009), Mediterranean (Fragopoulou et al., 2010) or low fat diets (Yang, Zhang, Lin et al., 2012).

Human intervention studies with significant effects on adiponectin, but without associated weight loss are limited (Silva et al., 2011; Bluher, Rudich, Klotting et al., 2012; Barbaresko, Koch, Schulze et al., 2013), and report increased adiponectin. Both improved insulin sensitivity (Galic et al., 2009; Kanazawa, Yamaguchi, Yamauchi et al., 2009) and lower diabetes risk (Sattar & Nelson, 2008) are associated with higher adiponectin levels but also bone loss (Richards et al., 2007; Basurto, Galvan, Cordova et al., 2009; Barbour et al., 2012) and risk of fractures (men) (Barbour, Zmuda, Boudreau et al., 2011; Johansson, Odén, Lerner et al., 2012). While conflicting evidence exists of adiponectin's actions to reduce osteoclastogenesis (Tu, Zhang, Dong et al., 2011) and activate osteoblastogenesis (Oshima, Nampei, Matsuda et al., 2005), a recent meta-analysis showed an inverse relationship between adiponectin and BMD (Biver et al., 2011). Adiponectin increases osteoblast numbers and differentiation, while indirectly affecting osteoclastogenesis through up-regulation of RANKL activity and decreased production of osteoprotegerin (Luo, Guo, Xie et al., 2006). As adiponectin declined in all groups and particularly in women with osteoporosis (who tended to be slim), reduced levels may correlate with lowered inflammation that may positively affect bone but does not explain differing group changes in bone turnover markers.

The negative association of OPG with bone mass in this study at baseline, concurs with previous studies showing higher levels associated with both osteoporosis and loss of height (Grigorie, Neacsu, Marinescu et al., 2003; Jørgensen, Hansen, Brox et al., 2011) but conversely with research showing OPG inhibits osteoclastogenesis (Boyce et al., 2008). The reduced levels of OPG seen by the end of this study could indicate less in circulation as more is bound to RANKL to inhibit bone resorption (Reyes-Garcia et al., 2010), however this was not complemented by lowered resorption markers in all groups. As OPG levels reduced, they remained tightly correlated with reductions in adiponectin. The strong correlation of both these inflammatory markers at baseline with BMD and their subsequent reduction during the study suggest their activity is interrelated.

Lifestyle factors such as weight and levels of exercise were unchanged in our study (although anecdotally there was loss of inches around the waist in the intervention groups). However, potassium urinary excretion tended to indicate, but not confirm the dietary changes reflected in the increased urinary potassium excretion, resulted in the significant reductions in inflammatory cytokines associated with bone in all groups. Unlike the other inflammatory markers, CRP did not reduce significantly during this intervention. CRP is produced by the liver and adipose tissue and is associated with acute infection/inflammation. Previous work suggests CRP and other inflammatory markers may not share the same regulatory pathways. Therefore the lack of change in CRP alongside significant reductions in other inflammatory cytokines IL-6, IL-10 and TNF in this study, supports the suggestion of regulation by different pathways for CRP (Bermudez, Rifai, Buring et al., 2002; Colbert, Visser, Simonsick et al., 2004).

An improved profile of potassium, magnesium, calcium and sodium dietary intake according to national and international nutrient reference intake values, was seen in intervention groups by the end of the study. However, despite compliance with ≥9 servings of fruit/vegetable/day, inclusion of 2 servings of green leafy vegetables/day and a reduction in breads/cereals food group, only one intervention group (SF) achieved national and internationally suggested dietary targets (SDT) for potassium (4700mg/day)(Ministry of Health (NZ), 2006; USDA et al., 2010) and the NZ SDT for

folate (SDT 600μg/day) was not reached by either intervention group. The reduction in sodium, while significant, is considerably more than the SDT (≤1600mg/day). These results suggest even greater change is required to current eating patterns to achieve recommended targets or targets are unattainable for the majority (Drewnowski, Maillot, & Rehm, 2012).

Reported dietary intake varied significantly between groups at end of study with reductions in food group servings of breads/cereals and increases in fruit/vegetables resulting in significantly lower estimated dietary acidity (PRAL) and sodium intake in intervention groups (Ministry of Agriculture and Fisheries, 2009). Macronutrient intakes remained the same, however micronutrient intakes of potassium, magnesium and calcium increased significantly in intervention groups.

85% of potassium consumed in the diet is absorbed and of that, 80-90% is excreted in the urine (K. Zhu et al., 2009). Therefore, increased urinary potassium excretion reflects increased potassium intake sourced predominantly in this study from vegetables and fruit in intervention groups who received specific counselling to increase this food group (Konstantinou, 2012). Increased potassium excretion was seen alongside reductions in urinary calcium excretion, particularly in Groups A and B.

Although urinary potassium excretion increased in all groups, significantly less (%) calcium was lost in urine by intervention groups compared to control (C). Whether the intervention groups prescriptive instructions to consume more vegetables (especially green leafy) which are more alkaline forming than fruits, or lowered PRAL contributed to the reduced loss of urinary calcium cannot be determined, only the intervention groups lowered estimated dietary PRAL and demonstrated increased alkalinity of urinary pH (refer Figure 18).

The inverse relationship that exists between potassium consumption and calcium excretion has been attributed to lowered intestinal absorption of calcium. Lowered intestinal absorption of calcium was observed in research with participants who were deriving most of their potassium intake from dairy, meat and cereal grains, rather than fruit and vegetable sources (Rafferty, Heaney, & Davies, 2005).

Analysis of Foodworks data from three day diet diaries determined our intervention groups derived most of their potassium intake from vegetables and fruit food group.

The urine calcium lowering effect observed when dietary acidity reduces is considered the main reason for lower calcium excretion rather than increased potassium intake (Maurer et al., 2003; Dawson-Hughes et al., 2009). Recent research has shown bicarbonate allows for increased calcium uptake in intestine but prevents its loss in the urine (Shi et al., 2012) while others have shown when dietary acid (PRAL) is higher there will be increased calcium lost from the urine (Pizzorno et al., 2010). The dietary acid load of groups A and B was significantly reduced in this study, therefore it is possible the significantly lowered urinary calcium excretion was due to the reduction in PRAL as compared with Group C, who had an associated increase in potassium intake as measured by increased urinary potassium excretion, but no significant change in their dietary acid load or urine pH.

Sodium intake also may account for obligatory calcium loss as both share the same reabsorption transporter in the proximal tubule (Heaney, 2006), however since all groups had significant reductions in urinary sodium output, reduced intake cannot account for differences in urinary calcium excretion between groups. A decrease in dietary acidity reduces not only calcium but also magnesium excretion (Carrera- Bastos, Fontes-Villalba, IO'Keefe et al., 2011) and while increased dietary intake of magnesium was reported in both intervention groups there was decreased urinary magnesium output. The control group's dietary magnesium intake and dietary acidity remained unchanged with a non-significant increase in urinary magnesium excretion. Previous work suggests acid-base status affects urinary excretion of magnesium regardless of intake (Rylander, Remer, Berkemeyer et al., 2006) therefore the reduction in urinary magnesium excretion in the intervention groups may be attributed to the reduction in dietary acidity in both these groups compared to Group C who had no reduction in dietary acidity.

The effect of the Scarborough Fair combination of vegetables/herbs/fruit on bone turnover and adiponectin seen in this study would need to be confirmed by further studies. Future trials should

stratify for BMD, ensuring sufficient participants with osteoporosis, and be sufficiently powered to allow analysis between all BMD groups. A longer study time period of at least two years, would allow for bone health assessment using DXA and/or peripheral QCT, to determine if changes occurred in bone mineral density, strength or quality (Weaver et al., 2013). Although we measured total adiponectin which may be more influential on bone cells (Sodi et al., 2009), measurement of high/low molecular weight ratio adiponectin may be more representative of other physiological activity (Silva et al., 2011) and may allow comparison with central fat loss (which numerous participants in this study suggested occurred despite weight maintenance). Central fat mass measurements such as waist circumference could be done, and this would enhance adiponectin analysis, as large central adipocytes poorly synthesise adiponectin's active high molecular weight oligomer (H. Lin & Li, 2012). Urinary vitamin C measured as an indicator of vegetables/herbs/fruit intake would further add to the confidence that any increase in urinary potassium increase in all groups was due to increased vegetables/herbs/fruit.

The strengths of the study are the following: randomisation to differing selections of vegetables, herbs and fruit with one combination specifically linked to bone health; community-based setting involving participants purchasing, storing and cooking additional vegetables, herbs and fruit allowing generalization of results to free living populations; control of potential confounders such as dietary calcium/vitamin K by specifying minimum intakes of servings dairy/green leafy vegetables; and no additional calcium/vitamin D supplementation, fortification or requirement to eat large amounts of a single food. It is likely that plant-based diets with high intake of plant phytochemicals will have beneficial effects for an aging population, as inflammation is central to the ageing process, and phytochemicals have been demonstrated to improve age-related degenerative diseases due to a global decrease in inflammation (Salminen et al., 2012).

In conclusion, this is the first study showing increased intake of a selection of vegetables/herbs/fruit reduced bone formation (P1NP) and resorptive (CTX) markers (osteopenic women). Lowered dietary PRAL resulted in urinary calcium conservation in intervention groups A and B compared to group (C)

while a high potassium/low inflammatory diet modulated inflammatory markers (TNF, IL-6, IL-10 and OPG) significantly downwards in all groups. Decreased adiponectin levels resulting from this dietary intervention are contrary to findings of epidemiological studies positively associating adiponectin with higher plant/polyphenol rich diets.

CHAPTER 7 DISCUSSION

The main purpose of this thesis was to investigate the question posed initially "Is it possible to reduce bone loss at midlife by a dietary modification increasing vegetables and fruit and emphasising particular vegetables/herbs/fruit?"

Maintenance of independence and health during the latter years of life is one of the hallmarks of an ideal society. Bone loss, particularly in midlife women can lead to fragility, pain and reduced quality of life. Estimates are that one in two women will sustain some form of osteoporotic fracture during their lifetime and one in three men (P. Brown et al., 2007; P. Brown et al., 2011). Fracture of the hip carries an even greater societal and economic burden with reduction in mobility and earlier dependence on carers. Over 50 % of older people will require longer term care after sustaining a hip fracture, however, more alarming is the greatly increased mortality with an estimated 25% dying within the first year (P. Brown et al., 2011). Most hip fractures are seen in women (90%) rather than men (Ashwell et al., 2008) and numbers are projected to increase with a rapidly ageing population. Estimates in New Zealand are for an increase of 37% in osteoporotic fractures due to our ageing population (P. Brown et al., 2011).

In New Zealand there is a growing public health concern about bone loss, osteoporosis and fracture risk, not only for loss of quality of life and premature death, but also the public health burden with rapidly escalating costs from the needs of an ageing population and also other chronic diseases developing in younger adults such as diabetes. Osteoporosis and fractures are predicted to cost New Zealand over $458 million in 2020, far in excess of other diseases typically associated with women such a breast cancer (P. Brown et al., 2011).

There is a number of contributing factors influencing bone health including the main unmodifiable aspect of genetics. Relevant factors that can be adapted by lifestyle changes however, include diet, exercise and sun exposure. Exercise and diet may account for up to 25% of the variance in bone mineral density (Anderson, Garner, & Klemmer, 2012a), and while both provide excellent research

angles, this study investigated dietary aspects of modifying bone loss at midlife with a primary focus on increased vegetables and fruit and the various ways this food group and its properties can contribute to reduction of bone loss particularly at the menopause transition (Hunter et al., 2008).

An essential part of the attractiveness of vegetables/herbs/fruit as a vehicle to research bone health is their favourable effects on general health. By promoting increased consumption during the two studies it was considered and determined that increased consumption of this food group was not only unlikely to cause adverse consequences, but may provide additional benefits to participant's health (Tobias et al., 2006) as well as possible effects on bone health.

