

**Studies on the Relationship between Dietary Calcium
and Bone Mineral Homeostasis in
Post-menopausal Women**

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Abstract

There is a considerable body of evidence to suggest that osteoporosis is the index disease of calcium deficiency, in both humans and animals. In addition, menopause or oophorectomy has been shown to affect bone metabolism such that a rise in the rate of bone resorption leads to post-menopausal osteoporosis. If this is the case then increasing dietary calcium intake or supplementation with calcium will result in reducing the incidence of osteoporosis in post-menopausal women. The current study was conducted to investigate the relationship between habitual calcium intake and the rate of bone loss in post-menopausal women.

Volunteers were recruited from the community to participate in a 2 year prospective study. A validated self-administered questionnaire was used to estimate total daily calcium intake in the subjects. Subjects were divided into tertiles according to calcium intakes. Six monthly bone density scans were performed of the lumbar spine, hip and forearm and the rate of decline in bone density for each site was calculated. Biochemical markers of bone metabolism at baseline were measured.

The mean calcium intake was 1114 mg/d (300 – 2589mg/d). Only 10% of the women met their recommended daily intake (RDI) of calcium from their diet alone. When calcium supplements were considered (42% were taking supplements) 31% of women met their RDI. About 84% had serum 25OHD levels >50 nmol/L. Serum PTH was related to calcium intake, C-terminal telopeptides (CTX) and age. Although the relationship between serum PTH and 25OHD was not significant, PTH and CTX tended to be higher in those with low serum 25OHD within each tertile of calcium intake. In addition, women with lower calcium intake were heavier, while an inverse relationship was observed between 25OHD and fasting glucose.

The prevalence of osteoporosis in the study group was much lower than that reported in previous surveys in Australian post-menopausal women, however, is consistent with the prevalence reported in health statistics for the wider community. The rate of

loss of bone density at the ultradistal forearm fell significantly in women on lower calcium diets compared to higher calcium diets although no significant relationship was demonstrated between the rate of bone loss at the lumbar spine or the hip and calcium intake. This significant relationship at the ultradistal forearm site may be because of the greater proportion of trabecular bone at this site.

Feedback on the bone density result was given to the subjects immediately after the baseline bone density measurement. The change in calcium intake after 12 months for women with osteoporosis or osteopenia was not significant suggesting that the feedback on bone density did not have an impact on dietary intakes or habits.

The acute biochemical effects of calcium supplementation with or without vitamin D were examined in a group of vitamin D deficient women (serum 25OHD <50 nmol/L). Suppression of PTH and CTX was more effective when calcium was given with vitamin D. When the acute biochemical effects of calcium carbonate and citrate supplements were compared, citrate was found to be more effective than carbonate in suppressing bone resorption markers.

Women with metabolic syndrome appear to be able to respond more favourably to a higher dietary calcium content than those without the metabolic syndrome.

The findings of this study confirm that calcium intake, even in a relatively healthy and health conscious cohort of postmenopausal women is linked to the rate of bone loss, at least in trabecular bone (ultradistal forearm). However the influence of calcium intake on the bone density at other sites may be influenced by a myriad of other metabolic and endocrine factors, including metabolic syndrome. Data from this study confirm a probable link between energy and bone mineral metabolism. This knowledge may be harnessed to exploit the potential of adequate dietary calcium to improve bone health.

Thesis Declaration

I declare that this work contains no material which has been accepted for the award of any other degree or diploma in my name, in any university or other tertiary institution and, to the best of my knowledge and belief, contains no material previously published or written by another person, except when due reference has been made in the text. In addition, I certify that no part of this work will, in the future, be used in a submission in my name, for any other degree or diploma in any university or other tertiary institution without the prior approval of the University of Adelaide and where applicable, any partner institution responsible for the joint-award of this degree.

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.....

SDC Thomas

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Publications and Presentations

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Chapter 1

Dietary calcium and post-menopausal osteoporosis

1. Osteoporosis

Osteoporosis is the most prevalent metabolic bone disease in the developed world. With an aging population, the impact of osteoporosis and resultant fractures is expected to rise throughout the world. Osteoporotic fractures increase with age with at least a two-fold higher incidence among women compared to men for all age-related fracture sites.

1.1 The costs and burden of osteoporosis

The social and economic burden of osteoporosis and consequent fractures is increasing worldwide. In the USA, it was estimated that in 2004 more than 10 million individuals suffered from osteoporosis (1). This number is expected to rise with each year. There were 692,000 Australians, 3.4% of the population, diagnosed with osteoporosis in 2007-08, and 89% of these were women (2). However, osteoporosis remains the most undetected and under treated national health priority in Australia accounting for only 0.6% of conditions managed by general practitioners (GPs) (3). Between the periods 1998-99 to 2007-08, the rate of advice from GPs for over the counter medication increased from 1% of osteoporosis related problems to 10% (3). This is likely to be due to the availability and promotion of calcium and vitamin D supplements.

According to Osteoporosis Australia's latest report, an estimated 4.7 million Australians over the age of 50 currently have osteoporosis or osteopenia, with associated 144,000 fragility fractures in 2013 (4) This has been equated to one fracture every 3.6 minutes in Australia. Although the rate of osteoporotic fractures were estimated to rise by another 30% by 2022 (equating to one fracture every 2.9 minutes) with an estimated cost of \$33 billion (4) more recent data suggest that the rate of hip fractures is decreasing in the developed world (5,6,7) and this is likely to

reduce the estimated future fracture rates and associated costs, however, no such data is available for fractures at other sites.

Re-fracture following an initial osteoporotic fracture, especially in the first 5 to 10 years has been recognised as an important contributing factor to excess mortality observed in both men and women with osteoporosis (8). Of men and women followed up for over 20 years by the Dubbo Osteoporosis Study, the rates of re-fracture within the initial 5 years of a fragility fracture were 24% for women and 20% for men. Mortality rates were higher for those who re-fractured (50% for women and 75% for men) compared to those fracture-free (26% for women and 37% for men) (8). Therefore prevention of osteoporosis and fractures will have a significant impact not only on the quality of life but on reducing premature death in both men and women.

It is clear that while primary prevention of osteoporosis and fracture is an important aspect of community and public health, the secondary prevention of fracture deserves equal if not more attention. All fracture patients present to tertiary hospitals, and elderly patients especially with hip fractures require surgery and hospitalisation, providing an opportunity to enrol them in a falls and future fracture prevention program. To this end, the International Osteoporosis Federation has implemented a program to prevent secondary fractures worldwide ('Capture the Fracture') (9). The program facilitates the implementation of Fracture Liaison Services within local health networks. The program has been well received globally, including the Australia. The Australian and New Zealand Hip Fracture Registry produced Hip Fracture Guidelines for the initial care as well as post-discharge implementation of strategies aimed at secondary prevention of fractures (10). The Royal Adelaide Hospital and Flinders Medical Centre uses a Falls Risk Assessment Tool on all patients admitted with hip and other fractures and a Post-Discharge Assessment is performed to implement a multidisciplinary care plan and community involvement in future fracture prevention, via a Fracture Liaison Service run by the Statewide Orthopaedic Clinical Network (11). Other examples of local care models include the NSW Model of Care (12) and WA Osteoporosis Model of Care (13).

In addition, the education of the public and raising awareness of adequate calcium intake is likely to contribute to preventing osteoporosis and fractures. The recently launched Calcium Calculator by the International Osteoporosis Foundation is an application that can be downloaded to a smart phone or used on the website to calculate daily calcium intake based on a typical weekly intake (14). The application is user-friendly, takes only a few minutes to complete and includes several ethnic and 'new' food items (kale, naan bread, rice milk, almond milk) that are not mentioned in traditional questionnaires. Globally, recent publications bear evidence for the success of secondary fracture prevention programs (15,16).

In addition to the disease burden and the cost on quality of life, the economic burden due to osteoporotic fractures is a significant proportion of the National Health Budget. In 2012 the total of osteoporosis and related fractures was \$2.75 billion and the total cost from 2013 to 2022 is estimated to be \$33 billion (4). Therefore the prevention of osteoporosis and its consequent fractures is a major public health priority.

1.2 Aetiology of osteoporosis

Osteoporosis is a heterogeneous disorder with a multifactorial aetiology. Age and oestrogen deficiency at the menopause have been shown to play major etiologic roles. Other factors include hereditary predisposition, nutrition, medication such as glucocorticoids, age-associated oxidative stress (17) and limited physical activity or mechanical loading as well as chronic inflammatory status (18,19). This review will be restricted to age, oestrogen deficiency and nutritional factors in the causation of osteoporosis. However, the multiple aetiologies of osteoporosis are acknowledged, but a discussion of all factors is beyond the scope of this review.

1.2.1. Nutritional factors

Nutrition plays a central role in the pathogenesis, prevention and treatment of osteoporosis (20). Calcium homeostasis is the major nutritional factor in maintaining the skeleton; however, numerous other nutrients that contribute to bone health have come to light. Food and Nutrition Board of the Institutes of Medicine have nominated

calcium, phosphorous, vitamin D, magnesium and fluoride as bone-related nutrients. The index disease associated with the deficiency in any of these nutrients is primarily a bone disease (21). Although in the past the recommended intakes of these nutrients were calculated on the basis of the minimum amount needed to prevent the index disease, the reference intakes took into account the amounts needed to prevent chronic disease and promote public health benefit (22).

The fact that numerous other nutrients are involved in maintaining bone health has been used to explain the inconsistencies between results of calcium and vitamin D intervention trials (23). Other nutrients that have been emphasised are vitamins C, K and B, potassium and carotenoids. The role of protein has been contentious, with proponents arguing the need for greater protein intakes in the elderly to maintain bone structure (23), and opponents arguing the fact that the greater acidic load introduced through animal protein may lead to a loss of calcium (24) and in turn, loss of bone mass. However a more recent longitudinal population bases study suggests that a high level of dietary protein intake, within the range commonly consumed does not result in bone loss in older people (25).

Bone loss has been attributed to regular intakes of caffeine and cola drinks (26) while consumption of alcoholic beverages may have an inconsistent effect on bone (27). Chronic alcoholism has been identified as an independent risk factor for osteoporosis, associated with a 2.8-fold increase in the risk of hip fractures (28,29) and lower bone density (30,31,32) but these conclusions were drawn from small studies on men whose regular intake of alcohol was more than 27 units/day for up to 24 years. Other studies suggest that moderate alcohol use may decrease fracture rates and increase bone density (33,34). In the Framingham cohort, women who consumed 7 oz (207 ml) per week or more had higher BMD at all sites compared to those who drank less than 1 oz (30 ml) per week, when averaged over 20 years (34). However, re-analysis of the data failed to show this relationship with current rates of alcohol consumption (35). A more recent meta-analysis suggests that those who consume 0.5 to 1.0 standard drinks per day have a lower risk of hip fracture; however a precise range of alcohol intake beneficial to bone density could not be determined (36).

1.2.1.1 Calcium and bone health

Bone acts as a reserve store of calcium which can be given up when needed. Insufficient dietary calcium intakes can lead to bone resorption to provide calcium resulting in a fall in bone mass and density. Any fall in extracellular calcium concentration leads to the secretion of parathyroid hormone, which increases bone resorption to release calcium. External calcium balance is determined by the relation between calcium intake and absorption and the losses of calcium through the skin, kidney and bowel.

Therefore the calcium economy is a dynamic state influenced by fluxes in dietary calcium intake, intestinal calcium absorption, renal calcium reabsorption and bone resorption. Other nutrients interact with calcium and affect the calcium economy in a positive or a negative manner.

1.2.1.2 Vitamin D

Vitamin D is considered an important nutritional factor in the prevention of osteoporosis by consumer groups such as Osteoporosis Australia (AO) (37) and professional groups such as International Osteoporosis Federation (IOF) (38). One in four (23%, 4 million) Australians are reported to be vitamin D deficient (serum 25OHD <50 nmol/L) in the National Health Measures Survey 2011-2012, report released in April 2014 (39). Seasonal variation in the level of sunlight exposure results in a variation of serum 25OHD. In South Australia, the rate of vitamin D deficiency is 15% in summer and 42% in winter (39).

Vitamin D has a clear and established effect on calcium and phosphate homeostasis (40). It appears that vitamin D can be implicated not only in bone health but muscle function (41). Evidence suggests that vitamin D is an independent predictor of falls in the elderly (41,42,43) presumably due to impaired balance and muscle weakness as a direct or indirect result of low vitamin D status.

In older adults, supplementation of vitamin D has been shown to attenuate secondary hyperparathyroidism (44,45). The lower PTH is associated with reduced bone turnover (45,46) and increased BMD (47). When calcium was added to vitamin D supplementation the reduction in PTH was greater than with vitamin D alone (44). To suppress bone resorption, as demonstrated by a significant reduction in bone resorption markers, however, supplemental calcium is required in addition to vitamin D, in both vitamin D sufficient (47) and vitamin D deficient (48) postmenopausal women. Furthermore supplementation of calcium with vitamin D reduces the incidence of fractures in the elderly (49,50,51,52). In a study in 1586 community dwelling subjects 65 to 71 years of age, supplementation with 800 IU of cholecalciferol and 1000 mg of calcium carbonate over 3 years reduced the rate of any fracture by 17%, non-vertebral fracture by 13% and distal forearm fracture by 30% but did not reduce the rate of lower extremity fractures (52). None of the fracture reduction rates in this study reached statistical significance. However in a meta-analysis of 29 studies, calcium supplementation significantly reduced fracture rates but adding at least 800 IU of vitamin D to the supplement reduced the fracture rates even further (51).

The DIPART study also showed a significant reduction in hip fracture risk when vitamin D was added (53). In this study, pooled data from 68,500 patients demonstrated that when 10ug of vitamin D was added to the calcium supplement, the hazard ratios for any fracture and hip fracture were 0.92 (0.86 – 0.99) and 0.84 (0.70 – 1.01) respectively. Doubling the vitamin D dose had no significant effect on the fracture rates. Their analysis also showed that supplementation with vitamin D alone was not effective in fracture reduction further validating the need for both calcium and vitamin D to prevent fractures (53,54).

While there has been no demonstrable benefit from vitamin D supplementation alone, using calcium and vitamin D together has been shown to reduce the fracture risk over and above that provided by calcium alone. The variable or inconsistent results obtained by various studies may be partly due to the pooling of data of heterogeneous groups of patients. It appears that, individuals with documented osteoporosis, at risk of osteoporosis or vitamin D deficiency may benefit more from supplementation with

calcium with or without vitamin D than community dwelling individuals without these characteristics (55). The recommended serum 25OHD level in Australian adults is ≥ 50 nmol/L at the end of winter and 10 – 20 nmol/L higher at the end of summer (54) for optimal musculoskeletal health.

1.2.2. Age

After the age of 60 years in men and after the menopause in women, there is progressive loss of bone, at the rate of about 0.5% pa in men and 1% pa in women. This is due to a rise in bone resorption in women and a fall in bone formation in men possibly secondary to a fall in androgens. The different mechanisms involved in the bone loss in men and women are reflected by histomorphometric features of trabecular bone. In older men, the trabeculae are generally thinner, while in older women the trabeculae are less in number, suggesting a total removal of trabeculae (56). However, the proposed ‘unitary model of estrogen deficiency’ suggests that, in both men and women, the mechanism underlying osteoporosis is estrogen deficiency (57).

The age related component of bone loss is associated with a decline in the number of osteoblasts present in the bone multicellular unit (BMU) (58). The number of osteoblasts in the BMU is a function of the generation of new osteoblasts from local progenitor cells and the life span of mature osteoblasts. Osteoblasts are generated from multipotential mesenchymal stem cells. With age, the self-renewal capacity of mesenchymal stem cells decline, leading to reduced numbers of osteoblasts, and more mesenchymal cells being diverted to the adipocyte lineage (59).

There is both a menopausal and age related decline in intestinal calcium absorption. It has been reported that Ca absorption decreases at the menopause with further decline with advancing age (60). This is not related to vitamin D status; there is an apparent decrease in gastrointestinal responsiveness to 1,25(OH)₂D in these subjects (61). In aged rats (24 months), immunofluorescence and Western blot studies have demonstrated a diminished number of vitamin D receptors (VDR) compared to young rats (3 months) (62). In addition, competition binding assays using ³H-1 α 25(OH)₂D₃ and 1 α 25(OH)₂D₃ demonstrated an age related decline in ligand binding

to VDR in rats (62). Although this model may explain at least partly, the age related decline in calcium absorption, the decline in VDR numbers or ligand binding has not been demonstrated in human enterocytes.

1.2.3. Menopause

Bone loss in post menopausal women was thought to occur in 2 phases (63,64). Both these phases were shown to occur in association with a falling estrogen level (65). Revision of the accepted involutional theory of osteoporosis suggests that estrogen deficiency is the underlying cause of bone loss in all phases in menopausal women and the continuous phase of bone loss in aging men (57). During the first 5 to 10 years after the menopause there is a phase of rapid bone loss (about 3% per year in the lumbar vertebrae) after which the loss of bone slows to about 0.5% per year (66). The rapid phase of bone loss may account for up to 50% of loss of spinal bone mass across life in women (67). This accelerated phase of bone loss during which a disproportionate loss of trabecular bone is demonstrated, has been attributed to the loss of direct effects of oestrogen on bone cell function (57). During the slow phase that follows, proportionate losses of trabecular and cortical bone occurs (57).

There are 3 proposed mechanisms to explain bone loss in menopause. One proposes that oestrogen and androgens influence the lifespan of osteoblasts and osteocytes by altering the redox balance, and reducing oxidative stress (68). Studies in animal models have demonstrated a steady increase in oxidative stress in the skeleton (69). Reactive oxygen species (ROS) have been shown to attenuate osteoblastogenesis and shorten the lifespan of osteocytes, while generating functional osteoclasts, with resultant net bone resorption (68). Oestrogen and androgen deficiency with menopause and advancing age has been proposed to lead to increased ROS generation. Evidence proving this theory has been published in the oestrogen or androgen deficient murine model, whose bone resorption was reversed by the administration of antioxidants (68).

The second proposed theory is an inflammatory milieu leading to an increased rate of bone resorption after the menopause (70). Oestrogen withdrawal results in an increase in inflammatory cytokines (IL-7 and TNF). These cytokines inhibit the

activity of mature osteoblasts and increase bone resorption. The features of the resultant bone are those described in the rapid phase of bone loss in early menopause (trabecular thinning, perforation, loss of connectivity between trabeculae) (71). The slower phase of bone loss that follows is attributed, at least in part, to apoptosis of osteoblasts (70).

Within the inflammatory milieu, NF- κ B ligand (RANKL), a membrane protein and a member of the tumor necrosis factor superfamily has been recognised as a key osteoclastogenic cytokine and the final downstream activator that drives osteoclastogenesis leading to bone resorption. Osteoprotegerin (OPG) is a decoy receptor for RANKL and modulates its activity. Both RANKL and OPG are synthesised by osteoblasts. More recent data suggests that there are other sources of RANKL. Cells of B cell lineage isolated from postmenopausal women were shown to express RANKL (72). B-cell lineage cells were found to be more abundant in ovariectomised mice compared to sham-operated littermates (73) which suggests that RANKL expression may be upregulated in the absence of estrogen. Cytokines IL-7 and TNF are proposed to induce bone loss via stimulating B and T cells to express RANKL, directly inducing osteoclasts and inhibition of osteoblasts generation (74,75). All these mechanisms have been shown to be enhanced in ovariectomised mice and in gene knock-out mouse models. This may explain the inflammatory milieu created by estrogen deficiency in post-,menopausal women leading to net bone resorption.