Within a population the disease level is said to vary by 30% between the 20% who eat the least vegetables and fruit and the 20% who eat the most (Ekman & Patterson, 2010). A dietary pattern favouring a majority of vegetables and fruit will be high in antioxidants and phytochemicals, particularly flavonoids (Weaver et al., 2012) with inflammation lowering properties and ability to influence cell signalling, both of which can delay the onset of several chronic disorders including osteoporosis (Lanham-New, 2006; Calabrese et al., 2008; Hunter et al., 2008; Salminen et al., 2012). The micronutrients vitamins K, C and E, minerals potassium (Macdonald et al., 2005) magnesium and calcium will also be amply supplied as raw materials for bone growth and repair (Heaney, 2009; Schulman, Weiss, & Mechanick, 2011). Additional calcium can be garnered from a diet with a lowered renal acid load provided by bicarbonate precursors only found in vegetables and fruit which reduce hypercalciuria (Shi et al., 2012) and fermentation of dietary fibre also can increase calcium uptake in the large intestine (Kruger et al., 2013).

While there are numerous mechanisms by which vegetables and fruit may reduce bone loss, there have been few human intervention studies using vegetables and fruit (P. Lin et al., 2003; Macdonald et al., 2008; Nowson, Patchett, et al., 2009) to ascertain effects on bone turnover markers and of those, several are limited to single fruits (Arjmandi et al., 2002) or unintentionally result in more fruit (Dragsted et al., 2004; Macdonald et al., 2008), rather than vegetables being consumed (Ashwell et

al., 2008; Chapman et al., 2012). Generic instructions to increase fruit/vegetables can result predominantly in increases in fruit, as they are sweeter and more convenient to eat, don't require cooking and can be eaten in isolation, whereas most vegetables are eaten with other foods. The DASH study, was the first large scale dietary intervention which included increased vegetables and fruit (9 servings/day), as part of dietary change which demonstrated unexpected positive results for bone turnover markers (P. Lin et al., 2003; Doyle et al., 2004). Both bone marker of resorption CTX, and urinary calcium were reduced and the results were attributed to a combination of effects of lowered renal acid load, increased potassium intake and lower sodium diet. The DASH study had several hallmarks deemed useful as a model for our two studies. One feature was the number of vegetables and fruit (9 servings/day) which we adapted to 6 servings/day of vegetables and 3 servings/day of fruit. The other features included the increased potassium intake and lowered sodium intake, which we hoped would result in lowered calcium excretion in our group of midlife women. No instructions were given as to low salt options, rather a reliance was placed on the higher intake of vegetables and fruit lowering intake of other foods, particularly processed food and possibly breads/cereals food group (high salt), while increasing starchy vegetable intake to reach 6 servings/day. A significant difference between our studies and the DASH study for implementing the increase in the vegetables and fruit was that we were not sourcing and supplying free fruit/vegetables to participants, rather the women would have to do this themselves.

Considerable readiness for behaviour change is required to increase vegetables and fruit intake and therefore the question of whether dietary changes can affect bone markers was addressed in 2 stages with separate studies.

The first study was a feasibility study to gather evidence that this type of eating plan was possible and achievable in a community setting where the women had to source, purchase, store and cook the extra vegetables and fruit. The dietary pattern is low acid and is created primarily by increasing the amount of base precursors rather than reducing acid by limiting protein based foods such as meat and dairy (Sellmeyer, 2013), and should result in a physiological change reflecting the shift from a state of mild

metabolic acidosis to one of mild alkalosis with consequent increase in urine pH (alkalinity), which the women monitored by self-checking fasting urine pH.

The daily monitoring of urine pH served several purposes: it can be a measure of compliance with the increased intake, the degree of change in urine pH would give an indication whether this number of serving vegetables and fruit was sufficient to change bone markers (0.5 pH unit as assessed using alkaline mineral water) (Wynn, Krieg, Aeschlimann, et al., 2009) and for some women knowing the pH should increase, served as added impetus to increase and maintain the increased intake of vegetables and fruit.

Additionally, this study investigated whether participants could consume specific vegetables with bone resorption inhibiting properties e.g. onions, broccoli (Mühlbauer et al., 2002) and dark green leafy vegetables (Bullo et al., 2011) on a daily basis or most days per week (5/7days/week), and what whole diet changes resulted with this type of dietary pattern e.g. was the nutrient intake profile improved or were there deficiencies.

It was also deemed useful to determine the change in estimated dietary PRAL and NEAP. We estimated a shift of 20 mEq/day would be required in dietary acid load to have sufficient physiological effect on urine pH and therefore indirectly, bone markers. If successful, it was hoped strategies implemented by the women would provide a useful model for the participants in the second study. The feasibility study was characterised by extensive monitoring of all participants, use of motivational techniques and questionnaires that were designed to garner information on how to manage the increase, strategies implemented and costs to participants personally in extra time for shopping, preparation and cooking and importantly, financial outlay.

The second study involved a very similar eating strategy but with specific culinary herbs and fruit included and less frequent self-monitoring of urine pH (twice a week). There were considerably more participants (148), time (3 months trial period staged over 6 months in 3 different regions) and financial investment ($71,000) from a larger research team involving DXA technicians,

phlebotomists, laboratory staff both at Massey University and external laboratories (Christchurch Endocrine laboratory and Eastern Institute of Technology- Wine Science laboratory) as all women were offered DXA screening (142 women), all women had anthropometric data recorded and intervention women had three fasting blood tests during the study, while control women had two (baseline and end).

Approximately half the women in each group completed two 24 hour urine collections (baseline and end) and all women completed two 3 Day Diet Diaries. The intervention women once again had the additional task of recording on two days each week their vegetable/herb and fruit intake as well as measuring and recording their fasting urine pH (second void) with dipsticks.

These two studies were the first to our knowledge where: increased vegetables rather than fruit intake was prescribed, specific vegetables and fruit with evidence based bone resorption inhibiting properties (animal model only) were consumed daily and urine pH dipsticks were used as a tool to self-monitor changes in urine pH and provide increased motivation. It is also the first time a dietary intervention using increased vegetables/herbs and fruit has been used to monitor changes in bone and inflammatory cytokines including adiponectin in a healthy population of post-menopausal women.

7.1 Main findings from study 1: A feasibility study

The main findings from study 1 were confirmation that a group of midlife women can significantly increase intake of vegetable servings to ≥ 6 servings/day and include specific vegetables and fruit regularly (≥ 5 times/week). A 76% compliance rate with the study's dietary requirements was achieved by this first group of 21 midlife women.

One important effect of this dietary change on food group serving numbers was while protein/meat and dairy food groups remained unchanged a reduction was seen in the number of servings from the bread /cereal food group. This food group contributes several key nutrients to the diet, but the corresponding reduction in nutrient intake from this food group was more than compensated for by

the increased contribution of nutrients from the vegetables and fruit food group. Sodium is associated with processed food in the New Zealand diet and this key nutrient (WHO., 2012) demonstrated significant reduction in dietary intake. The increased dietary intake of potassium from the vegetables and fruit combined with the reduction in sodium due to less breads/cereals being consumed, favourably changed the potassium/sodium ratio to align with current recommendations (Ministry of Health and the University of Auckland, 2003; Ministry of Health (NZ), 2006; Sebastian, Frassetto, Sellmeyer et al., 2006). The dietary changes had a large, reliable effect on estimated PRAL and NEAP with a reduction of over 20 mEq/day achieved as well as an increase in alkalinity of mean urine pH of 0.68.

As records of daily urine pH measurements along with daily intake of fruit/vegetables were available, regression analysis was used to determine whether a model could be found for the effect of the previous day's intake of vegetables and fruit on the following day's urine pH. This was useful only for a group of six participants who shared several common characteristics such as normal body mass index (BMI) and lifestyle similarities including moderate alcohol consumption levels and daily exercise levels but not for the group as a whole with disparate BMI, exercise levels and alcohol intakes. We determined therefore, that other dietary and lifestyle factors must also be important determinants in the variations seen in daily urine pH. Urine pH was shown to be highly variable for most of the participants, and while we were able to see a steady increase over the 8 weeks with daily measurements, a single measurement was determined not to be a useful indicator of renal acid load.

The questionnaires, emails and interviews with the women in this first study enabled us to more successfully implement the dietary change in study 2, including what strategies to recommend to participants. Several major adaptations in lifestyle were made by many of the women in Study 1 to accommodate the increased intake such as shopping habits. Several participants changed where they shopped for vegetables and fruit by switching to Farmers markets as they found them generally cheaper than supermarkets. More vegetable/fruit was bought at one time so required extra storage space (particularly refrigerator space) and extra time meal planning and cooking (Havas, Treiman,

Langenberg et al., 1998; Dixon, Mullins, Wakefield et al., 2004; Maclellan, Gottschall-Pass, & Larsen, 2004; Pomerleau, Lock, Knai et al., 2005; Kapur, Kapur, Ramachandran et al., 2008; Shaikh, Yaroch, Nebeling et al., 2008). Participants developed ways of incorporating extra vegetable families e.g. allium group into current familiar recipes and many shared freely recipes and tips which worked well for them and which could then be sent out to all participants via email.

In conclusion the feasibility study showed this increase in fruit/vegetables was achievable in midlife women in a community setting, that urine pH could be increased over the 8 week period by at least 0.5 pH units and provided a lifestyle snapshot of how to successfully achieve this dietary change in a free living population. Strategies implemented by the women, as well as results of dietary data and urine pH were very helpful in informing and confirming the design of the second study.

7.2 Main findings from the second intervention study: The Scarborough Fair Study

This larger group of women afforded the opportunity to assess baseline levels of bone and inflammatory markers and compare them with baseline dietary intake data and measures of bone mineral density including body fat. This was the first time a group of healthy, post-menopausal New Zealand women had these bone and inflammatory markers assessed and correlated with their BMD, both before and after a dietary intervention with increased vegetables and fruit.

A significant finding with baseline data was a difference in circulating OPG levels between the women, when stratified according to their bone mineral density. This circulating cytokine recptor is associated with lowered bone resorption (Mezquita-Raya, Higuera, Garcia et al., 2005), but in our study the highest levels were seen in the women with the most bone loss i.e. osteoporosis. Controversy still exists over what higher levels of this cytokine receptor mean in post-menopausal women. Some studies have proposed higher levels of OPG in circulation may be indicative of less osteoclastogenesis (Mezquita-Raya et al., 2005) while others suggest it represents the amount of OPG not bound to RANKL, therefore higher levels mean increased osteoclastogenesis is occurring (Reyes-

Garcia et al., 2010). Endorsement of this position is found with studies showing increased loss of height with higher levels of OPG (Jørgensen et al., 2011) and higher levels of osteoporosis (Grigorie et al., 2003).

Baseline dietary data showed the following nutrients; protein, vitamin B12, zinc, potassium and dairy food group were positively correlated with higher BMD, while dairy and potassium intakes also inversely correlated with CTX. Many previous studies have shown the close connection between dairy food (Heaney, 2009) and potassium intake and bone health (Sebastian et al., 2006; Sellmeyer, 2013) with potassium a main component of vegetables and fruit. However the inverse correlation of dairy and potassium with resorption marker CTX shown in this study is less well documented (Rahbar, Larijani, Nabipour et al., 2009).

In our study, the finding of strong positive associations between all measures of body composition (weight, BMI and fat/lean mass) and BMD is also controversial. (Gilsanz et al., 2009; Reid, 2010; Kawai et al., 2012). Increasingly fat is seen as a source of inflammatory cytokines which impact bone negatively leading to increased bone loss (Bhupathiraju et al., 2011; Kawai et al., 2012). While subcutaneous fat may be beneficial to bone, visceral fat is thought to be a reservoir for inflammatory cytokines (Gilsanz et al., 2009), however, our results did not reflect this as android fat (visceral fat) was not negatively correlated with bone mass nor with inflammatory markers except adiponectin. Instead BMI, body fat mass and body weight were all positively associated with adequate bone mass in this group of post-menopausal women which concurs with Reid, who states that "any inverse relationship between fat mass and BMD is usually accounted for by inappropriate treatment of highly collinear variables such as fat mass and weight" (Reid, 2008). Multiple regression analysis showed body weight, potassium and dairy intake were predictors of increased BMD in PM women and explained 39% (r^2=0.39, $p< 0.003$) of variance. In conclusion, these baseline correlates in study 2 highlight the importance of maintaining adequate body weight and emphasising dairy and potassium predominantly sourced from fruit/vegetables to reduce bone loss at midlife.