The third theory suggests that the calcium economy is disrupted in menopause, without the involvement of PTH. In postmenopausal women, oestrogen administration has been shown to lower urinary calcium excretion, lower urinary excretion of products of bone collagen metabolism (hydroxyproline), and lower serum alkaline phosphatase activity (76). These may be direct effects of oestrogen via bone specific oestrogen receptors on bone (discussed below) (77). Furthermore, oestrogen has long since thought to modulate the sensitivity of bone to PTH (78,79) or the calcium set point in the parathyroid gland (80,81).

The discovery of oestrogen receptors in the kidney (82) gave rise to the hypothesis that oestrogen may play a direct role in calcium reabsorption in the renal tubules. Thus, with oestrogen deficiency, such as following menopause, more calcium is lost in the urine due to inefficient reabsorption of calcium in the tubules as demonstrated by previous studies (83,84).

Evidence suggests that calcium absorption declines at the menopause and is reversible with replacement of oestrogen suggesting a direct role of oestrogen in intestinal calcium absorption (85). Calcium absorption is regarded as the main determinant of calcium balance, accounting for nearly 60% of the variance in calcium balance (86). The oestrogen deficiency caused by menopause then in effect, leads to a negative calcium balance and affects the calcium economy increasing the risk of osteoporosis. Furthermore, the rate of calcium absorption mainly via active transport, when adjusted for age, was shown to be significantly reduced in postmenopausal women with prevalent fractures (87).

1.2.3.1 The Role of Oestrogen in bone

Oestrogen has been shown to have 3 fundamental roles in bone metabolism. Oestrogen inhibits bone remodelling and the initiation of new BMUs, it inhibits differentiation and promotes apoptosis of osteoclasts, and promotes the differentiation of osteoblasts (57). Oestrogen receptors (ER) are members of the superfamily of ligand-regulated nuclear transcription factors. There are 2 isoforms of ER, α and β , both expressed in osteoclasts, osteoblasts and their precursors (88). At a cellular level, oestrogen acts via ER α , to activate endothelial nitric oxide synthase (eNOS) to produce nitric oxide (NO) (89). NO has been shown to regulate osteoclastic activity resulting in osteoclast apoptosis (90). Furthermore, oestrogen directly modifies osteoblast synthesis of osteoclast regulatory cytokines including osteoprotegerin (OPG) and receptor activator of NF- κ B ligand (RANKL) as well as up regulation of both α and β oestrogen receptors (91). The direct effect of oestrogen on osteoclast formation may be mediated via non genomic mechanisms (89). Oestrogen may exert an anti-resorptive effect on bone by stimulating oestrogen receptor, OPG and RANKL expression in osteoblasts. In addition there is evidence that oestrogen may exert its effects on bone metabolism via Dlk1/FA1 (Delta-like

1/Fetal Antigen 1). Soluble Dlk1/FA1 (sDlk1/FA1) is an endocrine factor that regulates bone mass, and higher circulating levels were reported in ovariectomised mice demonstrating enhanced bone resorption and inhibited bone formation (92). In postmenopausal women, replacement of oestrogen led to a fall in serum sDlk1/FA1 and bone turnover markers (93).

Androgens such as testosterone may be converted to oestrogen before they affect skeletal tissue. Therefore when there is a lack of androgens as in gonadal failure in males there is also a deficiency of oestrogen. This results in an up regulation of follicle stimulating hormone (FSH). FSH has been shown to have a direct effect on bone metabolism mediated via non gonadal FSH receptors and TNF α production (94,95).

1.2.4 Negative calcium balance in postmenopausal women

There is evidence for a negative calcium balance during and after menopause resulting from a fall in intestinal calcium absorption and an increase in the urinary calcium loss (96). Urinary losses of calcium were shown to be higher in postmenopausal women compared to premenopausal women (97,98). Together with the lowered calcium absorption in the gut (99) this leads to a negative calcium balance. Therefore it is reasonable to postulate that bone resorption occurs in these women to maintain the physiological calcium status (60,99). In addition, a high calcium intake has been shown to slow the rate of bone loss (100,101,102,103,104).

A review of 32 clinical trials of calcium supplementation in over 3000 postmenopausal women showed a significant loss of bone (>1% per annum) in the controls with no significant loss of bone in the calcium treated subjects (105). Twenty eight of these studies reported diet histories, which showed that, when the total calcium intake was less than 1300 mg a day the mean loss was 0.9% per annum ($P<0.001$) and when the intake was greater than 1300 mg a day the loss was 0.2% per annum (ns).

1.3 Calcium requirements

Calcium requirements are the intakes above which no further calcium can be retained (106). Calcium requirements are determined by balance studies, factorial estimates of requirements or assessment of changes in bone mineral density and bone mineral content. Balance studies estimate calcium requirement as the intake at which the excreted calcium equals the net absorbed calcium, adding an allowance for insensible skin losses (107). The rise in the calcium requirement after the menopause is likely to be at least an extra 200 mg a day (60).

The recommended daily allowance (RDA) of a nutrient is a value which is the estimated intake to meet the needs of 95% of the population. Intakes below the RDA will be adequate for some individuals but an intake less than two thirds of the RDA is considered to be inadequate. The National Institute of Health (NIH) Consensus Conference estimated the daily requirement of calcium to be 800 mg to 1000 mg in childhood, 1200 to 1500 mg from ages 12 to 24 years and 1000 mg from age 25 years to menopause and 1500 mg thereafter. (108). Taking into consideration the National Academy of Science recommendations to compensate for calcium losses in sweat, composite RDAs were estimated to be 600 mg for ages 1 to 3 years; 1000 mg for ages 4 to 8 years; 1600 mg for ages 9 to 18 years; 1200 mg for ages 19 to 50 years; 1400 mg for ages over 50 years and 1200mg during pregnancy and lactation (108). This revision of American RDAs and the FAO/WHO recommendations (109) resulted in a revision of the Australian/New Zealand recommendations. The recommended daily intakes (RDI) for the Australian and New Zealand population were estimated to be 500 mg for ages 1 to 3 years; 700 mg for ages 4 to 7 years; 1000 mg for ages 9 to 11 years; 1300 mg for ages 12 to 18 years; 1000 mg for ages 19 to 50 years; 1300 mg for women over 50 years; 1000 mg for men 50 to 70 years and 1300 mg for men over 70 years (110).

The position statement published by the Australian and New Zealand Bone and Mineral Society, Osteoporosis Australia and the Endocrine Society of Australia states that, in people with baseline calcium intakes between 500 and 900 mg a day, increasing the intake by a further 500 to 1000 mg a day has beneficial effects on bone

mineral density. However calcium intakes significantly above this recommended level is unlikely to lead to additional benefit for bone health (111). The Institute of Medicine recommended intake for postmenopausal women is 1200 mg a day with the upper level of intake set at 2000 mg a day (106). The US Preventive Services Task Force recommendation states that while there is insufficient evidence to assess the balance of benefits and harms of daily calcium supplementation greater than 1000 mg for the primary prevention of fractures in noninstitutionalised postmenopausal women, a daily supplementation of 1000 mg or less of calcium is not beneficial (112). This implies that to demonstrate the benefit of calcium on bone, at least 1000mg/day is required but current evidence is insufficient to delineate the possible adverse effects of calcium at this dose.

1.3.1 Factors affecting optimal calcium intakes

1.3.1.1 Content of calcium in dietary sources and type of diet

Dietary calcium is predominantly obtained from dairy products, with smaller amounts in bony fish, legumes, nuts, fortified soy products and cereals. Thus, the type and variety of diet will determine the content of calcium in the diet. Elimination of dairy from the diet as in lactose intolerance and veganism, severely curtails the amount of available dietary calcium but ovo-lacto-vegetarians appear to have similar calcium intakes to omnivores (113) suggesting that both ovo-lacto-vegetarians and omnivores obtain their calcium from dairy.:-

1.3.1.2 Bioavailability of calcium

Typically a vegetarian diet contains high amounts of phytates and oxalates which may bind to calcium in the intestine and reduce the efficacy of calcium absorption. This may increase the individual requirement for calcium. Foods rich in oxalic acid are spinach, rhubarb and beans. Seeds, nuts, grains and some beans including soy beans contain high levels of phytates. Although it is estimated that the bioavailability of calcium in soy milk is comparable to that of cows' milk, this is not always the case. Compared to cows' milk, the bioavailability of calcium in beans is 50% and from spinach is 10% (110).

Bioavailability of calcium from supplements depends on the dosage and the stomach contents at the time of ingestion. It has been shown that 250 mg calcium carbonate given with breakfast had a bioavailability comparable to that of calcium in milk (99,114). The proportion of calcium absorbed is an inverse function of load (115). At high intakes, the active transport mechanism becomes saturated and passive diffusion only accounts for about 5 – 10 % of additional absorbed calcium (115,116).

1.3.1.3 Sodium intake

Calcium requirement increases in proportion to sodium in the diet because the excretion of calcium in the urine is influenced by the sodium output (97,117). Every 100 mmol of sodium was shown to cause a loss of about 1 mmol of calcium in the urine. Salt loading in animals has been shown to result in bone loss (118). Restricting dietary sodium lowers bone turnover markers, suggesting a bone protective effect (119).

1.3.1.4 Dietary protein

Each gram of dietary protein has been shown to increase the urinary calcium excretion by 1 mg (120) but the benefit of additional calcium in dairy products outweighs the possible deleterious effects of extra protein (121). However a diet very low in protein has been shown to lower intestinal absorption of calcium (35,122,123) and the role of protein in calcium balance is controversial.

1.3.2 Average calcium intakes

Average Australian and New Zealand adult intake of calcium is estimated to be about 850 mg a day, and 40% of this calcium is obtained from non-dairy sources (110). The reported dietary calcium intakes for various cohorts in the United Kingdom, Italy and the United States show that it varies from as little as 198 mg a day to over 1000 mg a day in adults (124,125,126). In these studies, the majority of subjects had calcium intakes that fell below the RDA. In Australia the average habitual dietary calcium intake in over a 1000 randomly selected women was 631 mg with a range between 170 and 2071 mg (127). Less than 7% of this cohort used calcium supplementation. Dairy food provided up to 80% of the calcium. Seventy six percent

of the women aged 20 to 54 years and 87% of the women older than 54 years had calcium intakes below the recommended daily intake. These data suggests that in the general population, an average adult may obtain 60% of his or her calcium from dairy sources but some women may obtain up to 80% of their calcium from dairy.

1.3.3 Toxic effects of high calcium in the diet

There exists an inverse relationship between dietary calcium and fractional intestinal absorption of calcium. Therefore additional intakes of calcium to a typical diet would only marginally increase the urinary excreted calcium. It follows that the risk of developing nephrolithiasis from increased urinary calcium is very small. However milk alkali syndrome (MAS) is a well defined syndrome where hypercalcaemia and alkalosis can follow excessive intake of milk. Using MAS as the end point of toxicity the lowest observed adverse effect level is about 5 g of calcium in adult humans (128). The hypercalcuria of male stone formers has been shown to be due to hyperabsorption of calcium and less related to intake (129) and there is little danger of hypercalcuria from calcium supplements because intestinal fractional calcium absorption is an inverse function of intake (114). However, excess dietary calcium has been implicated in interfering with the absorption of zinc and iron in susceptible individuals. Therefore the upper level of intake for calcium has been set at 2500 mg a day for both men and women of all ages (109).

There has been considerable interest in the effect of calcium supplementation on the cardiovascular system. There is evidence that Ca supplementation exerts beneficial effects on systolic blood pressure (130) and serum lipids (131). The latter may be explained by the hypothesis that lowers circulating calcitrophic hormones secondary to high calcium intakes favouring lipolysis (132). However a meta-analysis of Ca supplementation in postmenopausal women with cardiovascular events as secondary endpoints demonstrated a 27% increased risk of myocardial infarction (MI), a non-significant increase in stroke and death in those receiving supplements (133). The Women's Health Initiative investigators however reported no effect of calcium supplement on cardiovascular risk (134). Meta-analysis of studies using either Ca or vitamin D reported a 26% increased risk of MI and a 19% increase in risk of stroke (135). More recent studies have also demonstrated a higher risk of coronary heart

disease/MI (136,137). The mechanism proposed to explain this phenomenon is that a high Ca milieu may result in extracellular deposition of Ca in vascular smooth muscle leading to calcified plaque (138,139). However the Canadian Multicentre Osteoporosis Study (CaMos) demonstrated that in both men and women, calcium supplementation up to 1000mg/d and higher intakes of calcium from dietary sources reduced the risk of all-cause mortality (140). The Melbourne Collaborative Cohort Study demonstrated that daily dietary Ca intakes up to 1348mg reduced the hazard ratio of fracture, non-fatal MI, stroke and all-cause mortality (141).

It appears that while Ca supplements cause serum Ca levels to spike to supra-physiological levels (when taken in 600 – 1200mg doses at once), dietary Ca may lead to a slower rise in serum Ca preventing the high Ca milieu that promotes vascular calcification. It is also possible that, other nutrients (including fibre) has an effect on the absorption of dietary Ca. Phytates, phosphates, fibre and fats may bind to Ca either preventing or slowing absorption. Therefore the actual absorbed Ca may be much less than estimated dietary intakes, while supplemented elemental Ca may be more readily absorbed.

1.3.4 Effects of calcium on bone mineral density

Ensuring adequate calcium intake, with supplementation when the diet is deficient, has been suggested as an inexpensive strategy to prevent osteoporosis and consequent fractures (142). The fact that calcium and oestrogen deficiency are important factors in the causation of post menopausal osteoporosis has long been accepted. An oophorectomised animal model using histomorphometric data and bone turnover markers showed that bone loss was maximum in those that were oestrogen and calcium deficient (143). In this study, dietary calcium deficiency induced bone loss in both cancellous and cortical bones, while the bone loss in oestrogen deficiency was confined to cancellous bone. This suggests that there may be additive effects of oestrogen and calcium deficiency, and the resultant bone loss from calcium deficiency is more widespread than that of oestrogen deficiency.

There have been a number of observational and interventional studies of calcium intake and its effects on bone density in post-menopausal women. One of these studies was a 2 year longitudinal study in postmenopausal women which showed that increasing dietary calcium protected against bone loss at the hip and ankle sites (117). The change in calcium intake was positively correlated with the change in bone density at the hip and ankle, suggesting that a lower calcium intake was associated with a greater rate of bone loss. Stable hip densities were reported at an intake of 1700 mg a day. However the amount of daily calcium intake to achieve calcium balance was also shown to be dependent on the sodium intake (117) such that, lower calcium intakes would achieve a balance if the sodium intakes were lower. This then leads to the concept that, the bone protective dose of calcium should be tailored for each individual. Another study conducted in Italy showed that post menopausal women with lower calcium intakes were more likely to have osteoporosis demonstrable by Dual Energy X ray Absorptiometry (DXA) scanning, with a T score below -2.5 when the measurements were corrected for body weight (144). This and other such studies suggest that on a population basis, the prevalence of osteoporosis will be higher in populations that have lower dietary calcium intakes, unless calcium is supplemented.

In a more comprehensive study, albeit in a smaller number of post- menopausal women Dawson-Hughes et al showed that those who had a calcium intake below one-half of the RDA appeared to lose bone more rapidly in their lumbar spine than those whose intake exceeded the RDA, when corrected for baseline bone density, years since menopause and body weight (126). They projected the annual rate of bone loss for post menopausal women to be 1.1%. Those on lower calcium intakes suffered a loss of bone up to 4% per annum while those whose calcium intake exceeded the RDA had a gain of bone of up to 6%. The results of this small study are encouraging because it demonstrates that, in post-menopausal women, calcium supplementation alone may be sufficient to increase their bone density. However a 2 year prospective non-interventional study failed to demonstrate a rise in BMD with increasing total calcium intake (dietary and supplemental) during the early postmenopausal period in women up to 60 years of age (145). The early phase of rapid bone loss after the menopause may not be amenable to calcium

supplementation alone and may require oestrogen replacement. In this study, on a calcium supplement of 500 mg, those in early menopause showed a statistically significant loss of bone regardless of the form of calcium taken. The placebo arm of this group also showed a significant rate of bone loss. In the late menopausal group, those who ingested the calcium in the form of citrate malate showed no bone loss in the lumbar spine during the study period of 2 years. In contrast, those who used calcium in the form of carbonate or those who were on the placebo showed a significant decline in bone density (101). A more recent study showed that 500 mg of calcium citrate is as effective as 1000 mg of calcium carbonate in suppressing bone markers (146) and other studies have demonstrated that calcium citrate has greater bioavailability and better absorbed than calcium carbonate (147,148,149) which may explain the bone protective effect of calcium citrate.

The variable, often conflicting results of calcium intake with or without supplements on bone density may be due to the heterogeneity of the cohort, other uncontrolled dietary factors such as high sodium intake, the form of calcium supplemented and other uncontrolled but important factors impacting on bone density such as physical activity.

In general, randomized controlled trials assessing bone mineral density show beneficial effects of calcium in both men and women (103,150,151,152,153). On average these studies showed that adequate calcium intake can result in a sustained reduction in bone loss of 50 – 60% (103,150,151,152). Furthermore in women who had a high calcium intake through their diet, a transient increase in BMD was observed when calcium was supplemented, an effect attributed to the suppression of parathyroid hormone (153,154). The effect on cortical bone is more sustained and cumulative than that on cancellous bone, which may be explained by the slower turnover of cortical bone.

1.3.5 Calcium and fracture reduction

With the information provided by studies such as those mentioned above, that showed preservation or a rise in bone density with high calcium intake, the next step

was to ascertain if a high calcium intake prevented or at least reduced the risk of fractures. Therefore randomised controlled trials were designed with fracture as the end point. An example of a study in this category is a randomised double blind study of 930 subjects of both sexes over 4 years which showed that the overall risk of a minimal trauma fracture was significantly lower in those who received 1200 mg of elemental calcium when compared to those who received the placebo (155). (hazard ratio 0.28 (95% CI: 0.09, 0.85). However the hazard ratio reverted to 1.10 (95% CI: 0.71, 1.69) during the post treatment 10 year follow up when the subjects were informed about the type of treatment they received and had the choice to take or not to take calcium.

A meta-analysis of 29 randomised controlled trials showed that the use of calcium, either alone or in combination with vitamin D in people over 50 years of age was associated with a significant reduction in fractures of all types (risk ratio 0.88; 95% CI: 0.83, 0.95; $p= 0.0004$) (51). Randomised controlled trials show that the rate of fracture risk reduction is significant even when the absolute differences in BMD change between the placebo and calcium supplemented groups are modest (153,156,157). This is now thought to be due to the beneficial effects of suppressing bone turnover.