Discussion of the changes seen during the intervention study takes into consideration the "Hawthorne Effect" (McCarney, Warner, Iliffe et al., 2007; Konstantinou, 2012). The Hawthorne effect was noted in Group C whereby although they were included in the study as a separate negative control group and instructed to consume their usual diet they appeared to have also increased vegetables and fruit as ending 24 hour potassium urinary excretion levels were higher than either intervention group. We have treated them as another group increasing potassium intake (most likely through increases in fruit/vegetables) but without specific dietary counselling to: include more vegetables than fruit, include specific vegetables/herb/fruit or reduce PRAL.

The main changes seen in the Scarborough Fair study were lowered plasma bone turnover markers in the group consuming the SF assortment of vegetable/herbs/fruit. Additionally, all inflammatory markers except CRP were characterised by down-regulation with significant reduction of TNF, IL-6, IL-10 and adiponectin in all groups. Recently it has been suggested "there is little evidence for any effect of foods or particular nutrients or non-nutrient, molecules to modulate inflammation by lowering cytokines in healthy women" (Daly, 2013) though it was possible in a limited way with "unhealthy women". In our group of apparently healthy PM women the effect of increased intake of vegetables/herbs/fruit (Group A and B) or increased potassium intake (Group C) has resulted in lowered plasma markers for bone turnover and inflammation which cannot be explained by other environmental changes or confounded by weight loss.

The bone resorption marker CTX was reduced in the Scarborough Fair group compared to the two other groups, but this reduction was limited to women with increased bone loss (osteopenia), not those with normal bone mineral density. Women with osteopenia composed the largest group of all women in this study, while those with normal BMD comprised approximately a third and 12 % had osteoporosis. Although no significant main effect was seen for change in CTX with the two factors: intervention group or BMD, the interaction of these two factors (group and BMD) demonstrated a significant decrease in resorptive activity for osteopenic women consuming the SF range of vegetable/herbs/fruit. Serum resorption markers have not previously been shown in human studies to

decrease without recourse to high doses of phytochemical extracts (Mackinnon, Rao, et al., 2011) or additional supplements such as calcium and vitamin D (Hooshmand et al., 2011). Therefore, this is the first study using a general selection of foods (vegetables/herbs/fruit) without supplementation or without the need to consume other than normal serving quantities to demonstrate this reduction. TNF as a potent stimulator of osteoclastogenesis (Horcajada et al., 2013) showed a large decrease within the SF group and this also, may account for some of the reduction in bone turnover through its known effect on osteoclastogenesis and inflammatory bone loss. PINP also significantly reduced in the SF group and this was a main effect seen in all women in this group regardless of BMD. Reduced bone turnover activity is positive for bone health in PM women due to a slowing of increased bone loss seen with high bone turnover markers.

Adiponectin is negatively associated with BMD (Biver et al., 2011) and this concurs with our findings in this study, but it has been positively associated with a healthier plant based diet (Detopoulou et al., 2009; Bakker et al., 2010; Fragopoulou et al., 2010; Silva et al., 2011; Guo, Niu, Monma et al., 2012; Neuhouser et al., 2012).

The dietary changes in this study, of markedly increased potassium intake from vegetables and fruit and/or increased potassium excretion in 24 hour urines, demonstrated that a diet designed to reduce bone loss long term, also lowered adiponectin (total). Nearly a decade ago, Berner demonstrated that receptors for adiponectin (AdipoR1 and AdipoR2) were not just confined to fat cells, but also were located in bone cells (Berner et al., 2004). The higher levels of adiponectin seen in women with the most bone loss at baseline in this study may be the result of lower receptor availability in the bone cells, or less circulating adiponectin attaching to receptors on bone cells. Once bound, adiponectin may lead to expression of enzymes which result in cell signalling to reduce bone turnover. Higher reductions in adiponectin, in women with the highest existing bone loss at baseline, may be because; more adiponectin was available to interact with receptors due to the cell signalling from active agents in certain phytochemicals or the reduction in inflammation. Alternatively the receptors may be more receptive to adiponectin attachment, as a result of a reduction in inflammation.

Another cytokine significantly modulated downward was TNF. This inflammatory factor negatively impacts bone health and is a portent of mortality, with higher levels considered the best predictor of mortality in frail older populations (Bruunsgaard et al., 2003). TNF has a catabolic effect on bone through up-regulation of resorption activity and a reduction in bone formation.

Elevated levels of TNF increase bone loss at menopause and also increase risk of fracture (Daly, 2013). The reduction in TNF in our study was seen alongside similar reductions in IL-6 but also, particularly in the case of the Scarborough Fair group (Group B), a concomitant reduction in bone turnover marker P1NP and a significant decrease in CTX resorption marker in women with osteopenia. These results appear contradictory, as lowering of TNF should increase bone formation, however, due to the paradoxical nature of many bone regulatory mechanisms it is proposed that multiple systems may have been affected with this dietary change, therefore other factors particularly adiponectin may override the bone formative effect of lower TNF (Biver et al., 2011).

Another important benefit for long term bone health seen during this study was the reduction in 24 hour urinary calcium excretion. Decreased urinary loss of calcium is linked to the increased potassium intake and lowered renal acid load (Sellmeyer, 2013). The acid-base composition of the first two groups (intervention) was altered by specific instructions to increase vegetable consumption rather than fruit which are more alkalinizing. Additionally, acid-base composition was altered due to suggestions to increase starchy vegetable intake by replacing some servings from the breads/cereals food group (Feasibility study tip) to accommodate the high number of servings of vegetables prescribed. This dietary change significantly altered PRAL (Frassetto et al., 2006).

While we had a negative control group (C) who appeared to increase their general intake of fruit/vegetables due to the “Hawthorne Effect”, they did not receive any specific dietary instructions, so they did not have the increased vegetable changes seen in the intervention groups and also their dietary PRAL was not changed significantly. So while the increased potassium has reduced calcium loss (Sellmeyer, 2013) the percentage reduction in urinary calcium excretion was significantly more

in both intervention groups where dietary PRAL also changed significantly (Remer, Krupp, & Shi, 2013).

It is important to note that lowered calcium excretion was seen in women with an already low PRAL at baseline. The reduction in urinary calcium excretion seen in the intervention women with an already low PRAL is therefore, unexpectedly high and augments well for continuous improvements in bone maintenance at midlife with feasible dietary changes.

Positive changes in nutrient intake were another significant finding in both studies. In study 2, micronutrient intake increased for potassium, magnesium, calcium and vitamin A, C and E and reflected current dietary advice to reduce risk of chronic disease by increasing potassium, folate, magnesium and calcium and reducing sodium. An improved profile of nearly all micronutrients was seen with levels more in alignment with national and internationally suggested dietary targets (Ministry of Health (NZ), 2006; WHO., 2012).

7.3 Limitations

There were a number of important limitations with both the above studies. Firstly, there was obvious selection bias particularly with the second study where recruited women had a healthy diet (evident in the low estimated dietary PRAL at baseline), were highly motivated and predominantly white and middle class. The reasons for this were unavoidable, as women had to be healthy for inclusion, which tended to exclude many prospective Maori or Pacific Island women with health issues in this age band. Additionally, the extra cost of providing extra vegetables and fruits would have deterred those with less available disposable income.

The second study was powered sufficiently to show a main effect of change in bone markers, however, it was not sufficiently powered to demonstrate an interaction effect in the groups being divided into three groups according to bone mineral density (normal, osteopenic and osteoporosis). Therefore, the study was limited as the interaction effect between women with osteoporosis compared

to the other two categories: normal bone mineral density and osteopenia, was not able to be determined between the three groups because of insufficient numbers.

As most women in this study had relatively good diets prior to the intervention, we are limited in generalizing the results to healthy midlife women and therefore not generalizable to men, those with less healthy diets, or women of a wider age. However, the significant decreases seen in the inflammatory markers could reasonably be expected to show an even greater influence of this dietary change in the general population or those with a less ideal diet. Future studies could focus on less healthy populations of women where dietary changes on bone and inflammatory markers may be more pronounced. In a lower income group, adaptations may have to be made for provision of additional food and this may promote increased uptake of this dietary change.

Reliance on personal report of dietary intake involves some measure of inaccuracy and although we endeavoured to get as precise a picture of the women's diets as possible, both random and systematic errors may be involved. Although urinary potassium excretion was measured, the biological markers such as Vitamin C and nitrogen balance were not performed on urine samples; however if we had of done these tests, this would have further added to the study findings. This study was relatively short term (3 months) because of time and resource constraints. Future studies of longer time periods would be more determinative of the effects of dietary change on bone health as there would be increased opportunity for DXA or pQCT scanning. BMD also has its limitations and peripheral quantitative computed tomographic imaging (CT scan) offers better possibilities for detecting subtle changes in bone tissue from plant bioactives, such as in micro-architecture and strength (Weaver et al., 2013). A weakness identified in the study design involved a measure of "contamination" of the control group who were asked to maintain their usual dietary intake of fruit and vegetables (no change) with the intervention groups (increased dietary intake of fruit and vegetables). Evidence from the urinary potassium excretion results from the three different groups showed an increased potassium excretion in Groups C along with the other two intervention groups. Increased urinary potassium is derived mainly from diet and it was thought that this increase in potassium was most likely from fruit and

vegetables. This “contamination” may have occurred due a only a single clinic waiting room being available at each centre. The women presented at the clinic in a fasted state, between the hours of 7-10 am, with the visit usually taking half to one hour of their time including eating breakfast with other participants who had scheduled similar times. This time, awaiting blood and other tests to be carried out, or while consuming breakfast, provided an opportunity for some discussion between participants’ in both intervention groups and control group participants, and a degree of “contamination” of the control group was inevitable due to shared discussion while waiting for blood tests and while over breakfast. In addition, various written material was available and left out for the intervention group on the benefits of increased vegetable intake. Some control group participants saw this material and asked if they could take a copy. Any request for printed material was not refused. This may have further incentivised some of those participants in the control group to increase their fruit and vegetable intake, which was evident from the increased urinary potassium excretion values in the control group. In light of this I would recommend scheduling control group participants on a different day to intervention group participant’s to prevent any mingling and sharing of dietary changes or reading material.

7.4 Strengths

The community based approach of both these studies, with participants sourcing, storing and preparing their own increased intake of vegetables and fruit is a particular strength of the studies, as it indicates a strong application to “real world” situations and possible use as a model in other community-based interventions. Unique aspects of this study on bone health which differ from others using a dietary approach to reduce bone loss are: its reliance on food only, and in normal serving sizes amounts rather than fortification or additional dietary supplements, and its emphasis on a range of foods containing bone “friendly” phytochemicals rather than a single food. While certain foods are known to have functional properties with positive effects for bone turnover, long term consumption may ultimately depend on having a variety of foods to choose from. The Scarborough Fair group had a range of over 20 fruits/vegetables/herbs to choose from and participants showed good compliance with this range from which they chose at least half their servings of vegetables and fruit and herbs.

This is the first time a reduction in adiponectin has been reported as a result of a dietary intervention with increased vegetables and fruit. The extent of reductions in circulating pro inflammatory markers TNF and IL-6 is greater than previously shown as a result of a nutritional intervention (Ziccardi et al., 2002; Bakker et al., 2010). IL-6 decreased by -0.7 pg/ml (p<0.000) in Group A, -0.8 pg/ml (p<0.000) in Group B and -0.9 pg/ml (p<0.000) in Group C. TNF demonstrated similar sized reductions of -4.6 pg/ml (p<0.000) in Group A, -5.3 pg/ml (p<0.000) in Group B and -3.3 pg/ml (p<0.000) in Group C. It is also significant, as the effects of the dietary intervention are not confounded by weight loss.

The control group provided an opportunity to compare 3 groups of post-menopausal women with nutritious diets based on dietary intake data and increased urinary potassium excretion. The increased intakes of potassium/fruit/vegetables allowed determination that the SF range of vegetables/herbs/fruit had effects on bone markers over and above that of increased potassium (A and C), other phytochemicals, lowered PRAL (B), and lowered sodium urinary output (A and C) in this study. It would cautiously be suggested effects may be more pronounced compared to women consuming a more usual diet lower in fruit/vegetables.

7.5 Recommendations for future research

There are several avenues to further investigate the benefits of diet on human health, and in particular, specific actions in preventing or reducing bone loss and osteoporosis.

Future research should involve longer term randomised human intervention studies with control participants who are not influenced by the study aims. The study design should allow for the ability to determine the specific effects of polyphenols compared to other dietary components. The precise profile of phytochemicals in a food, as well as how the phytochemical interacts with the food matrix and its bioavailability may allow for development of specific dietary recommendations (Scalbert, Andres-Lacueva, Arita et al., 2011; Macdonald-Clarke & Macdonald, 2013). Some of the fruit and vegetable sources of plant phytochemicals that should be investigated further for bone health include: dried plum, allium vegetables (onion, garlic, leek and chives), berries, grapes, oranges, and some

herbs. A more complete understanding of the individual bioactive's mechanism of action, would assist with understanding the best outcome measures to be assessing during any intervention e.g. which inflammatory markers or phase 2 enzymes. There is a need for a more comprehensive database of dietary polyphenols accounting for the variation in composition in foods in various countries so there is more certainty of dietary intake. In addition, the absence of standard methods of analysis of phytochemicals means there is variation in reported amounts of any specific phytochemical between laboratories (Scalbert et al., 2011; Tomás-Barberán & Andrés-Lacueva, 2012) as well as non-extractable phytonutrients usually not being included e.g. bananas, kiwifruit (Tarascou, Souquet, Mazauric et al., 2010; Tomás-Barberán et al., 2012). Human intervention studies with whole foods will need to ascertain precise methods of cooking and consumption to determine the availability of phytochemicals and their metabolites. Cooking methods, whether the food was fresh or frozen and whether the food was eaten with skins on or peeled, all greatly influence levels of phytochemicals available (Scalbert et al., 2011; Saha, Hollands, Teucher et al., 2012). Better understanding of the metabolism of phytochemicals including: their absorption and uptake in the gut, how an individual's genetic makeup and microflora influence the action and availability of polyphenolic metabolites and their action in the body, will all increase the understanding of polyphenols action on inflammation and bone health (Tomás-Barberán et al., 2012; Shen et al., 2013). Furthermore, the field of nutrigenomics may allow further identification of the specific effects of phytochemicals action on specific genes, proteins, or metabolites and give a more complete understanding of their health benefits (Scalbert et al., 2011), Regarding acid-base balance and bone health, while recent work has demonstrated the benefits on calcium balance and bone turnover markers of a diet with a low acid load due to supplementation with potassium citrate (mimicking the effect of fruit and vegetables) (Moseley et al., 2013), there is a need for longer term studies with human participants to determine effects not only on calcium balance but also other bone outcome measures. Assessing bone strength, flexibility and microarchitectural changes in addition to the usual measures of bone turnover markers and BMD will allow a broader definition of bone quality improvements that may result from dietary changes (Horcajada & Offord, 2013) including lower dietary acid loads. Although high dietary acid loads have been shown to induce higher steady state blood hydrogen ion concentrations and lower plasma

bicarbonate concentrations (Kurtz, Maher, Hulter et al., 1983; Frassetto, Morris, & Sebastian, 2007), a healthy diet, low acid load coupled with healthy kidney function and glomerular filtration rate may not require significant buffering from bone or muscle to neutralise the endogenous acid production (Frassetto & Sebastian, 2013).

7.6 Conclusion

The main contribution to research this study demonstrated, is that diet can be altered in a feasible and sustainable way to increase intake of a selection of vegetables/herbs/fruit with phytochemicals, which reduce bone turnover markers in PM women, particularly those with increased bone loss. Reduction of urinary calcium loss via lowered dietary PRAL is another element in the diet/bone relationship and this study's low inflammatory diet has been demonstrated to benefit bone health in both ways. The anti-inflammatory properties of the vegetables/herbs/fruit lowered inflammatory markers (TNF, IL-6, IL-10 and OPG) and adiponectin levels significantly downwards in healthy, weight stable, post-menopausal women. TNF and IL-6 are powerful stimulators of osteoclastogenesis and a reduction in bone turnover markers was evident in the SF group. Bone loss may be reduced if the dietary change is maintained long term as well as the reduction in bone turnover markers.

A reduction in adiponectin is another mechanism which protects bone loss at midlife as higher levels indicate higher bone loss though its mechanism of action is still speculative. The reduction in adiponectin is a novel finding and is contrary to previous studies showing higher adiponectin is associated with higher plant and polyphenol rich diets. The final mechanism protective of bone in this study was the decrease in urinary calcium loss through reduced PRAL. While the decrease in calcium was seen in all groups, a greater decrease occurred in women with specific dietary counselling to consume greater amounts of vegetables/herbs in preference to fruit, lowered dietary acidity (PRAL) and with increased urinary pH compared to women also with increased potassium urinary excretion but no change in urine pH or dietary PRAL. A more pronounced effect of the dietary intervention on

bone (CTX, P1NP), and inflammatory markers (TNF, IL-6, IL-10, CRP) and urinary calcium loss was demonstrated in Group B (Scarborough Fair group) compared to Group A or Group C.

7.7 Concluding remarks

Animal studies show evidence of particular vegetables/herbs and fruit effects on bone turnover markers and bone mineral content but there is limited evidence from human interventions with very few showing effects on resorption markers. Vegetables/herbs and fruit offer a rich array of phytochemicals with potential bone protective properties and the mechanisms involved range from anti-inflammatory effects on bone turnover via molecular mechanisms to lowered calcium loss due to lowered renal acid load. The effect of adiponectin on bone is another area still to be elucidated. There are many exciting possibilities for future research into dietary strategies to minimize bone loss at midlife. Some consumers prefer natural approaches rather than supplementation and therefore this specific range of vegetables/herbs/fruit (parsley, sage, rosemary and thyme, onion, garlic, broccoli, green beans, mushrooms, Chinese cabbage, tomatoes, prunes and oranges) may warrant further investigation and confirmation of this study's preliminary findings of reduced bone turnover in PM women.

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APPENDIX A: THE FEASIBILITY STUDY

Vegetables, Fruit  and pH Study

Ph. D student

Caroline Gunn

Supervisors:

Associate Professor Jane Coad

Dr Janet Weber

Professor Marlena Kruger

A1. Feasibility study – concepts and procedures

A nutritional strategy designed to increase consumption of vegetables and fruit to enhance bone health in midlife women.

Design concept:

This feasibility study aims to determine if

- A nutritional strategy involving increased consumption of vegetables and fruit can be successfully implemented in a group of free-living women
- Whether this dietary change results in a reduced acid load of the diet (determined indirectly from three day diet diary) and increased urinary pH by at least 0.5 units.

This strategy will be aided by the use of pH dipsticks to enable the women to achieve an increase in urinary pH of 0.5 pH units thought to be required to affect bone markers.

Questions to be answered:

- Are the two aims achievable:
    - Can the women manage to sustain the dietary change required i.e increase their consumption of vegetables and fruit to 9 serves a day and the range suggested?
    - Will the dietary change result in an increase urinary pH by 0.5 of a pH unit or up to neutral pH (6.5-7.0) and sustain it for the duration of the study (6 weeks)?
- How long does it take for the dietary change to affect urine pH?
- How is the measuring of urinary pH useful as a motivator for dietary change?

What other aspects of the women's diet change with the increased consumption of vegetables and fruit (e.g. intentional/unintentional substitution derived from interview and food records)?

To what extent does PRAL change from baseline and how much change in PRAL is required to achieve the urine pH change expected? (Note: NEAP and PRAL calculated from the 3 day diet diary

completed by participants at the beginning and end of the study) A calculation model for the NEAP and PRAL is included.

What are the main barriers/obstacles to achieving an increased consumption of a range/quantity of selected vegetables?

Motivational factors e.g. who is willing to participate and why?

Sustainability -Would the participants be willing to eat this way for a longer period or indefinitely?

Compliance issues e.g. what barriers are there to implementing the increase in fruit and vegetable consumption-cost/preparation time/taste and family/ social issues involved in sustaining it as this is intended as long-term dietary strategy. Also compliance with pH testing.

It is expected that after reading the information sheets on the recommended dietary changes needed and having any questions they may have, answered, the women will be able to understand how to increase their dietary intake of vegetables and fruit (alkaline forming foods) and **balance this increased intake of vegetables and fruit with other foods in their diet to achieve a corresponding increase in urine pH.** Advice and support will be available via email and telephone as required and in addition the women will be contacted weekly for monitoring of pH levels. No caloric restriction will be involved

The use of the pH dipsticks will provide an immediate and visual representation of the daily dietary acid load and therefore will be a useful adjunct to the dietary information provided to the women in implementing the dietary and behavioural modification needed.

Evaluation of the Feasibility study will be based on

Achievement of 2 main aims:

Did the women achieve the required intake of fruit (3 serves) and vegetables (6 serves) and the range?

Did urine pH increase by the amount necessary (0.5 pH unit)?

Were questions 1-5 satisfactorily answered by the information provided to participants, questionnaires and 3 day diet diary?

Also

Were there any errors in procedures or clarity of instructions which are required to be ironed out in this pilot study so they are not duplicated in the larger scale study?

Were procedures for data collection and data management robust?

Specified Dietary intervention required to achieve aims:

The women will be required to have an increased proportion of vegetables and fruit in their diet consuming at least 6 serves of vegetables (300-500 grams) and 3 serves of fruit each day.

Including the following:

Green leafy vegetables

2 servings cooked or 1 cup/day e.g. silverbeet, spinach, bok choy, or

other salad vegetables e.g. rocket, watercress

1 serving of other cruciferous vegetables e.g. broccoli, cauliflower, cabbage, Brussel sprouts (1 serve= ½ cup/day)

1 serving of Onion type (allium) vegetables- white/red onions, spring onions. Shallots, garlic, or leeks

No caloric restriction is intended as a result of this dietary strategy

Fruit- any type (3 serves)

Testing of urine-Use of Dipsticks

To aid in achieving the needed increase in alkalinity and decrease in dietary PRAL, pH dipsticks will be provided for monitoring daily urine pH. A daily record will be kept of the second voided urine pH to determine how it changes with the increased vegetables and fruit.

Feasibility Study Procedures:

A sample of 21 women in the 40+ age group will be followed for 8 weeks

Recruitment

Advertise by flier around Massey University and word of mouth for volunteers to trial a dietary strategy involving a high consumption of fruit and vegetables and in particular, a selected range of vegetables

No calories need be counted or dietary deprivation involved

Urinary pH will need to be monitored and recorded

Weekly contact with student researcher

Participants are expected to be drawn from the Manawatu/Hawkes Bay regions and should be healthy, 40 years of age or older and currently not achieving the NZ dietary guideline of 5 serves a day of fruit and vegetables

Information Session

Once the participant is fully aware of the expectations involved in the study and has read the information sheet and informed consent has been obtained -they will be given training on

Fruit and vegetables- recipes and tips on how to include more fruit and vegetables into their daily dietary intake

Urine pH measurement: how /when to measure-and explanation of variables that may influence the results

Procedures and data collection

The participants will be advised to initially continuing eating their normal diet and record their dietary intake (3 day diary).

Urinary dipsticks will be provided and 3 urinary pH readings on successive days are taken to establish a baseline urine pH measurement.

Participants will be asked their height/weight if known/if not they will be asked to check and report back as it required for working out PRAL values and also will fill in a general lifestyle questionnaire on exercise levels/smoking/alcohol

If there is enough interest from the participants (5 or more) for group sessions or an internet chat group, this will be arranged. These sessions would be for additional information, sharing of recipes, tips and support.

Participants will need to be contactable on a weekly basis for follow up

NB. For the duration of this study the participants will be asked to maintain their normal level of activity

Inclusion criteria

Women 40 years of age and over who are in good health and not currently under medical treatment and

Currently consume less than NZ dietary recommendations of servings of fruit and vegetables daily (4 or less serves of fruit and vegetables)

A2. Flier - Vegetables, Fruit and pH Study



Women (40 years or over) are wanted for a unique study whereby they increase their consumption of vegetables and fruit to decrease the acid load of their diet.

The study will be over an 8 week period. 