A landmark study was conducted over 3 years on more than 3000 institutionalised women between the ages of 69 and 106 years who were randomly allocated to receive 1200 mg calcium with added vitamin D or placebo over a period of 18 months. (49). Women with vitamin D deficiency were normalised before the commencement of calcium. The number of hip fractures was shown to be 43% lower ($P = 0.043$) and the total number of non vertebral fractures were 32% ($P = 0.015$) lower in those who received calcium and vitamin D. A further 18 months of treatment resulted in a fall in the incidence of non-vertebral fractures by 24% and hip fractures by 29% ($P < 0.001$) in those who received calcium. (49) Other studies have shown results consistent with this study (158, 159,160,161). Some studies have not been successful in showing a significant effect of calcium on the rate of fractures (162,163,164) which may be explained by the heterogeneity of patient populations and their various baseline vitamin D status. A per-protocol analysis of 1500 older

women suggested that 1200 mg of calcium daily reduced the risk of fracture by 34% where an Intention to Treat analysis failed to show a significant effect (165). Pooled results from prospective cohort studies show that calcium intake is not associated with hip fracture risk in men and women while randomised controlled trials show no reduction in hip fracture risk when calcium supplements are given, and a possible increase in the risk, with a neutral effect on vertebral fracture risk (163). A longitudinal prospective study of 61,433 women followed up for 19 years showed that the relationship between the incidence of osteoporosis and minimal trauma fractures with dietary calcium intake was nonlinear (166). Vitamin D deficiency in women in the lowest quintile of calcium intake exacerbated the rate of fracture. The highest quintile of calcium intake did not reduce the incidence of osteoporosis or fracture.

Overall, the current evidence points to a beneficial effect of calcium on fracture prevention in both men and women. A meta analysis of 29 randomised controlled trials supports the use of calcium with or without vitamin D in the prevention of osteoporosis and subsequent fractures in women over the age of 50 (51).

1.3.6 Calcium supplementation in the community

In almost all long term studies subject compliance with taking calcium was reported as moderate to low, defined as 80% of the tablets taken by 50 to 58% of subjects (103,150,151,152,156,165). According to this observation, the effectiveness of any intervention with daily calcium doses will be limited by non-compliance (51,165). However, according to current consensus, even with anti resorptive therapy, calcium supplementation is regarded as an integral component in the treatment regime of osteoporosis (111). Whether calcium should be used as a preventative agent is still controversial (167).

1.3.7 Estimation of dietary calcium intake

Nutrient intake can be estimated by a retrospective assessment using a food frequency questionnaire (FFQ) or by a prospective method, by tracking intakes

longitudinally using a food diary. Some questionnaires evaluate a single nutrient, while others assess the intake of energy and other nutrients (168).

Nutrient intake estimation is useful for research as well as in the clinical setting. FFQs have been the method of choice for dietary intake data for epidemiological studies (168). They have been adapted for use in the clinical and research setting. For calcium intake data, FFQs have been designed to be completed independently by subjects and considered to be relatively inexpensive (169).

However, FFQs need to be updated every few years because the pattern of dietary intakes changes over time (170). The other potential problem with FFQs is the inability to include all types of available fortified products on the market (171) or may not be appropriate for the gender or the age (172) or the geographic and socioeconomic characteristics of the cohort studied (173). Some validated total diet estimate questionnaires are lengthy and may not be accurate when used to assess a single nutrient (168).

1.3.8 The link between energy metabolism and bone metabolism

When studying the effects of dietary calcium intake on bone density and bone metabolism, other components of the diet and their metabolism on bone should be considered. The concept that bone remodelling and energy metabolism may be regulated by the same hormones under the influence of the hypothalamus was proposed in 2000 (174). This section will discuss the link between bone and calcium metabolism and energy metabolism, the proposed hormones involved in linking these processes and the role of nutrients.

The net calcium absorbed via the gastrointestinal tract on a Western diet is about 150 mg per day. This regular influx of calcium into the extracellular space is balanced by renal excretion and exchange with the bone calcium pool. Calcium homeostasis is maintained by 3 hormones, parathyroid hormone (PTH), calcitonin and 1,25 dihydroxyvitamin D (1,25(OH)₂ D). PTH, an 84 amino acid peptide is synthesised and secreted by the parathyroid gland and is in turn, regulated by extracellular

ionised calcium concentration. The biological actions of PTH include stimulation of osteoclastic bone resorption, stimulation of calcium reabsorption and inhibition of phosphate reabsorption in the renal tubules and stimulation of renal synthesis of 1,25 (OH)₂ D, all aimed at raising the serum calcium level.

Increases in ionised calcium in extracellular fluid results in the release of calcitonin, a 32 amino acid peptide synthesised and secreted by the parafollicular cells of the thyroid. The precise biological role of calcitonin in humans is yet to be delineated, and debated (175) but its role in fish and reptiles appear to be significant in calcium homeostasis (176). Although at pharmacological doses, calcitonin has been shown to decrease osteoclastic bone resorption (177) and decrease calcium reabsorption at the renal tubule with resultant calciuria, at physiological concentrations these effects have not been observed. In vitro observations also suggest that calcitonin may induce the formation of 1,25 (OH)₂ vitamin D and enhance calcium absorption in the gut (178). Furthermore, clinical observations support the concept that calcitonin has little chronic effect because neither calcitonin deficient patients (post total thyroidectomy) nor patients with medullary thyroid carcinoma and excess calcitonin are reported to suffer altered calcium homeostasis (176).

More recent evidence arising from in vitro cell and organ cultures and in vivo studies suggest that calcitonin may arrest bone resorption by inhibiting RANKL (179). The calcitonic effect of calcitonin is partly due to increased renal excretion of calcium secondary to a decline in renal reabsorption (180). Note-worthy is the inhibitory action on bone formation, demonstrated in mice and investigated extensively in various gene knockout mice (175). This phenomenon has been seen especially during growth when bone mineral accrual is highest (181). The proposed mechanism is the modulation of production of sclerostin, an osteocyte-derived negative regulator of bone formation (182). Another potential mechanism is the regulation of bone formation via the brain, proposed following the demonstration of calcitonin receptor expression and calcitonin binding in the arcuate nucleus of the hypothalamus (183). It is possible that the inhibitory effects of calcitonin on bone formation may ultimately be mediated via efferent neural pathways and neural regulators such as leptin and neuropeptide Y (184,185). Given that calcitonin acting via CNS is known

to regulate appetite and gastric secretion, this may be another link between bone metabolism and energy metabolism.

The concept of gut-brain-bone axis has opened up avenues exploring the effect of nutrients on enteroendocrine cells, and the effect of their secretions (incretins or gut derived hormones) on bone and other tissue. The major functions of incretins are to regulate gut secretions (acid, bicarbonate in the stomach), food intake (satiety), insulin secretion, lipoprotein lipase activity in fat cells, contraction of the gall bladder, pancreatic secretions and gut motility, in response to nutrient content in the gut lumen (186). The sophisticated sensory mechanisms of these cells are able to differentiate between different types of nutrients within the lumen.

The proposed link between incretins and bone is an evolving concept in nutrient-dependent regulation of bone turnover. The postprandial fall in bone resorption markers and the rise in bone formation markers (albeit to a lesser extent) has been attributed to the effects of incretins GLP-1 mainly, with GLP-2 playing a minor role (187,188).

An adipocyte derived hormone, leptin was shown to be a major regulator of osteoblast function, and inhibits bone formation in vivo cultured cells (189) and in mice (190). Leptin-deficient and obese *ob/ob* mice and leptin receptor-deficient *db/db* mice display an osteopetrotic skeletal phenotype at an early age with high trabecular bone volume (191). Deficiency of leptin or its receptor in these mice resulted in a decreased rate of bone formation (BFR) but also decreased the rate of bone resorption (BRR) with a consequent high bone mass. Subcutaneous injection of leptin in these mice corrected the low bone formation and low bone resorption phenotype (191) demonstrating that peripherally administered leptin (as opposed to hypothalamic centrally regulated leptin) has an effect on BFR and BRR. However in humans, low circulating levels of leptin is associated with low bone density (192).

As in any other endocrine pathway adipocytes and leptin production in turn, may be under a feedback regulatory control of bone metabolism. Osteocalcin, an osteoblast-specific protein, was nominated as the candidate hormone involved in the regulation

of energy metabolism. Indeed, glucose intolerance and greater amounts of visceral fat were noted in osteocalcin knockout mice (*Osteocalcin*^{-/-}) (193). Deletion of other genes in osteoblasts (ie: *Esp* or *Ptprv* coding for a receptor- like protein tyrosine phosphatase OST-PTP) in mice was shown to increase pancreatic β cell proliferation, insulin secretion and sensitivity that protects against obesity and diabetes (193). This suggests that osteocalcin and other osteoblast-derived factors may influence insulin secretion and energy metabolism.

Another important factor in the link between energy metabolism and skeletal physiology is cortisol. Evidence suggests that in human and rat bone- glucocorticoids (GC) are essential for the differentiation of osteoblasts and formation of mineralized extracellular matrix (194). GC, particularly cortisol plays a major role in energy metabolism. While acting as an integral component of the stress response, making energy available by increasing glycolysis and lipolysis, chronic excess cortisol leads to impaired peripheral glucose uptake, insulin resistance, increased lipogenesis both in the liver (non-alcoholic steatohepatitis) and in the trunk (central abdominal obesity or Cushingoid phenotype) (195). Indeed, patients with Cushing's syndrome have a 2 to 5 fold increase in subcutaneous and visceral fat compared to healthy controls (196). Furthermore, several features of Cushing's syndrome overlap with the metabolic syndrome. Central or visceral obesity, insulin resistance and hyperglycaemia are both common to the 2 syndromes. Given their role in fat and carbohydrate metabolism, and the common features of Cushing's and metabolic syndrome, it is reasonable to postulate that glucocorticoids play a role in the development of the metabolic syndrome. Current evidence indeed, points to subtle abnormalities in the hypothalamic-pituitary- adrenal axis (HPA axis) in metabolic syndrome, rather than a simple elevation of circulating cortisol (197).

The role of cortisol in the differentiation of osteoblasts has been investigated extensively. It appears that precursor cells are directed towards differentiation by GC, during which genes stimulating and inhibiting bone formation are activated. The essential role of cortisol in this process has been demonstrated by the fact that, when cells are deprived of cortisol, they convert inactive cortisone to cortisol by expressing 11 β -hydroxy steroid dehydrogenase (198). However when administered at

pharmacological doses GC lead to well characterised adverse effects on bone, namely suppression of bone formation and increased rate of bone resorption. It is accepted that osteoblasts, osteoclasts and osteocytes are affected by GC.

It is clear that cortisol or GC have a central and pivotal role in energy metabolism. It is also clear that GC is essential for skeletal health and metabolism. Therefore it is possible that energy metabolism and bone metabolism are linked via GC, mediated by intermediary metabolites or mediators. Osteocalcin has been proposed as a likely candidate produced by osteoblasts, that regulates energy metabolism and may have a role in modulating effects of GC. Osteocalcin has been shown to regulate glucose metabolism by influencing insulin secretion and sensitivity, and pancreatic β cell proliferation (199). Treatment with GC has shown to significantly suppress circulating levels of osteocalcin and increase circulating insulin (200). Experiments using transgenic mice have shown that the disruption of GC signalling pathway of osteoblasts attenuates several features of Cushing's and metabolic syndromes such as obesity, hyperlipidemia, insulin resistance and hyperglycaemia (200) leading to the conclusion that GC-induced suppression of osteoblast activity plays a major role in mediating the adverse effects of GC on energy metabolism. More interestingly, when GC treated mice displaying features of metabolic syndrome were treated with osteocalcin, fat/lean mass ratio, hyperlipidemia, hyperglycaemia, insulin resistance and steatohepatitis were reversed. Furthermore when osteocalcin vectors were transfected in to the mice insulin signalling in target tissues were protected from disruption by GC (200).

The actions of GC on osteoblasts, evidence that GC-induced derangement of carbohydrate and fat metabolism may be reversed by osteocalcin and the potential protection afforded by osteocalcin to target tissues of insulin from disruption by GC suggests that osteoblasts are intimately associated with energy metabolism. Therefore by extension, patients with metabolic syndrome and obesity, or a predisposition (ie genetic) to metabolic syndrome or obesity, may carry either acquired or inherited gene activation or deletion within osteoblasts. It is possible that even in individuals that have not inherited genes that predispose to metabolic syndrome or obesity, various acquired or environmental factors may modify cellular and genetic

components. These individuals therefore, may also display variant phenotypes in bone metabolism. For decades, obesity was accepted as a factor that protects against osteoporosis (201). Furthermore a meta-analysis showed that there was an inverse relationship between BMI and fracture risk (202,203). It was thought that in obesity the mechanical stimulation of bone due to greater body weight lead to higher bone density.

Inconsistent results were reported on the association between fat distribution and BMD, and at least in some studies this was because no adjustment was made for BMI (204,205). A cross sectional study of over 500 postmenopausal women however, reported a positive correlation between bone mass and BMI, total body fat mass and abdominal fat mass measured by DXA (206). The close link between bone and energy metabolism may at least in part, explain this finding. It appears that the deranged energy metabolism in these individuals leading to fat accumulation factors associated with this may stimulate osteoblasts to attain greater bone mass. It should be noted, however, that the relationship between bone mass, fat mass and lean mass are complex and numerous as yet unidentified factors may be at play.

1.4 Assessment of bone loss

Osteoporosis is defined by the WHO as a systemic skeletal disease characterised by compromised bone strength predisposing to increased risk of fracture, where bone strength reflects the integration of bone density and bone quality. Bone quality reflects bone architecture, bone turnover, accumulation of micro-fracture and bone mineralisation. A simpler definition would be that it reflects “too little bone in the bone” (207). Osteoporosis results from loss of bone due to the imbalance between the rates of formation and resorption, also described as uncoupling of formation and resorption, in the process of bone remodelling that occurs during the skeletal life cycle.

1.4.1 Bone mineral density and fracture risk

Bone mineral density (BMD) is measured by dual energy x-ray absorptiometry (DXA) and is currently the most effective measure of bone density and estimation of fracture risk. It is a two-dimensional areal density rather than a volumetric density. It combines the trabecular and cortical measurements and is often affected by degenerative artefacts in the spine. Therefore, the total hip measurement is used for fracture risk assessment. It provides the reference standard for the diagnosis of osteoporosis. The measurements are reasonably precise, with a reproducibility of about 1% so changes over 2.8% are significant for a given population and 4% for individuals (208). It is easy and quick to perform. Skeletal assessment using DXA is typically made in the lumbar vertebrae and total hip. BMD is expressed by its relationship to two norms: the expected BMD for young normal adults of the same sex (standard deviations from the young normal mean; T score); and the expected BMD for the patient's age and sex (Z score). The difference between the patient's score and the norm is expressed in terms of standard deviations (SD) above or below the mean. In general 1SD represents about 12% of the bone density value. With age, the T scores decline in parallel to the loss of bone density. According to the World Health Organization definition, osteoporosis is a BMD T score of less than or equal to -2.5 when measured using DXA (208)

Quantitative ultrasound (qUS) and quantitative computed tomography (qCT) are other techniques used for assessing bone quality. Although qUS can be used to predict fracture risk it cannot be used to diagnose osteoporosis or to monitor effects of therapy because of poor precision. Common sites measured are the calcaneus, patella and the tibia. Quantitative US correlates only weakly with BMD measurements obtained from DXA and no population-based standards exist (209) so its effectiveness in predicting fracture risk is uncertain. Quantitative CT is mainly used in research. It provides a volumetric density measurement, and remains the best technique for assessing trabecular and cortical volumetric densities. This allows the detection of early bone loss in the vertebral body because CT is more sensitive to small changes than DXA. It is not used in the clinical setting because the emitted radiation is several fold higher than DXA and it is more expensive.

A landmark study showed that elderly women with low hip BMD ($T < -3.5$) had a two fold higher risk of hip fracture than the average risk for age matched women and the hip bone density measured by DXA was inversely related to low trauma fracture risk at any site (210). The population-based reference interval provides a more reliable basis for the diagnosis of osteoporosis if derived from the same geographical region or ethnic community. BMD fracture prediction is to some extent site specific; BMD at the hip is a better predictor of hip fractures than of fractures elsewhere (211). However the total hip density has the highest predictive value for fractures in general probably because it is least affected by degenerative changes and has been proposed as the standard site (212). A peripheral measurement of BMD (phalanx, forearm) together with a good clinical evaluation of the osteoporosis risk profile of the patient has been proposed for the diagnosis of osteoporosis when central DXA is not available (213).

Using DXA it is generally accepted that the fracture risk rises by a factor of 1.5 – 2 for every standard deviation reduction in BMD (211). However, the bone densities of those who develop low trauma or osteoporotic fractures overlap with those who do not. In other words, not all individuals with a low trauma fracture have a T score below -2.5, and the majority of people with a T score below -2.5 will not suffer osteoporotic fractures (211). Furthermore, the proportion of women with osteopenia is about 6 fold that of osteoporosis, therefore the absolute number of fractures will be greater in those with osteopenia (214,215). Although “risk” is widely used in this context it is generally odds ratio that is cited. The relation between odds and risk is given by the equation:

$$\text{Risk} = \text{odds} / (1 + \text{odds}) \quad (216,217)$$

A nomogram published in 2007 used femoral neck BMD T score with other clinical risk factors such as prior fracture, history of falls, postural sway and quadriceps strength to predict the 5 and 10 year hip fracture risk in men and women in the primary care setting (218). Evidently the incorporation of clinical risk factors enhanced the performance of BMD in the prediction of osteoporotic risk factors in

both men and women. A 10 year absolute risk estimating algorithm has been developed by University of Sheffield, UK (FRAX) which takes well known risk factors (age, race, sex, T score, prior fractures, current fracture, current smoking, glucocorticoid use, rheumatoid arthritis) as well as risk factors less utilised in the clinical setting (alcohol, body mass index or BMI) into account. This algorithm has been validated for several countries. It does not take biochemical markers into account due to lack of data so bone turnover rate is not considered in the risk calculation. This risk calculation model has gained general acceptance worldwide and the risk calculator can be freely accessed on the web for use on individual patients by clinicians (219).

1.4.2 Bone turnover markers

If bone quality is to be assessed, bone turnover markers (BTM) provide a more dynamic measurement than bone density. While bone density measurements are slower to change, BTM can be used as surrogate measures of acute changes in cellular activity. However, longitudinal data of bone markers can be used to predict structural change in bone. In addition BTM provide an important tool in the clinical and preclinical assessment of response to interventions.

BTM are proteins or enzymes released by osteoclasts and osteoblasts due to increased synthesis or metabolism or substances released during the formation or degradation of type I collagen. Smaller peptides are excreted in urine unchanged, while larger peptides may circulate in the serum with a longer half life. These markers provide a qualitative and a quantitative assessment of the rate of bone turnover or remodelling at any particular point in time but can be affected by a variety of physiological and pathological factors. Therefore bone turnover markers may provide a measure of bone quality and can be used to identify individuals with high bone turnover, which occurs in some metabolic bone diseases. In osteoporosis of postmenopausal women where bone resorption occurs at a greater rate than bone formation, bone resorption markers are expected to be high. In addition, BTM may be used to monitor treatment response in metabolic bone diseases (220) and prediction of fracture risk (221,222,223). BTM are particularly useful for the

assessment of efficacy of antiresorptive therapy in osteoporosis and also to identify non responders and non compliant patients to therapy (224,225).

Numerous studies link the usefulness of BTM in fracture risk prediction as well as monitoring progression of bone disease and treatment response. However BTM are measured using various immunoassay methods, which clearly lacks standardisation. Therefore the position of the International Osteoporosis Foundation (IOF) and the International Federation of Clinical Chemistry and Laboratory Medicine (IFCC) is that current knowledge is inadequate to make consensus statements or clinical guidelines for the use of BTM in the clinical arena (226,227). They recommend using serum procollagen type 1 N propeptide (s-P1NP) as a marker of bone formation and serum C-terminal telopeptide type 1 collagen (s-CTX) as reference markers of bone resorption in clinical trials in order to collect sufficient data on these markers in the context of their contribution to estimation of relative risk of fracture. It is hoped that the uncertainties with regards to their use in fracture prediction and monitoring therapy may be resolved with further evidence from observational and interventional studies measuring these markers using standardised analytical procedures.

1.4.2.1 Markers of bone formation

These are direct or indirect products of active osteoblasts that circulate in the serum.

1.4.2.1.1 Propeptides of type I procollagen

Osteoblasts play a major role in bone formation. They synthesize type I collagen which forms 90% of bone matrix, and is the most abundant structural protein in vertebrates. Collagen synthesis commences with the formation of the pre-pro-peptide which undergoes post translational modifications within the endoplasmic reticulum (ER) and then the Golgi apparatus. During post-translational modification within the ER lysine and proline residues are hydroxylated and hydroxyl groups on lysine are glycosylated with glucose or galactose monomers, resulting in a pro-peptide. Each pro-peptide folds to the left. Three such pro-peptides produce a triple helix forming a

procollagen molecule, which consists of α helical regions and non-helical regions. Proline hydroxylation is critical for the stabilisation of the triple helix. It is then transported into the Golgi apparatus for the addition of oligosaccharides, and the secreted into the extracellular matrix. Here the N- and C- terminal propeptides (the non-helical ends) are cleaved by ADAMTS (a disintegrin and metalloproteinase with thrombospondin motifs) and BMP 1 (bone morphogenic protein 1)/Tolloid-like proteinases respectively, resulting in mature type 1 collagen (228) and releasing amino terminal (PINP) and C- terminal propeptide of type I collagen (PICP) in a stoichiometric fashion. Circulating levels are considered quantitative measures of newly formed type I collagen. PICP is a globular protein but PINP can be found in the circulation as a trimeric molecule or a monomeric linear form (229). Mature type 1 collagen undergoes racemisation and isomerisation via spontaneous non-enzymatic reactions within the bone matrix yielding 4 isomers of C-terminal peptides that is released during degradation. Isomerised C-terminal peptides were therefore proposed as a marker of bone turnover (230). Indeed, the current assay for C-terminal telopeptide (CTX) detects the β isomerised C-terminal telopeptide, thereby quantifying the telopeptide released during degradation of mature type I collagen rather than the telopeptides released during collagen formation.

PINP has been recommended as the reference analyte for bone formation in clinical studies by the IOF and International Federation of Clinical Chemistry and Laboratory Medicine (IFCC) (227). Additionally PINP has wider global acceptance and is more convenient to use as a marker of bone formation than PICP because of the following reasons. PINP release kinetics are not affected by food intake or diurnal variation. In addition, the intraindividual variability of PINP is small and it is more stable at room temperature than PICP (225,231).

Although early assays used antibodies raised against a synthetic peptide that recognised trimeric and monomeric fragments, the automated immunoassays (Elecsys 2010 system, Roche Professional Diagnostics, Rotkreutz, Switzerland; IDS-iSYS, Immunodiagnostic Systems, UniQ PINP RIA Orion Diagnostica, Espoo, Finland) all identify the trimeric form (intact molecule) (225,231). The automated assays may also measure various fragments and thermal degradation products (total

P1NP). While the Elecsys (Roche Diagnostics) assay measures total P1NP (trimeric form and the monomeric form) the ID-iSYS and UniQ P1NP RIA assays measure only the intact trimeric molecule.

Some studies have reported that the monomeric fragment may accumulate in chronic renal failure (232) and in metastatic bone disease (233), suggesting the use of current assays that measure the trimeric form should not be affected by this phenomenon. A recent study comparing the intact trimeric P1NP assay (IDS iSYS) with the total P1NP assay (Roche) in patients with chronic kidney disease showed that when using the total P1NP assay, measured P1NP was markedly elevated in patients with KDIGO (kidney disease improving global outcome) stage 3 or worse (below an eGFR of 30mL/min/1.73m²), but levels remained within the reference interval when measured using the intact P1NP assay (234). These findings suggest that in later stages of kidney disease, the monomeric forms accumulate in the circulation. Therefore in renal disease patients, the trimeric form needs to be measured in order to assess bone resorption.

A meta-analysis of prospective studies demonstrated a significant association between serum P1NP and bone mineral density (223) suggesting that P1NP may reflect existing bone content and rate of bone formation in the bone remodelling cycle. In earlier studies, P1CP was shown not to rise after the menopause thus may not reflect bone formation in osteoporosis (235) but moderate correlations between elevations of serum P1CP and the rate of bone formation have been reported (236).

However the association between circulating intact P1NP and fracture risk independent of BMD was found not to be significant in one study (237). The practical and widely acceptable use for serum P1NP is to identify patients who respond to anti resorptive and anabolic treatment in osteoporosis (238) but is not routinely recommended for clinical practice.

1.4.2.1.2 Alkaline phosphatase

The total serum alkaline phosphatase (ALP) consists of several dimeric isoforms originating from liver, bone, intestine, spleen, kidney and placenta. In healthy adults

about 50% of the total ALP in the serum is regarded to be of hepatic origin and the rest of bone origin (239). ALP plays a role in osteoid formation and mineralization and thus reflects bone formation.

Liver and bone ALP isoforms can be differentiated on the basis of their physical properties (heat denaturation, electrophoresis, precipitation) or chemical properties (selective inhibition and immunoassay).

The OFELY study showed that the bone specific ALP (bALP) level was significantly associated with fracture risk regardless of BMD in postmenopausal women (239). Few centres measure bALP due to labour intensive time consuming procedures and/or the cost (in Australia, Medicare Benefits Schedule does not pay a rebate for bALP as a bone formation marker), while most measure total serum ALP as part of the liver function test panel. However, due to the low index of individuality of serum total ALP and the poor correlation with bone histomorphometric indices total ALP measurement may not reflect metabolic changes in bone (240). The use of immunoassay to more specifically measure bALP (Alkphae-B kit, Metra Biosystems, USA; Ostase, Beckman Access, Beckman-Coulter Inc, USA) (241) has been shown to better reflect bone turnover and response to antiresorptive therapy in osteoporosis (242).

1.4.2.1.3 Osteocalcin

Osteocalcin (Oc) is a protein synthesised by osteoblasts, and to a lesser extent by odontoblasts and hypertrophic chondrocytes. It binds to hydroxyapatite in the bone matrix. It contains 3 gamma carboxy glutamic acid (Gla) residues which confers Ca binding properties. Serum Oc is a specific marker of bone formation and a marker of osteoblastic activity (243).

Serum Oc is rapidly degraded resulting in several circulating fragments (244) yielding variable results from different immunoassays (245). Furthermore, smaller serum Oc fragment levels may rise in renal failure due to the impaired renal clearance. Therefore assays that detect the intact molecule and the N-mid fragment are more robust (N-MID Osteocalcin One Step enzyme linked immunosorbent assay [ELISA] kit, Osteometer Bio Tech, Denmark) (243).

There is evidence that lower circulating levels of Oc are associated with an increased risk in all types of fractures in retrospective trials (246,247). However, this relationship was not found to be significant in prospective trials (for review see 222).

1.4.2.2 Bone resorption markers

These are type I collagen degradation products or substances secreted by active osteoclasts and reflect the rate of resorption of the collagen matrix or the number and activity of osteoclasts depending on the specific marker measured. In general these markers are stable in vitro.

1.4.2.2.1 Hydroxyproline

The amino acid proline is hydroxylated in collagen. It constitutes 12 – 14% of the total amino acid content of mature collagen. However this is not specific to bone and is found in skin, CIq complement component and connective tissue. In addition dietary intake of proteins containing hydroxyproline (OHP) influences the amount excreted in urine. The majority of OHP in urine occurs as part of small peptides. Some of the OHP may be within larger peptides and proteins and need to be hydrolysed before analysis. A significant proportion of urinary OHP can result from bone formation rather than resorption (248) and from the degradation of elastin and CIq (249). Therefore, the specificity of OHP for bone resorption is poor. Furthermore colourimetric assays are cumbersome and nonspecific. An improved HPLC assay can be used to monitor post menopausal osteoporosis. The urinary OHP concentration cannot be used to diagnose metabolic bone disease because of the lack of a cut point that differentiates between healthy and diseased individuals. Although a decrease in the excretion rate of OHP can be used as an indicator of response to therapy the significant biological variation needs to be borne in mind (250). Owing to these reasons urinary OHP measurement has been replaced by more specific markers and only mentioned here as a historical oddity.

1.4.2.2.2 Pyridinoline crosslinks

Pyridinoline (PYD) and deoxypyridinoline (DPD) are accepted as more specific markers of bone resorption compared to hydroxyproline. These are crosslinks formed

between peptide chains of different collagen molecules during maturation of bone and cartilage to provide mechanical stability. DPD is more specific to bone while PYD is also released from the degradation of cartilage. They are found in both free (40%) and protein bound forms in the urine. The advantages of measuring DPD and PYD in the urine is that these molecules result from degradation of mature collagen, excreted without further degradation, the urinary levels are not influenced by diet (251) and correlates with radio-isotopic measurement of bone resorption (252) as well as histomorphometric and dynamic parameters of bone resorption (253,254). However the diurnal variation in the rate of excretion prevents the use of random urine samples.

The free fraction of PYD and DPD are measured using immunoassays and has been automated (Pyrilinks-D, Metra Biosystems USA; DPC Immulite, Siemens, USA). HPLC methods for total PYD and DPD have also been introduced (255). Although pre- fractionation and hydrolysis has been used prior to HPLC assay for total PYD and DPD, the measurement of the free fraction alone is likely to be adequate since the proportion of free fraction does not change significantly (256).

1.4.2.2.3 Telopeptides

The mature triple helix of collagen forms cross link at specific. Lysyl and hydroxylysyl residues of the collagen molecule are oxidised to form aldehyde groups during 'maturation' which aids in the formation of crosslinks at specific sites in the tropocollagen molecule. Once cross linked, the polymers of tropocollagen are referred to as collagen fibrils. Fragments of the fibrils with intact cross-linked peptides are released to the circulation during proteolytic degradation of collagen such as during bone resorption. The cross linking process is demonstrated in figure 1.1. N- and C- terminal telopeptides and their involvement in pyridinium crosslinks are shown in figure 1.2.

The early radioimmunassays for C-terminal cross-linked telopeptide of type I collagen used antibodies raised against collagen isolated from human bone and degraded using metalloproteinase (MMP) (257). This assay (CTX-MMP) also cross reacted with DYP, PYD or pyrrole crosslinks. Furthermore, cathepsin K cleaves the

telopeptide structure and may abolish immunoreactivity in metabolic bone diseases such as osteoporosis. However this assay is useful in pathological bone resorption as seen in multiple myeloma and metastatic bone disease (258).

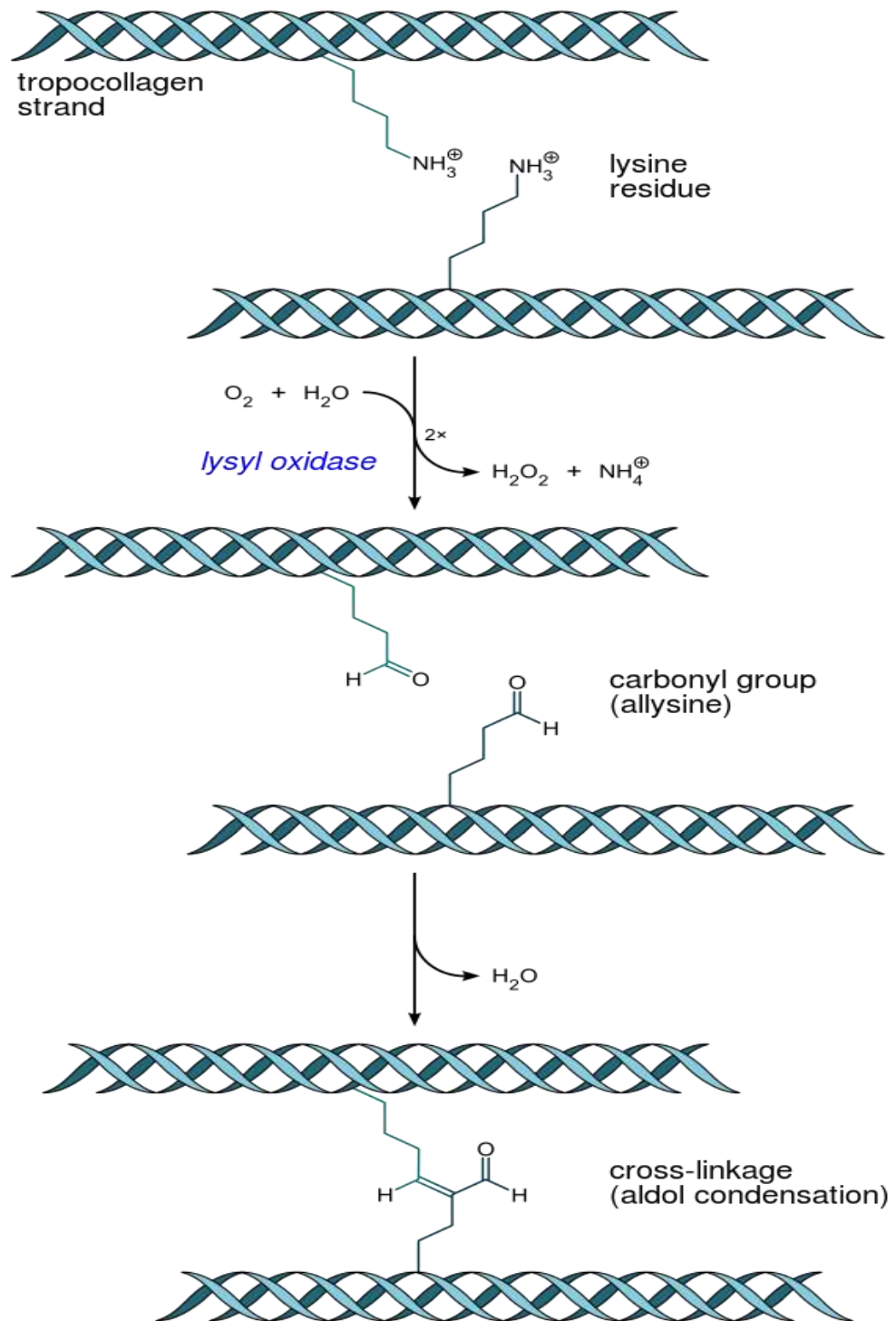


Figure 1.1 Cross linking of collagen fibrils.

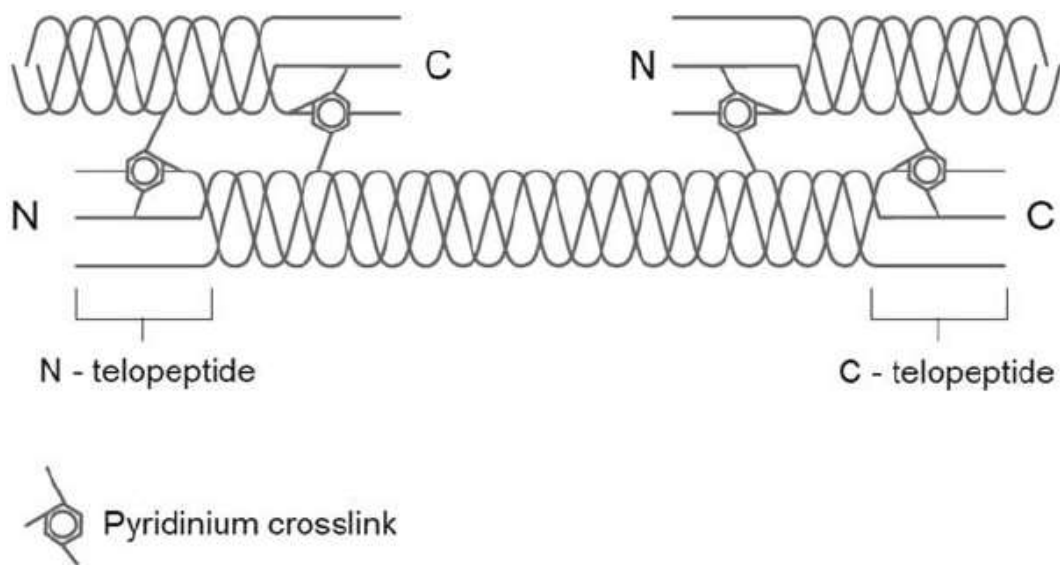


Figure 1.2. Cross linking between telopeptides and collagen fibrils

A monoclonal antibody against the C terminal telopeptide or $\alpha 1$ region of type I collagen (EKAHDGGR) allowed the development of assays for another bone resorption marker, the C-terminal cross-linking telopeptide of type I collagen (CTX). This octapeptide was originally purified from human urine. This antibody is specific for degradation products of type I collagen because it recognises the β isomer of aspartate, preceding glycine. The β isomerised Asp-Gly sequence increases the specificity of the assay for bone resorption (259). The most widely used assay in Australia is an automated assay (Beta-CrossLaps Roche Elecsys, Roche Diagnostics, Mannheim, Germany) which uses a monoclonal antibody against a synthetic polypeptide resembling the C terminal telopeptide or $\alpha 1$ region of type I collagen (EKAHD- β -GGR). IDS iSYS CTX-1 (CrossLaps) CLIA automated assay, also using a monoclonal antibody, has recently entered the market, produced by Immunodiagnostic Systems Nordic, Denmark.

Similarly, immunoassays have been developed to detect and quantify N-terminal telopeptides of type I collagen (NTX) (QYDGKGVG). The assays use a monoclonal antibody raised against the cross-linked N- terminal of $\alpha 2$ chain of type I collagen and use serum or urine (Osteomark Serum ELISA, Osteomark Urine ELISA, Alere; Ostex International, Seattle, Washington, USA), which has been automated. Dry chemistry systems (Vitros Eci Immunodiagnostic Systems, Ortho-Clinical

Diagnostics, New Jersey, USA) are also available. However these assays measure total NTX and are not specific for isomerised forms thus may reflect resorption as well as formation.