The study involves an increased consumption of vegetables  6 servings or more and fruit

 3 servings each day

If you are in good health, not currently under medical supervision for illness and don't currently meet the 5 serves of fruit and vegetables per day recommendation, then we would like to give you the opportunity to take part in this Nutrition study. For more information-please ring or email Caroline Gunn

Contact details: Ph.D. student: Caroline Gunn c.a.gunn@massey.ac.nz Massey University: 3569099 ext 2311

Supervisor Associate Professor: Jane Coad j.coad@massey.ac.nz 06 350 5962

A3. General Information Sheet - Vegetables, Fruit and pH Study

Thank you for your interest in this study which is being conducted by PhD student Caroline Gunn (Reg.Nutritionist) under the supervision of Associate Professor Jane Coad, Dr Janet Weber and Professor Marlena Kruger in the Institute of Food, Nutrition and Human Health (IFNHH), Massey University, Palmerston North.

Study description

This 8 week study investigates the effect of increased consumption of vegetables and fruit in the diet on urinary pH. Increasing fruit and vegetables should increase the proportion of alkaline forming foods and therefore decrease the acid load in the diet and increase urine pH. This is intended as a long term dietary strategy with beneficial effects on health and in particular for midlife women in reducing bone loss.

Participants will be expected to:

Consume 6 or more servings of vegetables per day (including specific types of vegetables) and 3 serves of fruit (any type).

Monitor and record their urine pH on a daily basis with pH dipsticks

There may be some additional food costs involved in buying more vegetables and fruit as well as food preparation

Participants will be recruited from advertisements around Massey University and in the community by word of mouth.

Places on the study are limited to 10

There are no known or expected detrimental effects or risks associated with participation in this trial.

Project Procedures

The main changes we would like you to make on a daily basis for 6 weeks involve:

Increasing your vegetable intake to 6 serves or more and fruit to 3 serves per day

Record your urine pH each morning with provided dipsticks

Also we would ask you to:

Have a brief interview on current vegetable/fruit consumption and lifestyle.

Keep a 3 day dietary diary at the beginning and conclusion of the 6 week study

Be contactable for weekly updates with the student researcher (phone)

Fill in an evaluation at the end of the study

Data Management

All data obtained from the participants including questionnaires, 3 day dietary diary and pH measurements will be kept strictly confidential

Data will be used to evaluate the viability of a larger study involving this same dietary strategy

All data will be treated as confidential and kept secure for a period of 5 years after which it will be disposed of

Any participant wanting a summary of the feasibility project findings will be provided with it

All participants names will be removed from any research findings.

Participant's 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 meet the following requirements

Female aged 40 years or over

In good health and not currently under medical supervision for illness

Currently don't meet recommendations for 5 servings of fruit and vegetables per day

Then we would like to hear from you

Project Contacts

If you wish to participate or learn more about the project please contact:

Caroline Gunn Ph.D. student researcher IFNHH, Massey University, Palmerston North

Ph 06 3569099 ext 2311 or c.a.gunn@massey.ac.nz

Or Associate Professor Jane Coad *Supervisor j.coad@massey.ac.nz*.

LOW RISK NOTIFICATIONS

"This project has been evaluated by peer review and judged to be low risk. Consequently, it has not been reviewed by one of the University's Human Ethics Committees. The researcher(s) named above are responsible for the ethical conduct of this research.

If you have any concerns about the conduct of this research that you wish to raise with someone other than the researcher(s), please contact Professor Sylvia Rumball, Assistant to the Vice-Chancellor (Research Ethics), telephone 06 350 5249, email humanethics@massey.ac.nz".

A4. Participants Information Sheet

Thank you for agreeing to participate in this study and increase your intake of fruit and vegetables.

Key requirements for this study are:

Eat at least 6 serves or more of vegetables daily

Eat 3 serves of fruit daily

Record your urine pH each morning using a dipstick

Specific vegetables are to be included on a daily basis

2 serves (of the 6) must be **green leafy vegetables** (spinach, silverbeet, watercress, bok choy)

1 serve of onion family (allium) vegetables e.g. spring onions, red/ yellow onions, shallots, leeks or garlic

1 serve of cruciferous vegetables e.g. broccoli, cauliflower, cabbage

At least 2 more serves of any other vegetables

Aims of this study:

Can you increase your consumption of vegetables and fruit to 9 serves a day and the range suggested. I am also interested in how you manage the dietary change required to increase your vegetable intake and finding out what motivators and barriers you experience.

You are provided with

Information on the study and guidelines

Notebook for recording urine pH/daily vegetable intake/exercise

3 Day dietary diary/ Questionnaire

If you have any queries please contact Caroline Gunn c.a.gunn@massey.ac.nz or phone 3569099 ext 2311

A5. Urine pH Information Sheet

Instructions

Record the pH of your second voided urine each day

Place the pH dipstick briefly in the midstream flow

Allow 10-15 seconds for the colour to develop.

Match it with the corresponding colour of the chart- if unsure use the bottom colour as the main guide.

Discard dipstick in the toilet (safe in septic tanks)

Write the number in your urine pH diary

Note: Fasting urine pH can vary between 4.5- 7.5 but commonly it is around 6.0.

Try to increase your pH by at least 0.5 pH unit and preferably to attain a pH reading consistently (or 5 out of 7 days in a week) of 6.5-7.0.

A change from 6.0-7.0 indicates a tenfold increase in alkalinity.

Factors which can affect urine pH

Besides diet, some medications, health conditions and certain activities can affect pH

A higher pH can be obtained by any of the following:

Kidney disease

Urinary tract infection

Recent sexual activity

Use of diuretics

Drugs to treat heartburn (antacids)

A lower pH can be due to any of the following

Lung disease

Diabetes complications

Starvation

Diarrhoea

Excessive exercise

Some medications- antibiotics, antihistamines, aspirin and other NSAID

pH, Food and Activity Diary

Each day after you have tested your urine with the dipstick, record the result in the table

Also each day fill in the other columns by recording your fruit and vegetable intake and your exercise levels FROM THE PREVIOUS DAY using the numbering system below

Food Intake

Record your previous day's vegetable and fruit intake using the scale 0-1, 2, 3… for total number of serves vege-

Exercise levels:

1 Sedentary Daily living activities only

2	Low active	30-60 mins moderate activity day
3	Active	More than an hour per day
4	Very active	More than 1hr moderate and 1 hour vigorous or 120 min moderate per day

Example only

ID	Day	Date	Urine pH	Veg serves total	Green veg	Allium vege	Fruit serves	Exercise Levels (1-4)
Week1	Monday	7/8	6.0	9	2	1	4	3
	Tuesday	8/8	6.5	8	2	1	1	4
	Wednesday	9/8	6.0	6	2	1	2	2
	Thursday	10/8	5.5	10	1	1	2	1
	Friday							
	Saturday							
	Sunday							

A6. Urine pH Diary

Urine pH diary: week one (RAMP UP)

ID	Date	Urine pH	Green leafy Aim : 1 serve/d	Onion Aim: 1 serve/d	Crucif type Aim:1 serve/d	Total Veg Aim: ≥4 serves/d	Fruit Aim: 2-3 serves/d	Significant Changes Exercise level or meds √
Week1	Mon							
	Tues							
	Wed							
	Thurs							
	Fri							
	Sat							
	Sun							

Urine pH diary: week two (RAMP UP)

ID	Date	Urine pH	Green leafy Aim : 1 serve/d	Onion Aim: 1 serve/d	Crucif type Aim:1 serve/d	Total Veg Aim: ≥4 serves/d	Fruit Aim: 2-3 serves/d	Significant Changes Exercise level or meds √
Week2	Mon							
	Tues							
	Wed							
	Thurs							
	Fri							
	Sat							
	Sun							

Urine pH diary: week three (Target levels)

ID	Date	Urine pH	Green leafy Aim : ≥2 serves/d	Onion Aim: 1 serve/d	Crucif type Aim:1 serve/d	Total Veg Aim: ≥6 serves/d	Fruit Aim: 3 serves/d	Significant Changes Exercise level or meds √
Week 3	Mon							
	Tues							
	Wed							
	Thurs							
	Fri							
	Sat							
	Sun							

A7. Supplementary information

Bone Health and diet

Bone health in midlife is the result of several interrelated factors including genetics, lifestyle (exercise) and diet.
Only exercise and diet are modifiable and can provide an effective strategy to avoid osteoporosis.
The dietary strategy presented in this study is based on supplying ample quantities of the nutrients that the body needs to maintain bone mineral mass through midlife years as the bone protective effect of oestrogen declines.
Vegetables and fruit contain minerals (e.g. calcium, potassium and magnesium) and vitamins (K and C) as well as variety of phytochemicals including antioxidants which have been shown by research studies to support bone health.

Other protective effects

Increased quantities of vegetables and fruit in the diet have also been shown to have a range of other health benefits associated with the provision of dietary fibre.

These include:

1) Slowing glucose absorption and reducing the insulin response thereby preventing or controlling diabetes
2) Lowering the risk of heart disease and high cholesterol
3) Reducing the risk of colorectal cancer
4) Increasing the uptake of calcium, magnesium and iron in the large intestine
5) Weight control

Additionally, vegetables and fruit are alkaline forming which may preserve muscle mass as the body ages as well as prevent bone loss.
As the proportion of vegetable and fruits in your diet increases there will be a corresponding change in your urine ph. This pH should increase, that means it is becoming less acidic. Aim to achieve a neutral or alkaline urine pH.

Serving Sizes Guidelines

Consume at least 6 serves vegetables each day and 3 serves of fruit.

What's a serve?

In general:

1 serve =½ cup cooked fruit or vege or 1 cup uncooked

Examples of vegetable serves

A single serving size (1/2 cup) is usually 50-80gms)

Broccoli	1/6 of a head =50grams or 1/2 cup
Brussel sprouts	4 small = 1/2 cup= 60 grams
Carrots	1 small = 1/2 cup = 1 serve
Pepper	1 large = 150 grams= 2 serves
Tomato	1 small = 80gram= 1 serve
Onion	1 medium 125grams= 2-3 serves
Cooked green leafy vegetable	½ cup cooked= 80grams= 1 serve
Mixed vegetables	½ cup =60 grams= 1 serve
Salad (uncooked vegetables)	1 cup= 1 serve
Potato, kumara, yam or taro	1 medium = 130grams

Fruit serve guide

If stewed/canned approximately ½ cup = 1 serve

Fresh fruit serves:

Medium size fruit	= 1 apple, 1 banana or pear
Smaller fruit	= 2 kiwifruit, 2 mandarins, 3 plums, 4 apricots
Very small fruit	= 20 strawberries, or 1½ cups of loquats,1 ⅔ cups berries
Fruit juice	= 160 mls = 1 small glass

Tips for increasing vegetable and fruit consumption:

Aim to have your meal plate comprise portion wise 70% vegetables and 30% other foods .

Make a large pot of soup, which you can keep in the refrigerator for 3-4 days. Each day take out a portion and add fresh vegetables

Make a big salad and store it in airtight container and it should last 3-4 days so long as you don't add any dressings or cut vegetables like tomatoes or cucumbers which can make things go soggy.

Prepare salads for lunch the night before or that morning as it may be difficult to think of doing this when you are hungry at lunchtime.

Cook more potatoes, kumara, pumpkin, taro, yams etc than you need for your evening meal so you can eat them the next day for lunch, breakfast or snacks

You may have to eat a lot more bulky foods to achieve fullness as well as eating more often than you are used to doing to achieve the kilojoule/kcalorie intake you are used to.

Always have snacks such as fruit handy.