Both CTX and NTX have been shown to rise significantly with the menopause (260,261). In addition urinary CTX levels above the premenopausal range (mean + 2SD) were shown to be associated with a 2 fold higher risk of hip fracture while urinary NTX levels were not associated with fracture risk (235,262). However these analytes show a high intra- and inter- individual variability, susceptible to change due to food intake and show a marked diurnal variation (263) requiring standardization of patient preparation, sample collection and handling.

The clinical utility of serum CTX and P1NP was emphasised in a recent publication with consensus reference intervals and treatment targets (264). In addition, a meta-analysis of prospective studies found a significant association between serum CTX and risk of fracture (223) although the use of serum CTX for this purpose has not been validated.

1.4.2.2.4 Tartrate resistant acid phosphatase

Tartrate resistant acid phosphatase (TRAP) belongs to the family of ubiquitously occurring acid phosphatases. Different isoforms are expressed in bone, prostate, spleen, liver and erythrocytes. The tartrate resistant isoform occurs in 2 subforms, 5a and 5b, and 5b is characteristic of osteoclasts possibly originating from macrophages (265). Although immunoassays have been developed to measure TRAP 5b in serum (266) the utility of this assay is limited due to the instability of TRAP in vitro (267).

1.4.2.3 Novel markers

Osteoprotegerin (OPG) and its ligand OPGL are cytokines regulating osteoclastogenesis. OPGL is better known as RANKL (Receptor Activator of Nuclear factor κ B Ligand). In addition to being an osteoclast differentiation factor (268), RANKL also has a role in T cell- dendritic cell communication and dendritic cell survival (269).

OPG is a soluble decoy receptor for RANKL (OPGL), and can neutralise its ability to bind to RANK. OPG is produced by osteoblasts. Although a large proportion of circulation OPG is derived from vascular smooth muscle and endothelial cells (270) it may have significant potential to inhibit bone resorption by suppressing differentiation of osteoclasts (271). Deficiency of OPG has been reported in juvenile Paget's disease (272).

RANKL, a member of TNF superfamily of ligands has been recognised as a critical second messenger in T cell activation and subsequent differentiation of osteoclasts, resulting in bone loss (273). RANKL is produced by osteoblast lineage cells which are thought to facilitate osteoclastogenesis in a paracrine manner (274). Expression of RANKL mRNA and OPG mRNA vary depending on the maturation stage of osteoblasts. In primary calvarial osteoblast cultures treated with $1,25(\text{OH})_2\text{D}_3$ RANKL mRNA expression rose during all differentiation stages except in mature osteoblasts (275). RANKL from activated T cells (276) may be responsible for focal bone erosions in inflammatory arthritis (277). Circulating RANKL has been shown to increase with stimulated bone resorption (277) and soluble recombinant RANKL administration lead to severe catabolic events in bone of rodents (278).

The receptor that mediates osteoclastogenic properties of RANKL is RANK (receptor activator of nuclear factor κB) and belongs to the TNF superfamily. The role of RANK in bone resorption was demonstrated by the high bone mass phenotype of RANK knockout mice which were devoid of mature osteoclasts (279). Binding of RANK to RANKL leads to the activation of a second messenger TRAF 6 (TNF receptor associated factor 6) which in turn activates various protein kinase pathways and transcription factors (280).

While OPG and RANKL play important roles in normal bone remodelling and maintenance of bone mass, bone resorptive properties of IL-1, IL-7, TNF- α , TGF- β , estrogen, PTH, PTHrP and vitamin D are now thought to be mediated via the regulation of OPG and/ or RANKL (280). In vitro studies demonstrate that calcitonin may exert its inhibitory effects on bone resorption by enhancing OPG mRNA

expression (281). OPG has been shown to rise with the menopause (282), but studies examining the relationship between estrogen and OPG report conflicting results (283). The evidence for a relationship between OPG and bone density, OPG and fracture (prevalent or future) or OPG and markers of bone turnover are conflicting (282). Therefore there is not yet evidence that OPG has any role in the clinical assessment in post menopausal osteoporosis.

Studies of serum RANKL in postmenopausal osteoporosis reported variable results, which raises the issue of the suitability of the currently available assay and the fact that circulating RANKL levels may not reflect those in the bone. Furthermore the changes in OPG and RANKL in response to various modes of anti-resorptive treatment have been inconsistent (282). For a comprehensive review of osteoblast physiology in normal and pathological conditions please see Ref 281.

1.4.2.4 General considerations of bone markers

Various bone markers detected in serum, plasma or urine are classified according to the metabolic process they are considered to reflect ie: bone formation or bone resorption. However some markers may be released during bone formation and resorption and these reflect the rate of bone turnover. Another classification reflects the biological location of the markers, ie: osteoblastic origin, osteoclastic origin or the organic matrix. But the fact that these markers are also present and released from tissue other than bone should be born in mind. Furthermore the rise in a particular marker may occur during non- skeletal pathological events as well as during normal physiological phenomena. The patterns of rise in bone markers are not disease specific. Therefore in a clinical sense, although bone markers reflect the rate of bone turnover, on their own, they cannot be used as diagnostic markers. However, bone marker concentrations and patterns can be used effectively to monitor treatment response and disease progression.

Available evidence for an association between risk of fracture and BTM are variable, which may be in part due to variable methodologies used to measure BTM and the heterogeneity in reporting fractures and fracture outcome (ie: hazard ratio for fracture

per SD rise in BTM, hazard ratio for fracture in the highest BTM tertile/quartile or in 2SD above reference population (284).

In an analytical sense, many of the bone markers are subject to biological variation (both within subject and inter – subject). Therefore the identification of a definitive cut-off point or an acceptable reference interval is difficult. Current cut off points are recommendations reflecting a desirable range, and these should be further refined using age and gender specific ranges. Secondly, most bone markers are susceptible to pre analytical factors such as time of collection, fasting status, physical activity, renal and liver function, haemolysis which necessitate the standardisation of collection practices. Following collection most markers need to be frozen because they are thermo labile even at room temperature (285). UV irradiation and other ambient influences have also been known to cause degradation. In addition they are also susceptible to freeze-thaw cycles. These issues necessitate strict standardisation of sample handling, storage and analysis. The currently available assays lack standardisation and external proficiency testing programs.

In order to address these vagaries, the IOF and IFCC Working Group on Bone Marker Standards have recommended reference markers for bone formation and resorption, respectively as discussed above. They are working together with manufacturers of assays to standardise the assays for the reference BTM (284). Furthermore it is anticipated that the harmonisation of methods for the reference markers will aid in the development of globally acceptable decision limits and treatment goals. Reference intervals for the nominated reference BTM for the Australian population have been published (286).

1.5 Conclusions and proposal for the current study

It is clear that with the menopause, there is an increased requirement for dietary calcium and a consequence of inadequate calcium intake may be - osteoporosis. Published studies to date suggest that adequate calcium intake, mainly in the form of supplemental intake, in institutionalised postmenopausal women lead to a reduction in bone loss, demonstrated by a fall in bone resorption markers. However

observational studies of community dwelling women on self-selected diets and bone loss have not been published. The current study proposes to ascertain if a higher calcium intake, through a self-selected diet leads to a reduced rate of bone loss compared to a lower calcium intake in community dwelling postmenopausal women.

1.6 The aims and Protocol of the current study

The following research project aims to establish if there is a difference between the rates of bone loss in post-menopausal women on self-selected low or high calcium diets. The rate of bone loss was assessed by serial bone density measurements. Baseline biochemistry allows the identification of any existing bone disease or other chronic disease and to investigate any relationships between these variables and dietary intake of calcium, as well as any relationship between these variables and the annual percentage bone loss. In addition, any relationship between the rate of fractional calcium absorption and biochemical variables in the serum and urine, and rate of bone loss were investigated.

1.7 Hypothesis

Self-selected diets high in calcium will prevent bone loss in community dwelling post-menopausal women, as demonstrated by a lower rate of fall in the bone density and lower levels of bone resorption markers

1.8 Aims

1.8.1. Validate a calcium intake questionnaire suitable for community dwelling women

1.8.2. Determine the baseline total calcium intakes, bone densities and biochemistry in the subjects

1.8.3. Determine any relationships between baseline measurements and dietary calcium

1.8.4. Determine rate of bone loss over 2 years using 6 monthly bone densities and compare the rates between quartiles of calcium intakes

1.8.5. Determine the acute effects of a calcium load on bone resorption and the effects of adding vitamin D to the calcium load (*published papers*)

1.8.6. Determine if metabolic syndrome as demonstrated by a greater amount of visceral fat influences a dietary calcium load (*The influence of metabolic syndrome on acute effects of a dietary calcium load on bone resorption markers*)

1.8.7. Determine the effects of feedback on results of bone density on calcium intakes

Chapter 2

Materials and Methods

2.1 Subject recruitment

Ethics approval for the present study was obtained from the Royal Adelaide Hospital Human Research Ethics Committee (RAH protocol number 090715a, b and c).

Advertisements (figure 2.1) were placed in the free community newspaper Messenger circulated in the Northern and North Eastern suburbs of Adelaide, encouraging postmenopausal women to participate in a study investigating the link between diet and bone density. In addition, messages were added to radio programs on Australian Broadcasting Cooperation on health and wellbeing, encouraging postmenopausal women to participate. All interested women were asked to contact a telephone message service and leave their name, address and telephone number. The messaging service then faxed these details each morning to the Clinical Chemistry office at IMVS/SA Pathology.

The selection criteria for the subjects were:

1. Postmenopausal women
2. Healthy and living in the community

Once the contact details of interested women were obtained, they were sent an information sheet (appendix 1), an accompanying letter (appendix 2), general health questionnaire including contact details and email address (appendix 3) and a dietary calcium questionnaire (appendix 4) via mail, with a stamped envelope so that the two questionnaires can be returned. Upon receipt of these surveys, some women were excluded from the study based on the following criteria:

1. Current hormone replacement therapy or hormone replacement therapy within the previous 6 months
2. Current anti-resorptive therapy or anti-resorptive therapy within the previous 6 months
3. Diagnosed bone diseases (Paget's disease, metastatic cancers, osteoporosis)

Have you passed through
MENOPAUSE
in the last 5 to 10 years?

Would you like to participate in a study looking at the link between
the calcium in your diet and your bone density?

All bone density measurements and blood tests will be
done at the Royal Adelaide Hospital.

If you are interested, please ring
8366 0642

This trial is conducted under the leadership of
Professor Chris Nordin and has been approved by the
Royal Adelaide Research Ethics Committee.

www.adelaidehospital.com.au

Figure 2.1. Advertisement as it appeared in the Messenger community newspaper in September 2009.

Of the selected women, those who had an email address were emailed the web link for the CSIRO diet survey at: (<http://calciumstudy.ofq.csiro.au/faces/Register.jsp>) with a username and a password created for individual subjects. The completed online surveys for each subject were accessed from the server and saved as Excel spreadsheets.

When the baseline dietary data were completed the subjects were invited via letter to visit the Endocrine Test Unit in the Royal Adelaide Hospital for baseline blood tests and urine tests including a calcium absorption test (appendix 5). At this visit, the investigator met with the subject, and answered any questions the subject may have. Informed consent was also obtained at this visit (appendix 6).

2.2 Baseline Blood and Urine Tests

The subjects were instructed via a letter (appendix 5) to fast for 8 – 10 hours prior to the blood and urine tests. Upon arrival, they were given a sterile 50ml container to collect a sample of fasting urine.

All blood specimens were obtained from the anti-cubital vein, after a minimal period of tourniquet application. Two serum specimens (plain gel tubes, 8 ml each) were collected for biochemistry, vitamin D, parathyroid hormone (PTH) and C-terminal telopeptides (β crosslaps, CTX). A single plasma specimen (into a lithium heparin tube, 8ml) was collected as the baseline specimen for the calcium absorption study (detailed below). All specimens were promptly sent to the laboratory.

2.3 Analysis of Specimens

Routine biochemical parameters were assayed on an automated Olympus AU 5700 instrument as per the manufacturer's instructions.

The specific methods used are given in table 2.1 below.

Table 2.1. Details of assay methods used in the biochemical analysis

Analyte	Assay method
Sodium, potassium and chloride	indirect ion selective electrodes
Bicarbonate	Enzymatic method using phosphoenol pyruvate carboxylase to convert HCO ₃ ⁻ and PEP and malate dehydrogenase as secondary enzyme
Glucose	Hexokinase and glucose 6 phosphate dehydrogenase
Urea	urease reaction with glutamate dehydrogenase as secondary enzyme
Creatinine	Jaffe reaction (yellow-orange compound with alkaline picrate)
Urate	Enzymatic method using uricase and peroxidase
Phosphate	Heteropolyacid complex formation with molybdite

Total calcium	Arsenazo III reaction to produce a purple complex
Albumin	Bromocresol purple complex formation
Total protein	cupric ions in alkaline solution to produce a blue violet complex
Total bilirubin	Diazo reagent in the presence of caffeine and surfactant
Gamma glutamyle transferase (GGT)	Szasz procedure – activity using gamma-glutamyl-3 carboxy 4-nitroanilide
Alkaline phosphatase (total) (ALP)	rate of conversion of pNPP to p-nitrophenol
Alanine amino transferase (ALT)	Enzyme activity using 2-oxoglutarate and L-alanine as substrates and lactate dehydrogenase as the secondary enzyme
Aspartate amino transferase (AST)	Enzyme activity using 2-oxaloacetate and L-aspartate with malate dehydrogenase as the secondary enzyme
Lactate dehydrogenase	Enzyme activity using lactate as substrate
Total cholesterol	cholesterol esterase and cholesterol oxidase
High density lipoprotein-cholesterol (HDL)	Anti-human β lipoprotein antibody is used to bind to lipoproteins other than HDL (LDL, VLDL and chylomicrons). HDL then reacts with cholesterol esterase and cholesterol oxidase
Triglycerides	lipase, glycerokinase, glycerol PO ₄ oxidase and peroxidase
CRP (C reactive protein)	Immunoturbidimetric assay

Calculated parameters:

Some of the biochemical variables were calculated using measured values and these with the equations are shown below.

$$\text{Anion gap} = ([\text{Na}^+] + [\text{K}^+]) - ([\text{Cl}^-] + [\text{HCO}_3^-])$$

Ionised Ca

$$= \text{total Ca} - \left\{ \frac{0.01257[\text{Ca}] \text{ albumin}}{1+0.01257[\text{Ca}]} \right\} - \left\{ \frac{0.0049[\text{Ca}] \text{ globulin}}{1+0.0049[\text{Ca}]} \right\} - \left\{ \frac{0.0835[\text{Ca}] \text{ anion gap}}{1+0.0835[\text{Ca}]} \right\} - \left\{ \frac{0.0759[\text{Ca}] \text{ bicarbonate}}{1+0.0759[\text{Ca}]} \right\}$$

Where [Ca] = ionised Ca (287)

$$\text{Globulins (g/L)} = \text{total protein (g/L)} - \text{albumin (g/L)}$$

The estimated glomerular filtrations rate was also available, calculated using the MDRD formula (288).

$$\text{eGFR (mL/min/1.73 m}^2\text{)} = 175 \times (\text{S}_{\text{cr}})^{-1.154} \times (\text{Age})^{-0.203} \times 0.742$$

LDL (low density lipoprotein) was calculated using the Friedeweld formula (289):

$$\text{LDL} = \text{total cholesterol} - (\text{HDL-cholesterol}) - \text{triglyceride} / 2.2$$

25 hydroxyvitamin D (25OHD)

The assay for 25OHD uses an automated competitive binding chemiluminescence method. Samples in duplicate are pre-treated to denature vitamin D binding protein then neutralised with assay buffer. Anti 25OHD antibody labelled acridinium is then added to the samples. After incubation magnetic particles bound to 25OHD are added to each sample followed by a further incubation step. The magnetic particles are captured using a magnet which removes unbound anti-25OHD antibody. A trigger reagent is added to catalyse the light emission from acridinium, which is inversely proportional to the concentration of 25OHD in the original sample. All reagents are supplied by Immunodiagnositics (IDS Inc, USA) and the assay is run on the IDS-iSYS Multi-Discipline Automated Analyser.

1, 25 dihydroxyvitamin D (1,25(OH)2D)

Serum 1,25(OH)2 D was measured using a radioimmunoassay using reagents supplied by Immunodiagnositics (IDS Inc, USA) according to manufacturer's

instructions. The radioactivity was read on a Perkin Elmer 2470 gamma counter and the concentrations were calculated.

Parathyroid hormone (PTH)

PTH was measured using a heterogeneous sandwich immunoassay using biotinylated antibodies and ruthenium labelled antibodies. The automated assay is run on Roche Modular Analytics E170 using Roche reagents (Roche Diagnostics GmbH, Mannheim).

β Crosslaps (CTX)

Serum CTX was measured using a 2 step electrochemiluminescence immunoassay using biotinylated monoclonal antibodies, streptavidin coated microparticles and anti CTX antibody labelled ruthenium complex. All reagents were supplied by Roche Diagnostics and the assay was run on Roche Modular Analytics E170 (Roche Diagnostics GmbH, Mannheim).

Calcium absorption

Active intestinal absorption of calcium was assessed using radiocalcium (^{45}Ca) with 20 mg CaCl_2 as carrier using the following protocol (290).

After an overnight fast, 2 x 10 ml of non-stasis venous plasma samples (into a Li heparin tube) were collected. The patient was then given a 250mL dose of distilled water with 5 μCi (in 3 mL of working solution prepared by diluting stock solution with an activity of 26.53mCi/mL) of ^{45}Ca isotope and 20mg of stable calcium. (3 mL of this solution was retained by the laboratory for the subsequent steps in the analysis). Another 10ml non stasis venous sample was collected one hour after drinking the dose.

Following centrifugation, 2mL of plasma from each of the samples were added to 12mL of scintillation fluid. 100 μL of the retained dose was added to one of the pre-dose plasma samples. The 3 vials were counted on a Packard Tri-Carb Scintillation Counter. Radiocalcium absorption was automatically calculated by the laboratory computer.

The fractional Ca absorption was calculated as follows:

Fraction of radioactive Ca dose per L of plasma = F_x/L

$$F_x/L = 0.01117 + 1.54 X (1/\text{body weight})$$

$$\text{Fractional Ca absorption} = FC$$

$$FC = F_x \text{ in } 10 L - [15.4 X (1/\text{body weight}) - 0.0154]$$

The baseline blood test included electrolytes (sodium, potassium, chloride, bicarbonate), anion gap, glucose, urea, creatinine, estimated glomerular filtration rate, total cholesterol, urate, phosphate, total and ionised calcium, albumin, globulin, total protein, total bilirubin, GGT, ALP, ALT, AST, lactate dehydrogenase, 25 OH vitamin D, CTX, PTH and radiocalcium absorption. These tests were repeated at 12 months with the addition of CRP and cortisol.

2.4 Bone density measurement

When the subject had completed the blood and urine tests, they were given appointments to attend the Endocrine and Metabolic Unit Bone Densitometry Service (Norland, Cooper Surgical Company) at the Royal Adelaide Hospital for assessment of bone density at three sites. Lumbar spine (L1 to L4), femoral neck, total hip, proximal and ultradistal forearm were measured. The bone density was reported in g/cm². T and Z scores were included in the reports. Body weight (kg) was obtained using digital scales, with the subjects wearing light clothing and no shoes. Height (cm) was assessed using a stadiometer and the body mass index (BMI) calculated as weight in kilograms divided by the square of height in meters.