N.B: The NZ Food and Nutrition Guidelines encourage consumption of foods that are low in fat, sugar and salt

Suggested Foods for Meals and Snacks

Breakfast:

Potatoes (warmed up in microwave or lightly steamed/fried)

Yoghurt/fruit-bananas,

Vegetable soup

Lunch:

Salad vegetables

Starchy vegetables such as potatoes, kumara, taro, yams, pumpkin

Soup with added vegetables

Dinner:

Salad and or vegetables

Starchy vegetables such as potatoes, yams pumpkin, kumara or taro

Snacks:

Fruit e.g. bananas, apples, citrus fruit

A8. Participants Daily To Do List

Vegetables

1) Eat at least 6 serves of vegetables every day

2 serves Green leafy vegetables (spinach, silverbeet, watercress, rocket,)

1 serve Onion family: yellow, red, spring onions, shallots, leeks and garlic

1 serving cruciferous vegetables (cauliflower, broccoli, cabbage)

At least 2 more serves of any other vegetables eg : carrot, peas, green beans

Fruit

2) Eat 3 serves a day of any type of fruit available that you have available and enjoy

Urine pH

3) Record the pH of your second voided urine each day and fill in record sheet line

A9. Questionnaires –initial, mid and end (Vegetables, Fruit and pH Study)

Initial interview (semi directed)

Introduction: These questions are to help establish your present intake of vegetables and fruit, lifestyle factors including barriers associated with their consumption, and what strategies you may use to increase your vegetable consumption

1). Would you usually consume vegetables on a daily basis Yes □ No □

If Yes, How many serves on average do you have per day (Photo guide book)

Usually One □

Two □

Three □

Four □

Five or more □

2. Are you aware of the MOH's food and nutrition guidelines for vegetables and fruit? (probe what it is)

3. How do you currently incorporate vegetables into your diet? (probe which meals, mixed dishes)

4. What ways can you see yourself using to increase your consumption of vegetables?

Shopping habits

5. Who does the fruit and vegetable shopping in your household?

Access to vegetables and fruit- how often are they always available in the house? (probe how easy it will be to increase F/V)

6. Where do you normally get your vegetables from? (probe how often you buy)

7. Who does the cooking?

8. Do other people you live with influence your meal menus? (Probe how they will cope with these increased vegetables)

9. Is cost a significant factor in buying vegetables and fruit?

10. Do you know of any health benefits associated with increased vegetable and fruit consumption?

11. Do you think personally, increasing your consumption will have any effect on you?

12. Do you eat fruit? Yes □ No □

If yes how much per day_____ serves

(probe how easy is it for you to increase fruit consumption)

13. Do you drink alcohol? Yes □ No □

If yes How any units per week_____

(Refer appendix i for measures)

14.Age:

□40-45 01

□ 46-5002

□ 51-5503

□ 56-6004

□ 61-6505

□ >65 06

15. What, if any, supplements are you taking? *(Frequency of consumption).*

Why are you taking it? *(Probe if looking to include particular nutrients)*

i)_____

_____ ii) Are you still taking it?

16. How would you describe your level of Physical Activity?

Sedentary daily living activities only □

Low active Plus 30-60 mins moderate activity day □

Active Plus more than an hour per day □

Very active Plus more than 1hr moderate and 1 hour vigorous

or 120 mins moderate /day □

17. Do you know your height? Yes □ No □

if yes what is it in metres_____m _____ ft.inches

Do you know your weight? Yes □ No □

if yes what is it in kilos _____kg

Smoking – Which category do you fit into?

Are a current smoker □

Use to be a smoker □

Never smoked □

Motivation –what is the main reason you want to participate in this study? (Probe what benefits participant sees as being part of this study)

Which ethnic group or groups do you identify with? *(Tick all that apply)*

□ NZ European 01

□ NZ Maori 02

□ NZ Asian 03

□ Pacific Islander 04

□ Indian 05

□ Chinese 06

□ Other European 07

□ Other Asian 08

□ Other (specify)… 09

Appendix i: Definition of 1 unit of alcohol

All of the drinks listed below contain one unit of alcohol:

1 single measure of spirits 25 mls

1 small glass of sherry

1 small glass of wine **(half 175 ml glass)**

1/2 pint of ordinary strength beer, lager or cider. (3.5-4%ABV)

Note: A large glass of wine = 3 units

Alcopops contain 1.4-1.5 units per bottle

1/2 pint of strong beer, lager or cider (4-5.5%ABV) = 1.5 units

It is important to note that when you are drinking at home a 'home measure', such as a glass of wine, beer or spirits, may be two or three times larger than a pub measure of 25 mls.

Mid Interview

Vegetables, Fruit and pH Study

4 Week Questionnaire:

Name:

Period covered Week (_____)

_____

Question 1.

How are you finding achieving the dietary modifications required?(probe achieving number of serves, types, shopping, refrigeration)

_____

Question 2.

Have you managed to record your urine pH on a daily basis.

Question 3.

Has your urine pH increased? (Discuss)

_____

Question 4.

Have you experienced side effects from eating more vegetables and fruit? (Discuss)

_____

Question 5.

Did you feel that you had any changes in yourself with consuming more vegetables (energy levels, body functioning)? (Discuss)

_____

End Interview

Vegetables, fruit and pH Study

End of Study Questions

Participant No._____

Was the information provided about diet and recording urine pH easy to understand?

Do you think the dipsticks were useful in motivating you to achieve and maintain a raised urine pH?

How effective were you in modifying your daily intake of vegetables? (Reasons for this)

What were the negative aspects of this dietary change on your lifestyle?

Did you experience any physical side effects e.g. Bowel changes/increased wind?

What were the positive aspects of this dietary change for you?

Was there a significant increase cost overall to your food bill?

Have you had to make significant changes in your lifestyle to accommodate the dietary change?

Did others you may live with eat more vegetables and fruit as a result of you participating in this study?

Did you experience difficulty with accessing, transporting or storing the extra vegetables as a result of you participating in this study? (E.g. fridge space, transport)

What advice would you give to participants in further trials?

A10. Participants Consent Form

Vegetables, Fruit and pH Study

Participant 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

Full Name:_____

Signature: _____

Date:_____

A11. 3 Day Dietary diary

Instructions for completing the Food Record

Record all that you eat and drink for three consecutive days. One day must be weekend day. Start each day at midnight and finish 24 hours later.

On the diet record sheet include:

The time of day the food was eaten

Where it was eaten

Description of the food (preparation)

Amount eaten (weighed or estimated)

Note:

Eat normally (You will not be assessed on the content of your diet.

Remember to record all drinks, ***including water***. If you drink from a sipper bottle throughout the day record it as such, ie. you don't need to record every sip,

Record cuts and types of meat, type of milk, type of bread, fruit varieties, brand names, cooking method used, etc.

Measure serving sizes where possible, e.g. how many cups of breakfast cereal, not "a bowl"; how many cups of ice cream, not "a dessert plate". A cup should be a metric measuring cup if possible.

Many purchased foods and drinks state the weight or volume on the labels, these can be used directly

If vitamin or mineral supplements are used, list the amount taken each day, the brand name and label information

Record the amounts of all foods and beverages in the form that they are consumed. For example, record the amount of a cooked piece of meat rather than the weight / size of a raw piece of meat.

Example only

Date_____

Time food was eaten	**Place eaten**	**Complete description of food** (preparation, variety, brand) If possible attach the recipe or the nutrition label (fortified foods).	**Amount consumed (units, measures, weight)**
*Exanple:*7: 55 AM	Home	Pizza, Hawaiian, homemade- (recipe attached) microwaved	1 slice (1/8 of pizza)
" "	" "	Orange juice (Citrus Tree with added calcium- nutrition label attached)	1 glass (275 ml)
10 AM	Car	Raw apple (Gala)	3/4 medium (*ideally you would weigh whole and leftovers)*
12 noon	Office	Sandwich – margarine, ham, lettuce, tomato, tasty cheese	Olivani margarine (Lite) - 2 tsp Ham (shaved) – 1 slice Lettuce – 1 leaf Tomato – 1 small Edam cheese – 25g grated

Day one
Date_____

Time food was eaten	**Place eaten**	**Complete description of food** (preparation, variety, brand) If possible attach the recipe or the nutrition label (fortified foods).	**Amount consumed**

Day two

Date_____

Time food was eaten	**Place eaten**	**Complete description of food** (preparation, variety, brand) If possible attach the recipe or the nutrition label (fortified foods).	**Amount consumed**

Day three

Date_____

Time food was eaten	**Place eaten**	**Complete description of food** (preparation, variety, brand) If possible attach the recipe or the nutrition label (fortified foods).	**Amount consumed**

A12. NEAP and PRAL Calculation Model

Reference: (Frassetto, Lanham-New, et al., 2007)

Agreed calculation models and terminology from the 2nd International Acid-Base Symposium held in Munich, (8-9 September 2006)

Remer and Manz (1995) estimated the Net acid load from average intestinal absorption rates of ingested protein and additional materials as well as an anthropometry-based estimate for organic acid excretion:

Estimated NEAP (mEq/d) =PRAL (mEq/d) + OA_{est} (mEq/d)

Whereby PRAL denotes dietary potential renal acid load and OA est denotes urinary organic anions with both components calculated as follows:

PRAL(mEq/d) = 0.49xprotein(g/d) +0.037xphosphorous (mg/d)-0.021x

potassium(mg/d)-0.026 x magnesium (mg/d) – 0.013 x

calcium (mg/d).

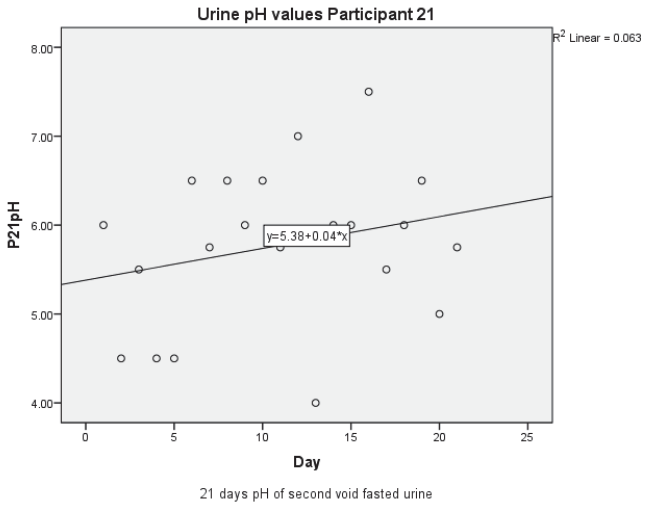
OA_{est} (mEq/d) = individual body surface area(a) x 41/1.73

a) Body surface area calculated according to the formula of Du Bois and Du Bois as follows:

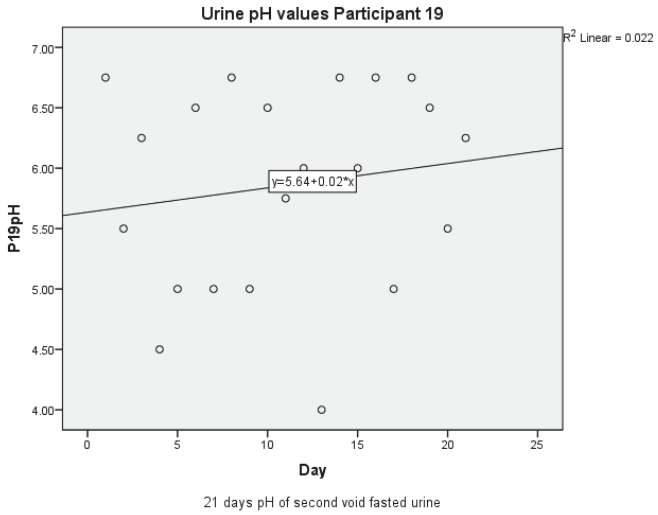
Body surface area (m^2)= 0.007184-height(cm) $^{0.725}$- weight(kg) $^{0.425}$

A13 Urine pH graphs from participants in Feasibility Study

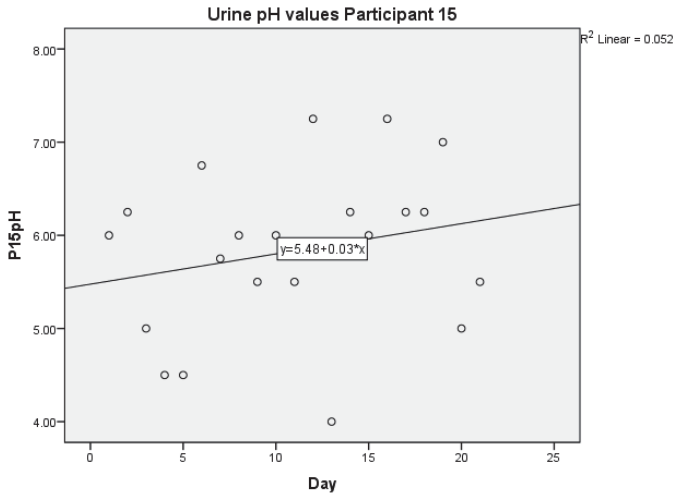
Urine pH readings from participants in feasibility study showing trends over the 8 weeks of study. A linear regression line models the trend to increased urine pH with time on the study consuming a diet high in fruit and vegetables which provide alkaline buffers upon metabolism.