2.5 Visceral fat measurement

The fat mass in the lumbar region was measured using DXA as a separate measure using Research Mode on the instrument (Norland, Cooper Surgical Company). Bone mineral density (g/cm²), bone mineral content (g), lean mass (g) and fat mass (g) were obtained. The waist circumference of the subjects was also obtained during this measurement using a centimetre tape, placed around the smallest circumference in the torso.

Upon the completion of the baseline bone density measurement, 6 monthly appointments for repeat bone density measurements were made for each subject (5 in total). At the 12 months' bone density appointment they were given the paper based

dairy food frequency questionnaire to complete. They were also sent emails with the link for the CSIRO online FFQ and a blood test request form was mailed out to the subjects at this point.

The 12 month blood test included the baseline blood tests except radiocalcium absorption. In addition to these, triglycerides, HDL, LDL, Cortisol and 1,25 (OH)₂ vitamin D were measured.

2.6 Validation of the Dietary Ca Questionnaire

The short calcium intake questionnaire was validated by using a 3 day weighed food records for 26 subjects who agreed to participate in the validation following baseline blood sample collection and bone density measurements. The short calcium questionnaire was compared with the previously validated online food frequency questionnaire for the same 26 subjects as further validation.

2.6.1 Introduction

Despite nation-wide attempts at estimating nutritional intakes of Australians, there is currently a gap in the knowledge of intakes of specific nutrients, especially calcium. The latest National Health Measures Survey (39) published extensive data on fruit and vegetable intakes and vitamin D intakes but calcium intakes were not estimated. Previously published data suggest that calcium intakes among both male and female Australians are, on average, well below the recommended daily intakes (RDI) (103). Particularly in postmenopausal women, the intakes appear to fall well short of the RDI. After the menopause, calcium absorption decreases with age (291) while urinary calcium losses rise (292) increasing the RDI. However an individual's dietary habits may not change to accommodate the increase in RDI to maintain calcium balance. The Geelong Osteoporosis Study demonstrated that 87% of older Australian women had intakes below the RDI (127).

The lack of data on nutrient intakes is partly due to the lack of validated and easy-to-use tools for the estimation of intakes. Food-Frequency Questionnaires (FFQ) are widely used in epidemiological studies to gather data on a variety of nutrients. They

are inexpensive and self-administered. However they are by nature, extensive and lengthy in order to include all consumed food items. This leads to inaccurate results due to subject boredom and lack of time. There is also inaccuracy introduced to the estimate due to cognitive challenges in estimating usual intakes over a period of months to a year. In addition, although validated for multiple nutrients, they are inadequate and less accurate in estimating the intake of a single nutrient (168). They may not be appropriate for all age groups, as many are only available online. Some may require analysis using a computer, therefore the result is not readily available. These are not suitable for use in the clinical setting where estimates need to be made during consultation, or in the research setting, where estimates need to be made during subject enrolment (293) particularly when subjects are screened based on their dietary intakes.

Various FFQs have been validated for estimating calcium intakes but these have been developed decades ago (127,294) although still in use (295), they may not be relevant today (296). Most studies published prior to 2000 do not include dietary supplements (168) and for a nutrient such as calcium, supplements may make up a significant proportion of intake. Due to various campaigns promoting supplementary calcium, a significant contribution from dietary supplements to total daily calcium is expected. In addition, the reproducibility of FFQs for calcium has not been assessed in the published validation studies. Despite weighed food records (WFR) being the method of choice for validating FFQs, only 2 (127,296) out of 4 validation studies of calcium FFQs used WFR. The other 2 used food records only (297,298). None of these published validation studies assessed reproducibility of the questionnaire.

Accurate, reproducible results obtained using an easy – to –use questionnaire for estimating calcium intake would contribute to the clinical and research fields. In addition, Australian dietary intakes have undergone considerable change with more multicultural food items becoming available and the high consumption of dietary supplements. The aims of the current study are to validate a short questionnaire of calcium intake against a 3-day WFR and an online FFQ of all nutrients in Australian postmenopausal women.

2.6.2 Methods

2.6.2.1 Participant Recruitment and Data Collection

Volunteer postmenopausal women participating in an observational, non-interventional prospective study of bone density were invited to participate in the short calcium questionnaire (SQ) validation study. Ethics approval was granted by the Royal Adelaide Human Research Ethics Committee (Protocol number 09071; 29th July 2009) and informed consent was obtained from each subjects. Demographic data, medical history and medication were recorded. Each subject met with a researcher, who gave them verbal instructions on how to complete the 3-day WFR, provided them with electronic kitchen scales (Homemaker Slimline Electronic Scale, China) and demonstrated how to use it. The SQ consisted of food items high in calcium (mainly dairy). A baseline intake of 300mg calcium was added to the total obtained from the SQ to account for non-dairy calcium intake derived from dietary modelling based on daily food intake of a 60 year old female (Appendix 4). After completing the baseline SQ on paper they were given access for an online FFQ (298) at home or when they revisited the dietician to submit the WFR. SQs which were completed by the subjects 24 and 12 months prior to this study as part of the observational study, were used to assess reproducibility. Calcium added to the daily intake by supplements was added to the intakes obtained from all methods.

2.6.2.2 Nutrient Analysis and Statistical Analysis

Dietary data were analyzed using a database of Australian foods (Foodworks, Xyris Software, Australia) for the SQ and WFR. The online FFQ was based on a previously published FFQ (298). Mean difference and standard deviation of differences were calculated for calcium intakes assessed by SQ and WFR, SQ and FFQ and FFQ and WFR. In order to ensure robustness of the validation, a range of statistical techniques were used. Wilcoxon paired samples tests were used to determine whether the estimates of intakes were significantly different between methods. Bland-Altman plots were used to assess the agreement between the 3 methods. A cross classification method was used to further assess the agreement between the methods, as follows: Subjects were ranked into tertiles according to calcium intakes for each method. The degree of agreement between the methods was determined using linear weighted

kappa (κ) statistic with 95% confidence intervals. The percentage of subjects classified as meeting or not meeting the RDI (1300 mg per day for postmenopausal women) by each method were determined. A Student's t test was used to determine if the current estimates of Ca intake using SQ was significantly different from estimates 24 and 12 months prior (reproducibility), collected as part of the observational study. Only dietary Ca intakes were considered for the reproducibility study because subjects may have started, ceased or changed calcium supplements. Statistical analysis was performed using Analyse-it for Microsoft Excel 3.60.5, 1997-2015 (Analyse-it Software Ltd, Leeds, UK).

2.6.3 Results

Twenty six women completed the validation study. Demographic data and Ca intakes from the 3 methods for the 26 subjects who completed the study are presented in table 26.1.

Table 2.6.1. Demographic data and Ca intakes by the 3 methods.

	Mean	SD	Range
Age(years)	62	4	55 - 68
Height (m)	1.61	0.04	1.53 – 1.69
Weight (kg)	66	14	47 - 106
BMI	25.5	4.9	19.1 – 38.2
Ca intakes (mg/day)			
FFQ	1180	488	460 - 2225
SQ	1080	488	360 - 2125
WFR	1034	517	383 - 2306

There were no significant differences between the WFR and SQ ($P=0.38$), WFR and FFQ ($P=0.186$) or SQ and FFQ ($P=0.36$) estimates of mean calcium intakes. The degree of agreement between these methods in ranking subjects to tertiles of intakes and in classifying into meeting or not meeting RDI is presented in table 2.6.2.

Table 2.6.2. The degree of agreement between methods in ranking subjects into tertiles of intakes and classifying into meeting or not meeting RDI.

	Degree of agreement in linear weighted kappa	
	Ranking of subjects into tertiles of Ca intake	Classifying whether meeting RDI
WFR vs SQ	0.38 (fair)	0.54(moderate)
WFR vs FFQ	0.38 (fair)	0.39 (fair)
SQ vs FFQ	0.66 (substantial)	0.59(moderate)

Bland Altman plots (figures 6.2.1, 6.2.2 and 6.2.3) depict the mean bias for calcium intakes between the methods.

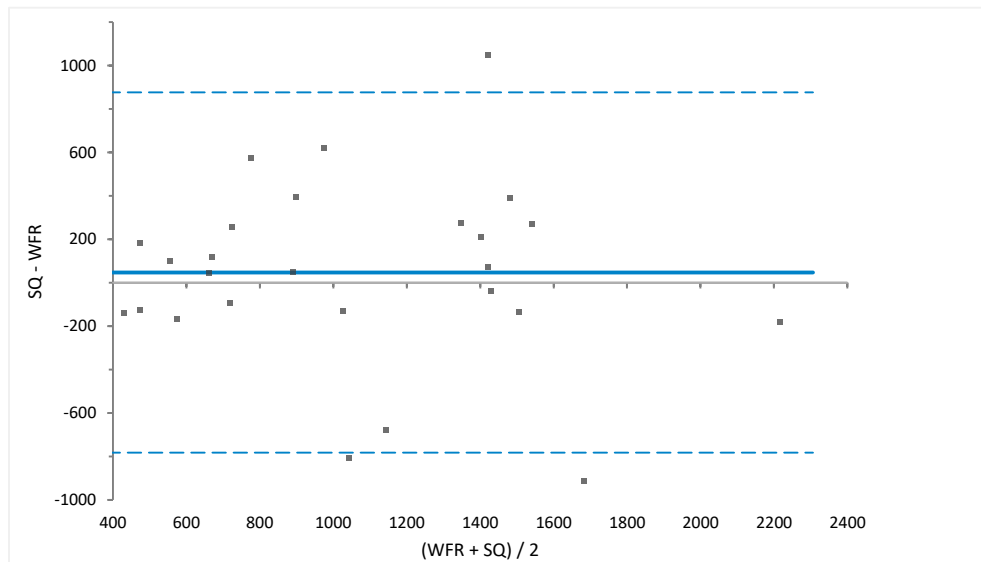


Figure 6.2.1. Bland-Altman plot, WFR (weighed food record) against SQ (short questionnaire). The mean bias was 46.2 mg/d (95% CI -783.5 to 876.0 mg/d).

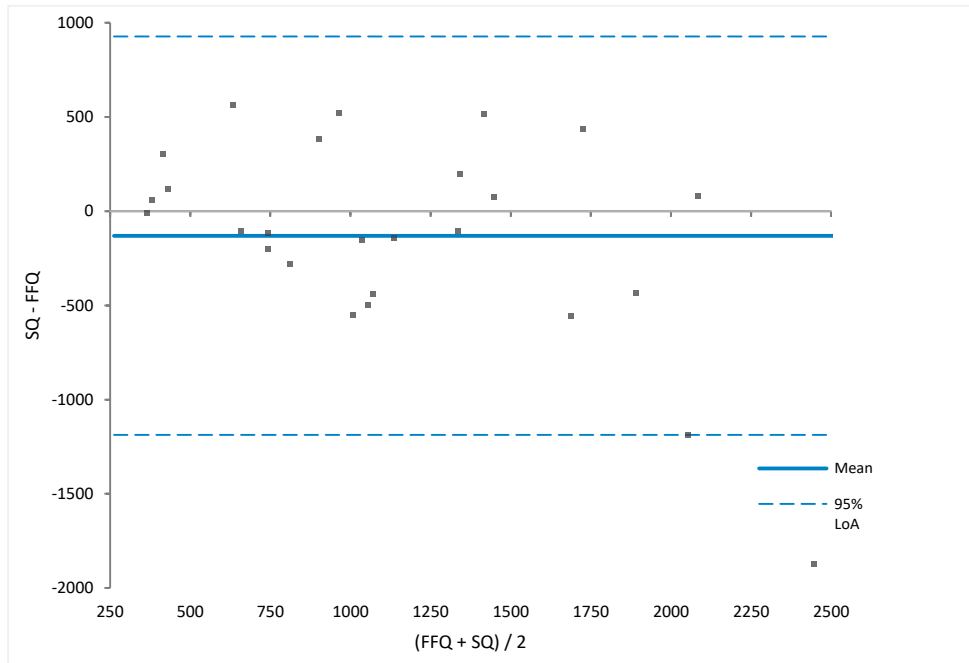


Figure 6.2.2. Bland-Altman plot, SQ against FFQ (online). Mean bias -130 mg/d (95% CI -1187.6 to 927.5 mg/d).

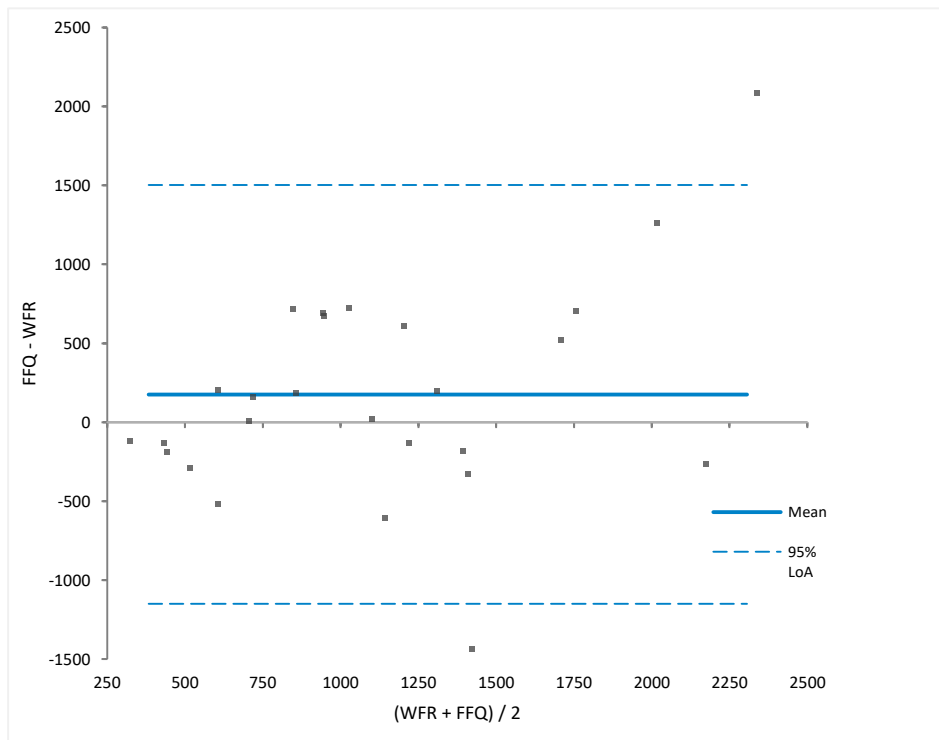


Figure 6.2.3. Bland-Altman plot, WFR against FFQ. Mean bias 176.3 mg/d (95% CI -1149.7 to 1502.2 mg/d).

There was considerable bias or disagreement between WFR and FFQ (mean 176.3 mg; 95% CI -1149.7 to 1502.2; 1.96SD) and between SQ and FFQ (mean -130.0 mg; 95% CI -1187.6 to 927.5). The bias between WFR and SQ was much less at 46.2 mg (95% CI -783.5 to 876.0 mg/d). However none of these biases were statistically significant.

With regards to the reproducibility of the SQ there was no significant difference between the current estimates and 24 months prior ($P=0.97$) and 12 months prior ($P=0.66$).

2.6.4 Discussion and conclusions

Despite substantial agreement between SQ and FFQ in ranking subjects into tertiles of intake, there was considerable overall bias, although not statistically significant, between the 2 methods of estimating Ca intakes. SQ showed moderate agreement with WFR for classifying subjects into meeting or not meeting RDI, and also showed the least bias. SQ was reproducible, as shown by yearly estimates over 3 years.

Generally the subjects in this study had a high intake of calcium perhaps due to the awareness of the study's objectives in terms of validating a questionnaire of calcium intakes and the observational study on bone density.

When estimating Ca intakes in a clinical or research setting, it is important to have a brief but accurate tool, to save time. The FFQ used here was developed for the estimation of multiple nutrients and was considerably biased when compared to WFR, generally overestimating intakes while SQ showed minimal bias and better agreement with WFR. The ability to complete the dietary questionnaire while waiting for or during consultation is an added advantage of an SQ. Immediate feedback given to the patient is of value because this is an effective strategy to increase Ca intakes in individuals with inadequate intakes (297). Amongst the subjects recruited for this study some 69% did not meet the RDI of calcium, consistent with previously published figures of up to 76% falling short of the RDI (127). Whether a patient meets RDI or not is important when advising them on their

requirement of Ca supplements and SQ may be used in the clinical setting for this purpose.

The reproducibility of the SQ in this study suggests that it is suitable to be used in the 2 year prospective observational trial the women were initially recruited to, and can be used to determine if their calcium intake varied significantly during the study.

The background or base intake of calcium was considered to be 300 mg/d from all non-dairy sources, derived from dietary modelling based on daily food intake of a 60 year old woman. Further refinement of the short questionnaire may be required in terms of including food items that contain dairy such as cheese toppings in pizza and Mexican food (nachos, tacos etc).

2.7 Statistical Analysis

2.7.1 Relationship between baseline biochemistry and habitual calcium intake

The correlation between baseline biochemistry (CTX, PTH, 25 OH vitamin D, ALP, radiocalcium absorption, rate of urinary Ca excretion) and habitual total Ca intake were assessed using linear regression. Additionally, maintenance of habitual calcium intake was assessed by re-administering SQ and FFQ at 12 months and 24 months to identify any significant change from baseline calcium intakes.

2.7.2 Relationship between baseline biochemistry and bone density

The relationship between baseline biochemical variables and baseline bone density of the proximal and ultradistal forearm, lumbar spine, femoral neck and total hip were analysed using linear regression and Pearson's correlation.

2.7.3 Rate of change in bone density

The subjects were divided into 3 groups using the tertiles of daily total Ca intake. The rate of change in bone density for each measured site for each quintile of daily total Ca intake was calculated by subtracting the latest value from the previous value. The rates of change in bone density for the 3 tertiles of Ca intakes were compared against each other.

The rate of change in bone density was correlated to baseline bone density of each site and to baseline biochemistry (CTX, PTH, 25 OH vitamin D, ALP, radiocalcium absorption, rate of urinary Ca excretion).

2.7.4 Relationship between visceral fat and other measured parameters

Visceral fat measurement was correlated with baseline biochemistry (CTX, PTH, 25 OH vitamin D, ALP, radiocalcium absorption, rate of urinary Ca excretion), baseline bone density of measured regions, daily total Ca intake and daily dietary Ca intake linear regression and multivariate ANOVA.

2.7.5 Relationship between metabolic syndrome and bone metabolism

Metabolic syndrome was defined as a waist circumference greater than or equal to 88cm (ATP criteria). The subjects were divided to 2 groups, those with metabolic syndrome and those without, based on the waist circumference. The waist circumference was correlated with the visceral fat measurement to confirm that a greater waist circumference was due to higher amounts of visceral fat. Baseline biochemistry (CTX, PTH, 25 OH vitamin D, ALP, radiocalcium absorption, rate of urinary Ca excretion), baseline bone density of measured sites, the rate of change in bone density and daily total and dietary Ca intake were compared between the groups.

The influence of overweight/obesity (ow/ob) on bone density, and the relationship between calcium intake and ow/ob were examined using odds ratios and relative risks.

Chapter 3

Recruitment and progression of subjects, baseline biochemistry, the prevalence of osteoporosis and osteopenia and calcium intakes

In order to evaluate the effects of calcium intake on changes in bone density in community – dwelling post-menopausal women, subjects were recruited to a 2 year observational prospective study. The methodology is given in detail in chapter 2 (2.1). Briefly, ethics approval was obtained to recruit community-dwelling post-menopausal women to participate in a prospective observational study of calcium intakes and bone densities. A detailed account of recruitment and progression of subjects during the 2 years of the study is described below. This chapter also contains the baseline biochemistry and the prevalence of osteoporosis and osteopenia in the subjects.

3.1 Recruitment of subjects

In response to the advertising, 273 women contacted the nominated call centre telephone number and provided their contact details. Out of these 12 women were excluded because they were pre or peri-menopausal. The remaining 261 (95.6%) women were mailed the basic health questionnaire (Appendix 3), the FFQ (Appendix 4), information for subjects (Appendix 1) and a letter (Appendix 2) inviting them to participate in the study. Only 180 (69%) were returned, out of which 5 were excluded (n=1 on a bisphosphonate; n=2 malignancy, n=2 chronic renal failure).

3.1.1 Progression of subjects

The remaining 175 women were then contacted and appointments were given for fasting blood tests and baseline bone density measurements. Figure 3.1 depicts the progression of subjects up to 12 months from baseline.

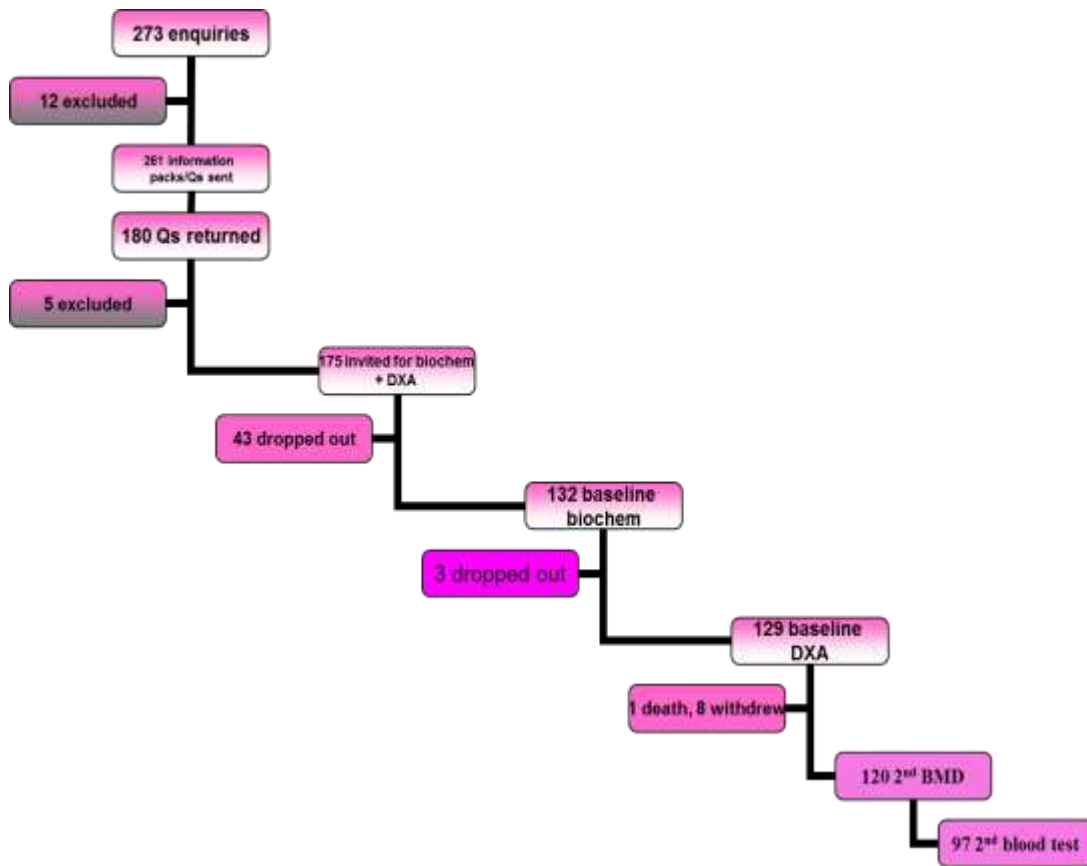


Figure 3.1. Progression of subjects

3.1.2 Number of subjects completing each stage of the study

Only 132 women arrived for their blood tests and 129 out of these for the baseline bone densities. All subjects received a copy of the bone density report, and those who had osteoporosis at least in one site were given the option of being referred for specialist treatment (n=16). All but 1 declined this invitation and continued to participate in the study. The number of subjects completing each stage is shown in table 3.1.

Table 3.1. The number of subjects completing each stage

	Number	percentage
Enquired	273	
Invited to participate	261	
Completed FFQ	180	
Recruited	175	100
Baseline biochemistry	132	75
Baseline BMD	129	74
2 nd BMD (6 months)	120	69
2 nd Biochemistry (12 months)	97	55

3.2 Baseline data

3.2.1 Demographic data

Demographic data are presented in table 2 for the 129 subjects that attended for their baseline bone density, where their height and weight were measured.

Table 3.2 Demographic data.

	Mean	range	SD
Age years	61	51 – 73	5
Height m	1.62	1.48–1.81	0.06
Weight kg	66	45 – 108	12
BMI kg/m ²	25	18 – 42	4
YSM years	10	1 – 39	7

YSM = years since menopause

3.2.2 The prevalence of osteoporosis and osteopenia in the subjects enrolled

Table 3.3 below lists the number of subjects who were diagnosed with osteopenia or osteoporosis at each measured site on the first (baseline) bone density scan, according to WHO criteria (bone density for each site below 2.5 standard deviations from the young normal average for Australian women classified as osteoporosis, and bone density between 1 and 2.5 standard deviations below the young normal average classified as osteopenia). (208).

Table 3.3 The numbers of subjects with osteopenia and osteoporosis at specific anatomical sites.

Site	Number scanned	Osteopenia		Osteoporosis	
		No	%	No	%
LS L2 – 4	129	39	30	11	9
FN	129	74	57	7	5
TH	129	45	35	4	3
PFA	48	45	37	12	10
UDFA	48	31	25	0	0

LS = lumbar spine, FN = femoral neck, TH = total hip, PFA = proximal forearm, UDFA = ultradistal forearm.

The prevalence of osteoporosis at any site in this group was 12% (n=16) and the prevalence of osteopenia at least at one site is 56% (n=72). The estimated prevalence of osteoporosis in community dwelling Australian adults aged 50 years and over is 9% (15% among women and 3% among men) based on self-reported surveys 2011-2012 (299).

The following histograms (figures 3.2 to 3.7) depict the overall prevalence of osteoporosis and osteopenia at each site measured in the subjects compared to that in the community as reported in Australian postmenopausal women in a previous study investigating calcium intakes and bone density, the Geelong Osteoporosis Study (GOS) (127). In addition the histograms also indicate the prevalence of each condition in the subjects of the present study with a total calcium intake that met the daily requirement of 1300mg (adequate Ca intake), in those who did not meet the requirement (low Ca intake) and in subjects that took a calcium supplement and those who did not. While the GOS reported a more than 20% prevalence of osteoporosis in females over the age of 50 years (127) which exceeds the AIHW 2014 reported prevalence (299), the prevalence of osteoporosis in the subjects of this study was 9% at the lumbar spine, 7% at the femoral neck and 4% at the hip. Osteopenia was reported in more than 70% of females over the age of 50 years (127). The prevalence

of osteopenia in the subjects of the present study was 30% at the lumbar spine, 74% at the femoral neck and 45% at the hip. The prevalence of osteoporosis in the subjects was much less than the reported prevalence in Australian community dwelling post-menopausal women at all sites measured while the prevalence of osteopenia at the femoral neck in the study group was consistent with that reported by GOS (127).

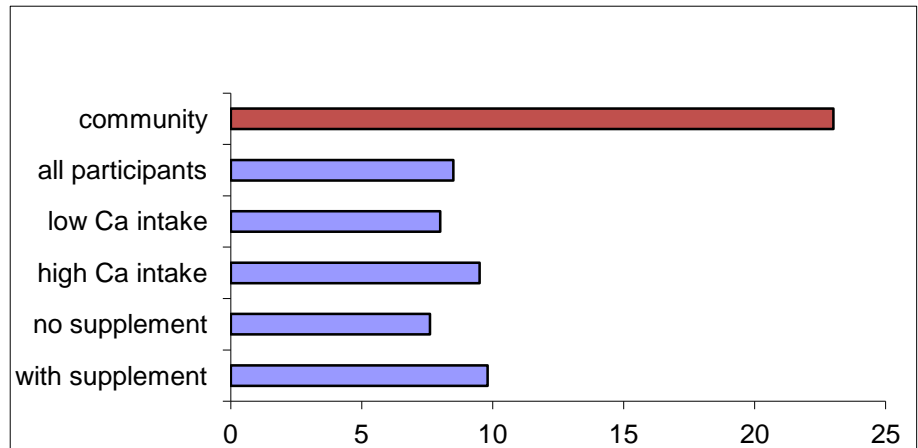


Figure 3.2. Prevalence of osteoporosis in the lumbar spine in the participants of the present study (blue bars); reported prevalence for the Australian female population over 50 years (red bar, Geelong Osteoporosis Study 2011, Ref 127). All participants = all subjects in the current study; High Ca intake = intakes ≥ 1300 mg, low Ca intake = intakes <1300 mg/d.

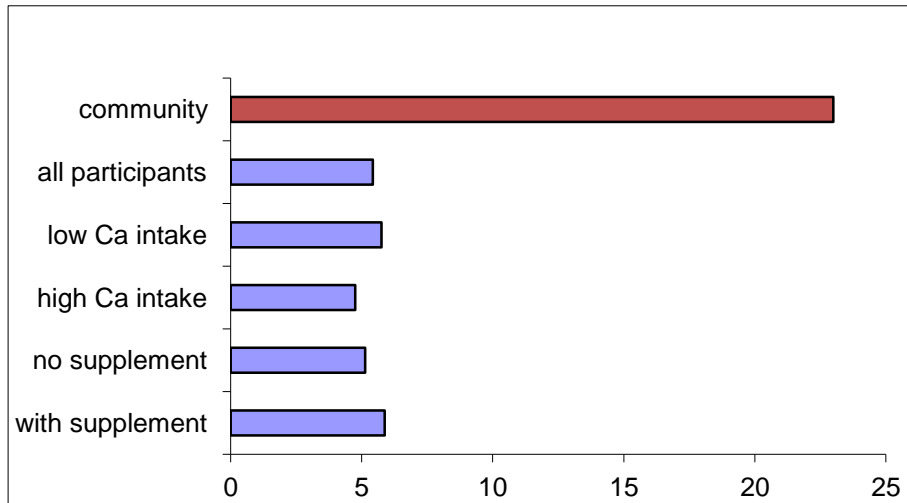


Figure 3.3 Prevalence of osteoporosis in the femoral neck in the participants of the current study (blue bars); prevalence for the Australian female population over 50 years (red bar, Geelong Osteoporosis Study 2011, Ref 127). All participants = all subjects in the current study; High Ca intake = intakes \geq 1300mg, low Ca intake = intakes $<$ 1300mg/d.

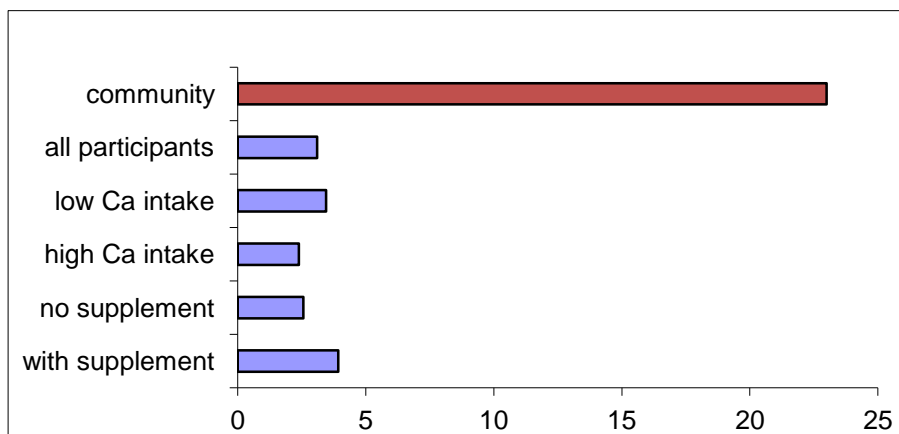


Figure 3.4 Prevalence of osteoporosis in the total hip in the participants of the current study (blue bars); prevalence for the Australian female population over 50 years (red bar, Geelong Osteoporosis Study 2011, Ref 127). All participants = all subjects in the current study; High Ca intake = intakes \geq 1300mg, low Ca intake = intakes $<$ 1300mg/d.

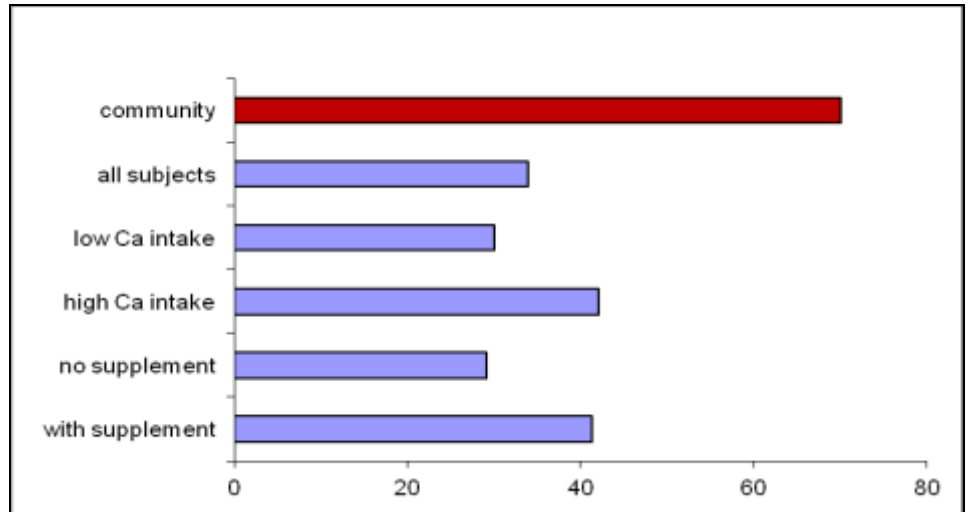


Figure 3.5. Prevalence of osteopenia in the lumbar spine in the participants of the current study (blue bars); prevalence for the Australian female population over 50 years (red bar, Geelong Osteoporosis Study 2011, Ref 127). All participants = all subjects in the current study; High Ca intake = intakes \geq 1300mg, low Ca intake = intakes $<$ 1300mg/d.

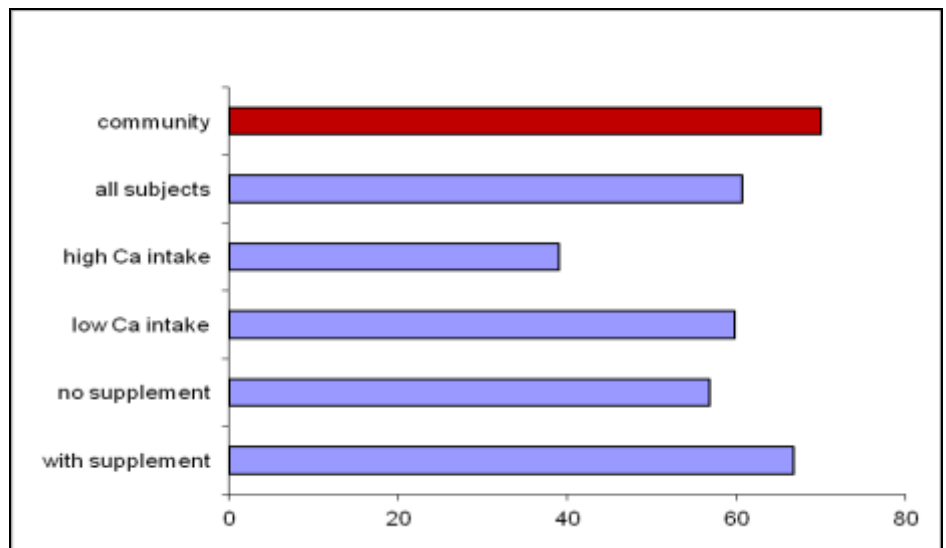


Figure 3.6. Prevalence of osteopenia in the femoral neck in the participants of the current study (blue bars); prevalence for the Australian female population over 50 years (red bar, Geelong Osteoporosis Study 2011, Ref 127). All participants = all subjects in the current study; High Ca intake = intakes \geq 1300mg, low Ca intake = intakes $<$ 1300mg/d.

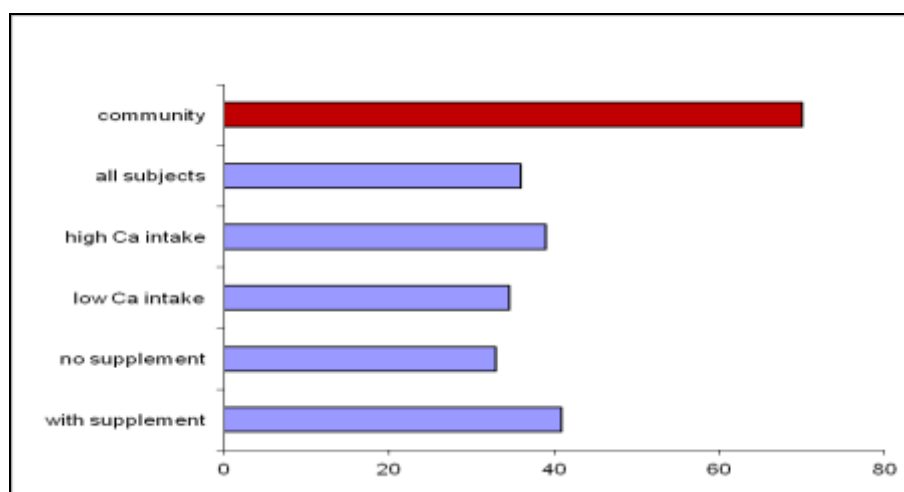


Figure 3.7. Prevalence of osteopenia in total hip in the participants of the study (blue bars); prevalence for the Australian female population over 50 years (red bar, Geelong Osteoporosis Study 2011, Ref 127) All participants = all subjects in the current study; High Ca intake = intakes \geq 1300mg, low Ca intake = intakes $<$ 1300mg/d.