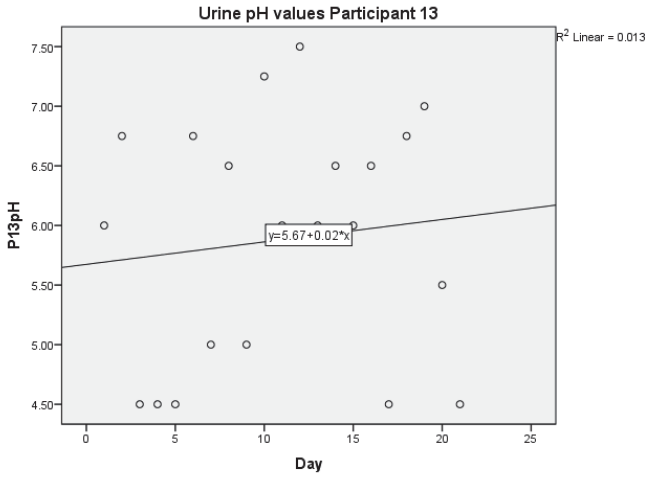
21 days pH of second void fasted urine



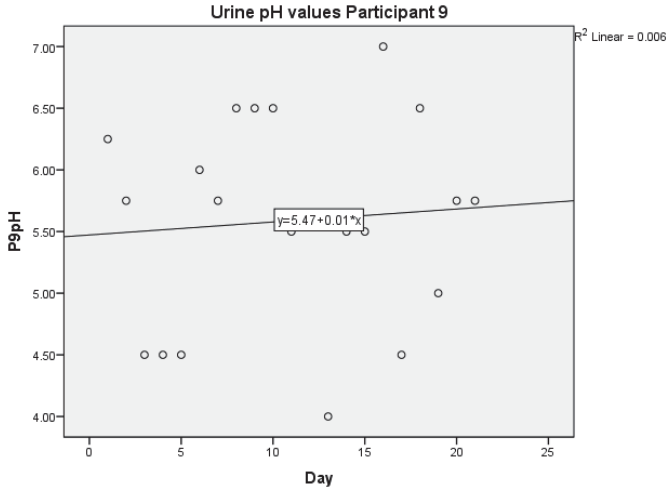
21 days pH of second void fasted urine



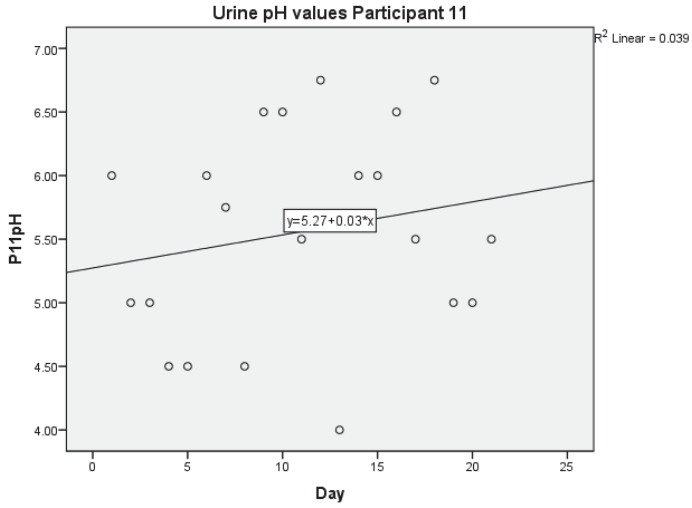
21 days pH of second void fasted urine



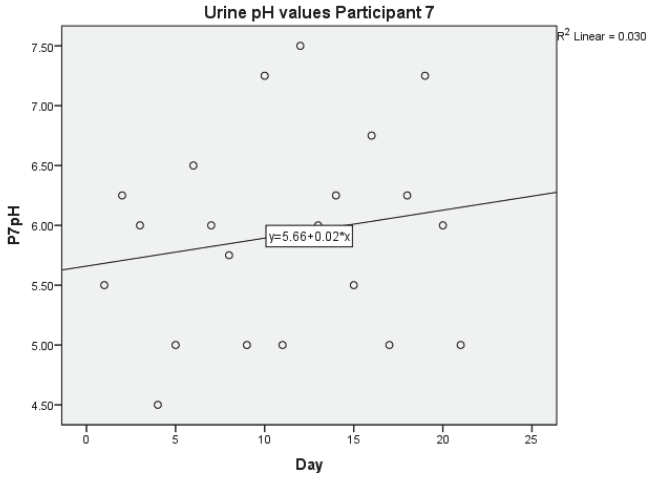
21 days pH of second void fasted urine



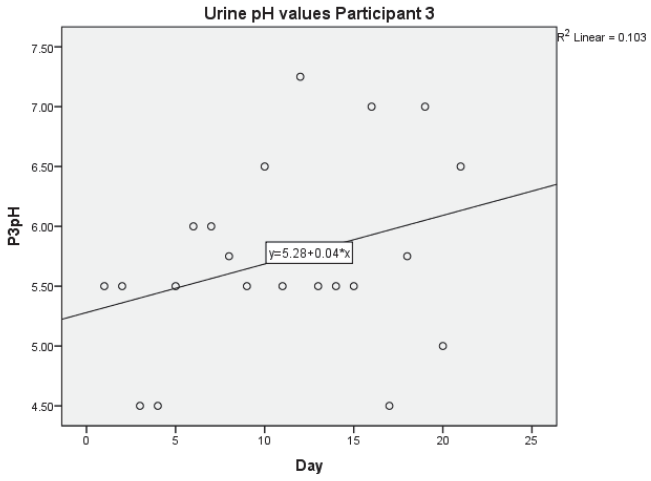
21 days pH of second void fasted urine



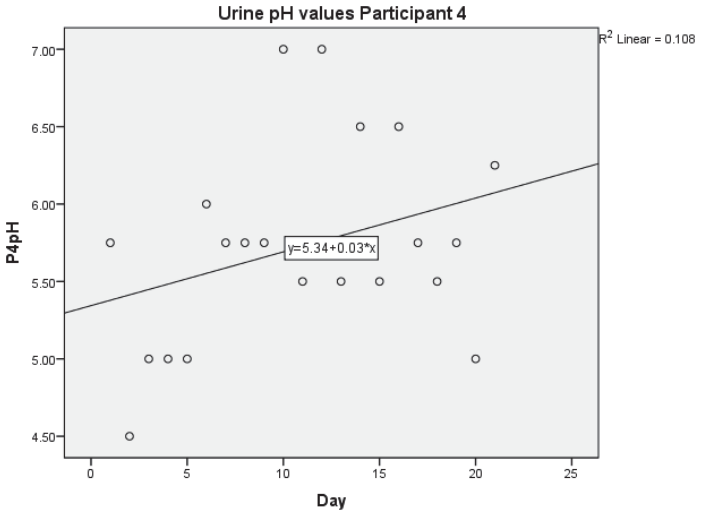
21 days pH of second void fasted urine



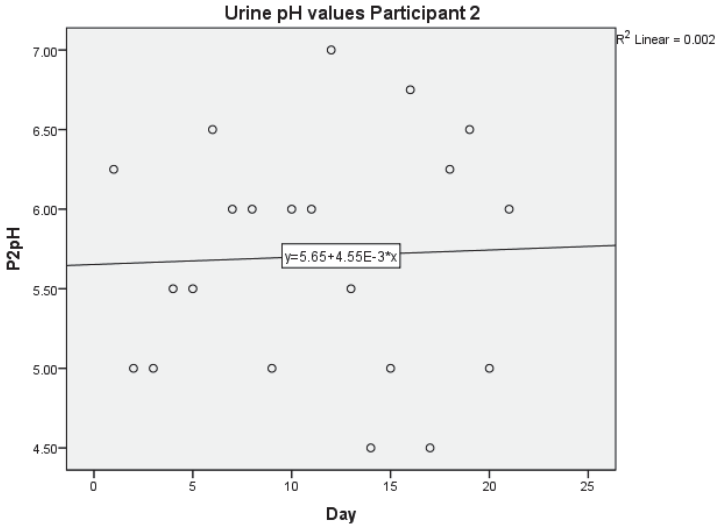
21 days pH of second void fasted urine



21 days pH of second void fasted urine



21 days pH of second void fasted urine

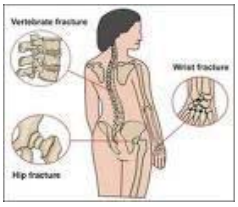


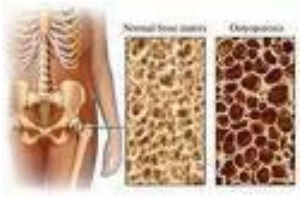
21 days pH of second void fasted urine

APPENDIX B: THE SCARBOROUGH FAIR STUDY

B1. Flier / Information Sheet

Bone Health, Vegetables, Herbs and Fruit Study





Women (5 years or more post-menopausal) are wanted for a unique study whereby the effects on bone health of increasing fruit and vegetable intake to 9 serves/day and including common culinary herbs are assessed.

The study will be over a 3 month period.

The study will involve you having your bone health assessed by a bone mineral density scan and blood samples taken to check bone and inflammatory markers at the Human Nutrition laboratory at

Massey University. Participants will be from Hawke's Bay, Palmerston North/Wanganui and Auckland regions.

You will be asked to increase consumption of vegetables and fruit to 9 serves each day and you may be asked to include some specific vegetables, fruit and common culinary herbs in your diet

If you are in good health, between 50 and 70 years of age and 5 years or more post-menopausal, then we would like to give you the opportunity to take part in this study investigating bone health and diet. For more information-please ring or email Caroline Gunn

Contact details: PhD student: Caroline Gunn c.a.gunn@massey.ac.nz

Massey University: 3569099 ext 2311 for PN and HB residents

Supervisor: Professor Marlena Kruger m.kruger@massey.ac.nz 06 3569099 ext 5905

B2. Consent Form

Institute of Food, Nutrition and Human Health

Bone Health, Vegetable, Herbs and Fruit

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

Full Name:_____

Signature: _____

Date:_____

This project has been reviewed and approved by the Massey University Human Ethics Committee: Southern A, Application _11_/_11. If you have any concerns about the conduct of this research, please contact Professor Julie Boddy, Chair, Massey University Human Ethics Committee: Southern A, telephone 06 350 5799 x 2541, email humanethicsoutha@massey.ac.nz.

B3. Participants diary, Groups A and B

Bone Health, Vegetables, Herbs and Fruit Intake Diary for Group IA

For participants in Dietary Intervention Group IA

Diet IA (3 months)

Key requirements for this study are:

Eat at least 6 serves or more of vegetables daily

Eat 3 serves of fruit daily

Record your urine pH twice a week in the morning (second void) using a dipstick

Vegetables

1) Try to eat at least 6 serves of vegetables every day including

1 serve Green leafy vegetables (particularly spinach, silver beet, green cabbage, watercress but any green vegetable is better than none)




At least 5 serves or more of any other vegetables you choose but try and <u>avoid</u>: mushrooms, green beans, broccoli, red cabbage, onions and garlic, tomato, leeks, cucumber

Fruit

2) Try to eat 3 serves a day of any type of fruit available and include a serve of apples 

or bananas  **if you can on some days each week but avoid oranges and prunes.**

Herbs

3) Try to include any one of the following herbs in your diet (in usual culinary quantities only) on some days each week.

Basil,  **Mint**  **and Oregano** 

but avoid garlic, parsley, sage, rosemary and thyme

Dairy

4) Try to have 2 serves of dairy products/day 

(or soy alternative) 

Stage the fruit and vegetable increase gradually over 2 weeks

This will help you adjust to the increased fibre intake

Week 1 aim to have at least 4 serves fruit and vegetables every day

Week 2 aim to have at least 6 serves fruit and vegetables every day

Week 3 aim to have at least 9 serves fruit and vegetables every day

Weekly Diary (any 2 days each week)

Urine pH: on 2 occasions each week record your urine pH (second void) with the dipsticks provided and record the result in the table

Also twice each week fill in the diary columns by recording your fruit and vegetable intake FROM THE PREVIOUS DAY (24 hour Fruit and Vegetable dietary record).

Note in the final column if you experience any change to your usual routine eg illness, have to take any medication or change your activity level substantially.

EXAMPLE ONLY

ID	Urine pH	Green Vege	Total Vegetable serves	Fruit serves	Herbs Type	Changes in health, meds.
Week 2 *Day 1*	6.0	1	4	2	-	-
Day 2	5.5	2	4	1	oregano	-
Week 3 *Day 1*	6.5	2	6	3	Basil	Headache-panadol
Day 2	6.25	1	6	2	-	-

Serving Sizes Guidelines

What's a serve?

In general:

1 serve =½ cup cooked fruit or vege or 1 cup uncooked

Vegetable serve guide

A single serving size (1/2 cup) is usually 50-80gms

Broccoli	1/6 of a head =50grams or 1/2 cup
Brussel sprouts	4 small = 1/2 cup= 60 grams
Carrots	1 small = 1/2 cup = 1 serve
Pepper	1 large = 150 grams= 2 serves
Tomato	1 small = 80gram= 1 serve
Onion	1 medium onion = 2-3 serves
	¼ cup of cooked onion =1 serve
Cooked green leafy vegetable	½ cup cooked= 80grams= 1 serve
Mixed vegetables	½ cup =60 grams= 1 serve
Salad (uncooked vegetables)	1 cup= 1 serve
Potato, kumara, yam or taro	1 medium = 130grams

Fruit serve guide

If stewed/canned approximately ½ cup = 1 serve

Fresh fruit serves

Medium size fruit	= 1 apple, 1 banana, 1 orange or pear
Smaller fruit-	= 2 kiwifruit, 2 mandarins, 3 plums, 4 apricots, 6 prunes
Very small fruit	= 1 cup strawberries, loquats, or berries
Fruit juice	= 160 mls= 1 small glass

Herbs

These are generally eaten in small quantities (1-2 tsps in a meal) however some fresh herbs may be eaten in slightly larger quantities.

.**Diary "Vegetables, Herbs and Fruit study".**

Record your <u>previous</u> day's vegetable and fruit intake and <u>today's</u> urine pH.

ID	Urine pH	Green Vege	Total Vegetable serves	Total Fruit serves	Herbs Type	If any change in health, meds
Week 1 *Day 1*						
Day 2						
Week 2 *Day 1*						
Day 2						
Week 3 *Day 1*						
Day 2						
Week 4 *Day 1*						
Day 2						
Week 5 *Day 1*						
Day 2						
Week 6 *Day 1*						
Day 2						
Week 7 *Day 1*						
Day 2						
Week 8 *Day 1*						

Day 2						
Week 9 *Day 1*						
Day 2						
Week 10 *Day 1*						
Day 2						
Week 11 *Day 1*						
Day 2						
Week 12 *Day 1*						
Day 2						
Week 13 *Day 1*						
Day 2						

Supplementary information

Bone Health and diet

Bone health in midlife is the result of several interrelated factors including genetics, lifestyle (exercise) and diet.

Only exercise and diet are modifiable and can provide an effective strategy to avoid osteoporosis. The dietary strategy presented in this study is based on supplying ample quantities of the nutrients that the body needs to maintain bone mineral mass through midlife years as the bone protective effect of oestrogen declines.

Vegetables and fruit contain minerals (e.g calcium, potassium and magnesium) and vitamins (K and C) as well as variety of phytochemicals including antioxidants which have been shown by research studies to support bone health.

Other protective effects

Increased quantities of vegetables and fruit in the diet have also been shown to have a range of other health benefits associated with the provision of dietary fibre.

These include:

1) Slowing glucose absorption and reducing the insulin response thereby preventing or controlling diabetes

2) Lowering the risk of heart disease and high cholesterol

3) Reducing the risk of colorectal cancer

4) Increasing the uptake of calcium, magnesium and iron in the large intestine

5) Weight control

6) Slowing cognitive decline

Additionally, vegetables and fruit are alkaline forming which may preserve muscle mass as the body ages as well as prevent bone loss.

As the proportion of vegetable and fruits in your diet increases there may be a corresponding change in your urine ph. This pH may increase, that means it is becoming less acidic though some people may not notice this happening for several weeks.

Tips for increasing vegetable and fruit consumption:

Aim to have your meal plate comprise portion wise 75% vegetables and 25% other foods.

Make a large pot of soup, which you can keep in the refrigerator for 3-4 days. Each day take out a portion and add fresh vegetables

Make a big salad and store it in airtight container and it should last 3-4 days so long as you don't add any dressings or cut vegetables like tomatoes or cucumbers which can make things go soggy.

Prepare salads for lunch the night before or that morning as it may be difficult to think of doing this when you are hungry at lunchtime.

Cook more potatoes, kumara, pumpkin, taro, yams etc than you need for your evening meal so you can eat them the next day for lunch, breakfast or snacks

You may have to eat a lot more bulky foods to achieve fullness as well as eating more often than you are used to doing, to achieve your usual kilojoules (kcalorie) intake.

Always have snacks such as fruit handy

Try juicing your vegetables as an easy way to have a serve or two

N.B: The NZ Food and Nutrition Guidelines encourage consumption of foods that are low in fat, sugar and salt

Bone Health, Vegetables, Herbs and Fruit Intake Diary for Group IB

Diary for Group IB

Diet IB (3 months)

Key requirements for this study are:

Eat at least 6 serves or more of vegetables daily

Eat 3 serves of fruit daily

Record your urine pH twice a week in the morning (second void) using a dipstick

Vegetables

1) Try to eat 6 serves of vegetables every day including

at least 1 serve Green leafy vegetables (particularly lettuce,  **Chinese cabbage such as Bok choy**  **or Soy Chum**  **or nappa**  **or rocket**

 **but any type of green leafy vegetable will be better than none)**

at least 2-3 serves or more of any of the following on most days : mushrooms,  **green beans,**  **broccoli,**  **red cabbage**  **,**

onions and garlic,  **tomato,**  **leeks**  **or cucumber** 

at least 2 serves of any other vegetables you like or have in the garden

Fruit

2) Try to eat 3 serves a day of any type of fruit available and include at least one serve of oranges  **or prunes.**  **on most days if you can (Avoid apples and bananas)**

Herbs

3) Try to include at least one of the following herbs in your diet (in usual culinary quantities only) on most days

Garlic, Parsley, Sage, Rosemary and Thyme (avoid basil, mint and oregano)

Dairy 

4) Try to have 2 serves of dairy products/day (or soy alternative 

Stage the fruit and vegetable increase gradually over 2 weeks

This will help you adjust to the increased fibre intake

Week 1 aim to have at least 4 serves fruit and vegetables every day

Week 2 aim to have at least 6 serves fruit and vegetables every day

Week 3 aim to have at least 9 serves fruit and vegetables every day

Weekly Diary (any 2 days each week)

Urine pH: on 2 occasions each week record your urine pH (second void) with the dipsticks provided and record the result in the table

Also twice each week fill in the diary columns by recording your fruit and vegetable intake FROM THE PREVIOUS DAY (24 hour Fruit and vegetable dietary record).

Note in the final column if you experience any change to your usual routine eg illness, have to take any medication or change your activity level substantially.

EXAMPLE ONLY

ID	Urine pH	Green Vege	Total Vegetable serves	Fruit serves	Herbs Type	Any changes health, meds
Week 2 *Day 1*	6.0	1	4	2	parsley	-
Day 2	5.5	2	4	1	Garlic	-
Week 3 *Day 1*	6.5	2	6	3	sage	Headache-panadol
Day 2	6.25	1	6	3	rosemary (stew)	-

Serving Sizes Guidelines

What's a serve?

In general:

1 serve =½ cup cooked fruit or vege or 1 cup uncooked

Vegetable serve guide

A single serving size (1/2 cup) is usually 50-80gms

Broccoli	1/6 of a head =50grams or 1/2 cup
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Mixed vegetables	½ cup =60 grams= 1 serve
Salad (uncooked vegetables)	1 cup= 1 serve
Potato, kumara, yam or taro	1 medium = 130grams

Fruit serve guide

If stewed/canned approximately ½ cup = 1 serve

Fresh fruit serves

Medium s(Bermudez et al., 2002) fruit	= 1 apple, 1 banana, 1 orange or pear
Smaller fruit-	= 2 kiwifruit, 2 mandarins, 3 plums, 4 apricots, 6 prunes
Very small fruit	= 1 cup strawberries, loquats, or berries
Fruit juice	= 160 mls= 1 small glass

Herbs

These are generally eaten in small quantities (1-2 tsps. in a meal) however some fresh herbs (e.g. parsley) may be eaten in slightly larger quantities e.g. up to ¼ cup depending on enjoyment.

Diary "Vegetables, Herbs and Fruit study".

Record your <u>previous</u> day's vegetable and fruit intake and <u>today's</u> urine pH.

ID	**Urine pH**	**Green Vege**	**Total Vegetable serves**	**Total Fruit serves**	**Herbs Type**	**Any change in health, meds**
Week1 *Day 1*						
Day 2						
Week2 *Day 1*						
Day 2						
Week3 *Day 1*						
Day 2						
Week4 *Day 1*						
Day 2						
Week5 *Day 1*						
Day 2						
Week6 *Day 1*						
Day 2						

Supplementary information

Bone Health and diet

Bone health in midlife is the result of several interrelated factors including genetics, lifestyle (exercise) and diet.

Only exercise and diet are modifiable and can provide an effective strategy to avoid osteoporosis.

The dietary strategy presented in this study is based on supplying ample quantities of the nutrients that the body needs to maintain bone mineral mass through midlife years as the bone protective effect of oestrogen declines.

Vegetables and fruit contain minerals (e.g calcium, potassium and magnesium) and vitamins (K and C) as well as variety of phytochemicals including antioxidants which have been shown by research studies to support bone health.

Other protective effects

Increased quantities of vegetables and fruit in the diet have also been shown to have a range of other health benefits associated with the provision of dietary fibre.

These include:

1) Slowing glucose absorption and reducing the insulin response thereby preventing or controlling diabetes

2) Lowering the risk of heart disease and high cholesterol

3) Reducing the risk of colorectal cancer

4) Increasing the uptake of calcium, magnesium and iron in the large intestine

5) Weight control

6) Slowing cognitive decline

Additionally, vegetables and fruit are alkaline forming which may preserve muscle mass as the body ages as well as prevent bone loss.

As the proportion of vegetable and fruits in your diet increases there may be a corresponding change in your urine ph. This pH may increase, that means it is becoming less acidic though some people may not notice this happening for several weeks.

Tips for increasing vegetable and fruit consumption

Aim to have your meal plate comprise portion wise 75% vegetables and 25% other foods.

Make a large pot of soup, which you can keep in the refrigerator for 3-4 days. Each day take out a portion and add fresh vegetables.

Make a big salad and store it in airtight container and it should last 3-4 days so long as you don't add any dressings or cut vegetables like tomatoes or cucumbers which can make things go soggy.

Prepare salads for lunch the night before or that morning as it may be difficult to think of doing this when you are hungry at lunchtime.

Cook more potatoes, kumara, pumpkin, taro, yams etc. than you need for your evening meal so you can eat them the next day for lunch, breakfast or snacks

You may have to eat a lot more bulky foods to achieve fullness as well as eating more often than you are used to doing, to achieve your usual kilojoules (kcalorie) intake.

Always have snacks such as fruit handy

Try juicing your vegetables as an easy way to have a serve or two

N.B: The NZ Food and Nutrition Guidelines encourage consumption of foods that are low in fat, sugar and salt

B4.Work station check list

Subject Number:

Treatment group **IA** ☐ **IB** ☐ **C** ☐

Bone Health,

Vegetables, Herbs and Fruit Study

STATION CHECK LIST

Visit 1

_____/_____

Subjects ID : _____

Introduction

3DDD ☐

Consent form signed ☐

Phlebotomy ☐

Urine pH ☐

Anthropometric data* ☐

Compliance diary ☐

Visual demo ☐

*over page

Weight (kg)_____

Height (cm)_____

Systolic (mmHg)_____

Diastolic (mmHg)_____

Urine pH_____

B5. Confidentiality Agreement

Institute of Food, Nutrition and Human Health

Bone Health, Vegetables, Herbs and Fruit Study

CONFIDENTIALITY AGREEMENT

I (Full Name - printed)

agree to keep confidential all information concerning the project (Title of Project).

I will not retain or copy any information involving the project.

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

APPENDIX C: ETHICS AND FUNDING