3.3 Baseline calcium intakes in the subjects

3.3.1 Baseline Calcium intakes as per validated short questionnaire

A self-administered short questionnaire (SQ) was validated in a subset of the subjects, against weighed in food records (WFR) (see 2.6; Chapter 2 Material and Methods for details of the validation process). A total of 175 women completed the SQ. SQ considers dairy and other calcium rich food and an extra 300mg/day was added to the total to account for dietary items not listed. This baseline intake of 300mg/day was determined by dietary modelling of the daily intakes of a 60 year old woman with a sedentary lifestyle. The mean age of this group was 60.6 (SD 5, range 51 – 78). Total daily Ca intake on average was 1114 mg (SD 498, range 300 – 2589) and the Ca obtained daily from diet alone on average was 860mg (SD 299, range 300 – 1790). Out of this 175, 73 subjects were taking Ca supplements (42%). When considering the total intake of Ca, only 31% of the women met their recommended daily intake of Ca of 1300mg. When dietary Ca only was considered (without supplements) only 9.7% met their daily requirement. Of those who were taking supplements, 59% took a daily dose between 500 and 999mg, 17% were on $>$ 1000mg and 24% were on a dose $<$ 500mg. The most commonly consumed supplement was

CaCO₃ with added vitamin D (Caltrate, Pfizer Inc, NC, USA). Figure 3.7 depicts the types of various Ca supplements, and the percentage of women taking them.

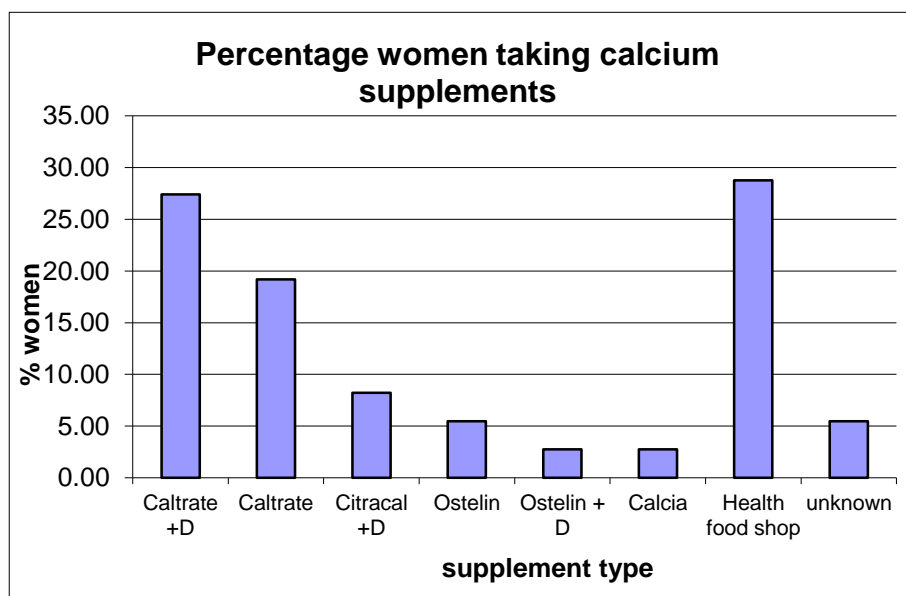


Figure 3.8. Types of Ca supplements and percentage of women consuming them.

3.3.2 Baseline Calcium intakes as per online questionnaire

The online CSIRO questionnaire is a food frequency questionnaire (FFQ) designed to estimate all nutrients using average intakes recalled from a period of 12 months, therefore includes seasonal fruit and vegetables. However it did not include any supplemental intakes. All 175 women invited to participate in the study were sent log in information (web link, log in name and a password) to complete the FFQ. Out of these 131 women completed it. The comparison between SQ with and without Ca supplements and the FFQ are given in table 3.4 and linear regression given in figure 3.9.

Table 3.4. Comparison between SQ total intake (including supplements), SQ diet only intake and online FFQ dietary Ca intake

	SQ total intake mg/d	SQ diet mg/d	FFQ mg/d
number	175	175	131
Age, mean(SD) y	60.6 (5)	60.6 (5)	60.6 (5)
range	51 - 78	51 - 78	51 - 78
Intake, mean(SD)	1114 (498)	860 (299)	1180 (478)
range	300-2589	300-1790	400 - 2552
% meeting RDI	31	9.7	30

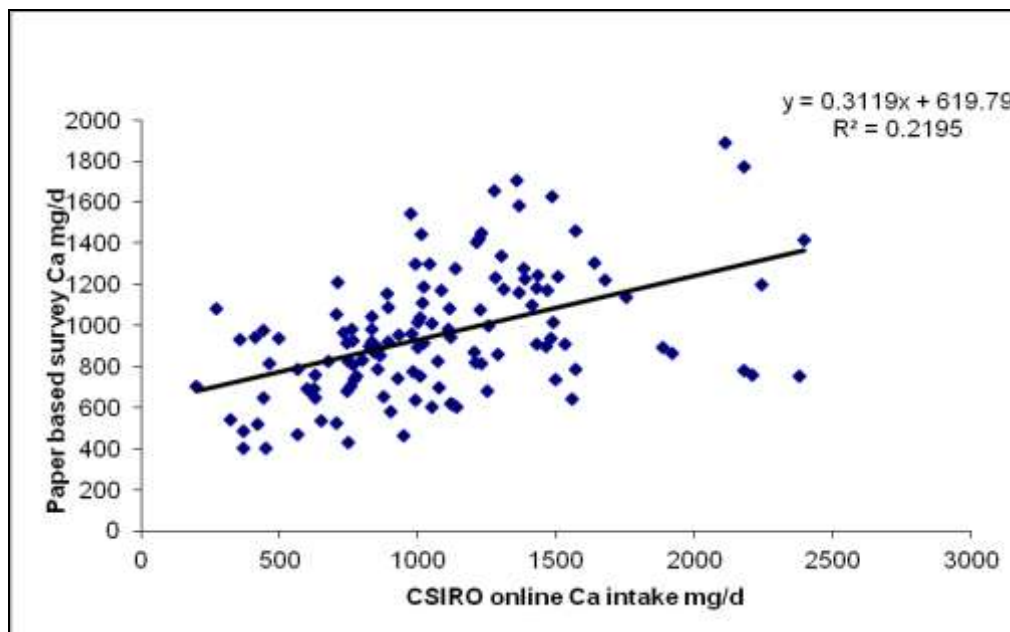


Figure 3.9. Linear regression between SQ and FFQ daily Ca intakes.

Linear regression between the SQ and the online FFQ showed a poor correlation with an R^2 of 0.22. The lack of agreement between these 2 questionnaires may be because the online FFQ was more extensive and included all food items consumed (estimated over a period of 1 year) whereas the SQ included dairy and fish. Although a ‘background’ calcium intake of 300mg/d was added to the SQ to account for non-dairy and non-fish calcium sources (modelled on a 60 year old women with a sedentary lifestyle by a senior dietician) this may not apply to the subjects of our study who appear to be more ‘health conscious’ and active. Therefore the SQ may

have underestimated their calcium intake. On the other hand, because the online FFQ was very extensive and the subjects were able to log in and complete the questionnaire over several days the intakes may not be estimated accurately, and may have overestimated their intakes. however it did not account for any supplements. Overall, the agreement between SQ and FFQ was low.

3.4 Baseline biochemistry

Fasting baseline biochemistry of serum including fractional calcium absorption and urine were completed by 131 subjects in order to identify any renal or hepatic disease or electrolyte imbalances including abnormal calcium homeostasis. The results are given in table 6 below.

Table 3.5 Baseline fasting serum and urine biochemistry

Analyte	mean	SD	range	RI
HCO ₃ ⁻ (mmol/L)	27	3	20 – 35	22 – 32
AG (mmol/L)	14	3	7 – 22	7 – 17
Creatinine(umol/L)	61	10	38 – 92	50 – 100
iCa (mmol/L)	1.22	0.04	1.15 – 1.40	1.10–1.30
tCa (mmol/L)	2.38	0.08	2.19 – 2.68	2.10–2.55
PO ₄ (mmol/L)	1.15	0.13	0.86 – 1.46	0.65–1.45
ALP (IU/L)	83	25	27 – 175	30 – 110
CTX (ng/L)	451	183	70 – 1064	<400
PTH (pmol/L)	4.4	1.7	0.6 – 11.2	0.8 – 5.5
25OHD (nmol/L)	73	25	21 – 157	60 – 160
UCa:Cr	0.27	0.21	0.03 – 1.40	0.03–0.40
UNa:Cr	10.5	7.5	1 – 48	
Ca abs	0.60	0.22	0.15 – 1.34	>0.5

There were no subjects with renal impairment or hepatic disease. Most other analytes including electrolytes were within the reference interval. The desirable range for bone resorption marker, C-terminal telopeptide (β CTX) was set at <400 ng/L by the local laboratory (Institute of Medical and Veterinary Science / SA Pathology)

following a survey of β CTX levels in healthy premenopausal women. When this cut point is used, 58% of the subjects had a greater than desirable level of bone resorption. The current Australian consensus reference interval for postmenopausal women on fasting morning serum β CTX (aged 50 to 70 years) is 50 – 800 ng/L. Based on this criterion, only 4% had an increased rate of bone turnover. CTX however is subject to acute changes in response to various factors therefore raised CTX was not included as in the exclusion criteria. There were 3 subjects with PTH above the upper limit of the reference interval, but all 3 had normal total and ionised calcium suggesting secondary hyperparathyroidism. A more detailed description of cross-sectional biochemistry data is presented in Chapter 4.

3.4.1 Vitamin D status

The proportion of subjects in each category of deficiency and the ABS reported proportions for the general population are given in table 3.6. The majority of this group of women were vitamin D sufficient (85% >50 nmol/L). Three subjects had levels below 30nmol/L but none were below 13nmol/L. According to NHMS 23% of Australian adults are vitamin D deficient but only 15% of adults aged 65 – 74 years are deficient (39). The mean serum 25OHD level in the subjects was slightly higher than average at 73nmol/L (SD 25). In the South Australian population 55 to 64 years and 65 to 74 years are 68.9 nmol/L (IQR 55.0, 81.0) and 69.8nmol/L (IQRI 56.4, 82.0) respectively. In South Australia, 15% of adults are vitamin D deficient in summer and the rate rises to 42% in winter (39).

Table 3.6 Serum 25OHD in the subjects of the current study and 25OHD levels reported for the Australian population (39)

Deficiency category 25OHD nmol/L	serum 25OHD Subjects (%)	Australian population (male and female) %	
		51 – 73 years	55 – 64 years 65 – 74 years
Sufficient ≥ 50	111 (85)	81.6	83.4
Mild deficiency 30 – 49	17 (13)	15.1	13.0
Moderate/severe <30	3 (2)	2.4	2.4
Total deficiency <50	20 (15)	17.5	15.4

Vitamin D status determined by serum 25OHD levels in the subjects of the current study are comparable to those reported by the NHMS (39) for the Australian population over the age of 55. Blood samples in the subjects for this study were collected as they were recruited throughout the year, therefore a distinction between winter and summer levels were not made.

3.4.2 Parathyroid hormone and its relationship to other analytes

The relationship of PTH to other analytes for the 129 subjects recruited for the study was examined using regression analysis and ANOVA (table 3.9).

Table 3.7. Relationship between baseline serum PTH and other analytes

Analyte	iCa	tCa	PO ₄	25OHD	ALP	CTX	Ca abs [#]	Ca:Cr [*]
Multiple R	0.063	0.069	0.109	0.077	0.109	0.303	0.147	0.109
R ²	0.004	0.005	0.012	0.006	0.012	0.092	0.022	0.012
Adjusted R ²	-0.004	-0.003	0.004	-0.002	0.004	0.085	0.014	0.004
SE	0.039	0.085	0.129	25.202	24.61	175.81	0.209	0.219
n	127	130	130	130	130	130	128	124
<u>ANOVA</u>								
F	0.505	0.604	1.553	0.769	1.528	12.918	2.796	1.483
P	0.478	0.439	0.215	0.382	0.219	0.004	0.097	0.226

[#] fractional Ca absorption; ^{*} urinary calcium excretion rate

Baseline serum PTH was significantly related to baseline CTX suggesting that a higher serum PTH may lead to a higher rate of bone turnover. No other measured analyte was related to serum PTH. PTH may be determined by dietary Ca intake and may not be explained by 25OHD status alone. It is possible that in those with low 25OHD, serum PTH may remain within the normal range if dietary Ca was adequate. The effects of PTH on other biochemical variables and the effect of Ca intake on PTH will be discussed in detail in Chapter 4.

3.5 Discussion

This is a cross sectional analysis of calcium intakes, prevalence of osteoporosis and biochemical parameters relating to calcium metabolism in a sample of community

dwelling post-menopausal women. Only 50% of eligible subjects enrolled in the study and 37% attended at 6 months.

The prevalence of osteoporosis and osteopenia of the subjects of the current study were comparable to those reported for healthy Australian older persons (over the age of 50) (299). A previous study (127) however reported higher rates of prevalence of osteoporosis in their subjects. In that study, all adults enrolled in the Commonwealth Electoral roll as a resident of the Geelong area fulfilled the criteria to participate. Therefore the women in the age categories of >50 years may have included residents of assisted-care living facilities, those with minimal mobility and those with chronic disease. This may have resulted in the higher prevalence of osteoporosis and osteopenia in their subjects compared to the reported prevalence for healthy Australian population reported by AIHW, where the prevalence was determined by general practitioner diagnosed osteoporosis in community dwelling otherwise healthy individuals, thus excluding people with chronic disease and other factors such as long term glucocorticoid treatment that may lead to osteoporosis (299). It is also relevant that this reported prevalence, while representative of 'doctor-diagnosed' osteoporosis, may underestimate the actual prevalence of osteoporosis in the community. This is because routine bone density screening for osteoporosis is not offered due to restricted funding by Health Insurance Commission (Medicare Benefit not available for screening of asymptomatic patients, and only available if there are signs and symptoms of conditions resulting in osteoporosis or if a minimal trauma fracture has occurred) (300) and diagnosis may only have been made in patients who may have suffered a minimal trauma fracture. The data of the current study however suggest that the prevalence of osteoporosis in this cohort is representative of that in the community dwelling healthy population.

Two questionnaires for calcium intakes were compared. Only 30% of the women met the RDI for calcium intake according to both questionnaires. However the more extensive online FFQ may have overestimated intakes while the SQ may have underestimated intakes because the correlation between the 2 was poor. Biochemistry of bone related analytes in fasting blood samples for all subjects were acceptable and those that fell outside the reference interval set by the laboratory were not clinically

significant. The percentage of subjects in the current study with various levels of vitamin D deficiency as estimated by serum 25OHD levels were comparable to those reported for healthy community dwelling Australians (39).

In this cross sectional data analysis, a significant relationship was seen between PTH and CTX. This by no means is a causative relationship but very probably is an association. It is likely that calcium intakes influence both CTX and PTH, and the expectation is that, both PTH and CTX will show an inverse relationship with calcium intake. The relationship between PTH, 25OHD and calcium intakes will be discussed further in chapter 4.

Chapter 4

Relationship between serum parathyroid hormone, vitamin D status and calcium intake

4.1 Introduction

Vitamin D has been recognised as an important factor in maintaining healthy bone. The position statement on vitamin D status in Australian and New Zealand adults suggests a serum 25OHD cut-point of 12.5nmol/L to define severe vitamin D deficiency. A cut-point of 13nmol/L has been used by the Australian Bureau of Statistics (ABS) in its National Health Measures Survey (NHMS) (2011-2012) because serum 25OHD levels are generally reported in whole numbers (39). Vitamin D deficiency and insufficiency are common in the community due to unavailability of vitamin D in food and the lack of exposure to sunlight. Pigmented skin types do not synthesise vitamin D in adequate amounts. These factors have led to vitamin D deficiency being recognised as a significant public health concern in Australia. Vitamin D deficiency or insufficiency has been reported in 31% of Australian adults (serum 25OHD levels <50 nmol/L). During winter, it is estimated that about 50% of women fall into the deficient or insufficient category (54).

Low serum 25OHD has been associated with a rise in serum PTH, such that the point at which the parathyroid hormone (PTH) starts to rise has been suggested as the cut-point for vitamin D insufficiency. Estimates of this inflection point has been reported as 50 nmol/L (301) and as high as 110 nmol/L (302). In South Australia, one study suggested a level of > 40 nmol/L to maintain bone health (303) while another demonstrated that in post-menopausal women, bone resorption markers rose significantly when serum 25OHD status fell below 60 nmol/L while PTH rose at a level of 50 nmol/L (304). The inflection point in serum 25OHD at which PTH rises may vary between populations due to the influence of calcium intake, although some argue that calcium intake does not influence serum PTH levels in subjects with a serum 25OHD above 25 nmol/L (305). A more recent study of post-menopausal women suggested that both serum 25OHD and calcium intake significantly influenced serum PTH levels, even at 25OHD levels below 19 nmol/L (125).

The current study aims to ascertain the influence of Ca intake on PTH, relationship between calcium intake and serum 25OHD, PTH and the bone resorption marker C-terminal telopeptide (CTX). The relationship between anthropometric measures and serum PTH is also explored.

4.2 Materials and Methods

Post-menopausal women from the community were recruited as described in Chapter 2. Data from post-menopausal women recruited for 3 other studies of shorter duration (these studies are presented in detail in chapters 7, 8 and 10) are also included here. The baseline biochemistry results obtained from all subjects from these studies were combined in this cross-sectional study. All women resided in the South Australian community. Women with chronic disease, on regular prescription medication, previously diagnosed bone disease and malignancy were excluded. A total of 201 women were included in this study.

Each woman completed a basic health questionnaire (appendix 3) and a calcium intake questionnaire (appendix 4). They were then invited to attend the Endocrine Test Unit at the Royal Adelaide Hospital for fasting blood tests, following a 8 to 10 hour fast. Blood was collected for biochemistry (electrolytes, creatinine, urea, liver function tests, calcium and phosphate), 25OHD, CTX and PTH. Calcium absorption was measured as detailed in Chapter 2.

Calcium intakes were calculated using the validated questionnaire (see Chapter 2.6 for validation study). When subjects were taking calcium supplements, the total intake (dietary and supplemental) was considered. The intakes were then divided into tertiles and within tertiles of intake, the subjects were further categorised according to serum 25OHD. The categories were serum 25OHD <30 nmol/L, 30 – 60 nmol/L and > 60 nmol/L. This provided an even distribution of subjects across the tertiles while retaining clinical significance. The serum variables between tertiles of calcium intake as well as for each level of 25OHD within tertiles were compared using a Student t test. Relationship between serum measures and calcium intake were explored using multiple regression and correlation using Analyse-It statistical package. $P < 0.05$ was considered statistically significant.

4.3 Results

Demographic and biochemistry data for all subjects are listed in table 4.1.

Table 4.1. Demographic and biochemistry data for all subjects (n=201).

	Mean	SD	SE	range	n
age (y)	61.4	5.3	0.4	50 – 75	201
Height (m)	1.6	0.06	0	1.48–1.84	173
Weight (kg)	68.3	16.7	1.3	45 – 210	172
BMI (kg/m ²)	25.9	5.7	0.3	17.8-70.1	172
Ca intake (mg/d)	1016	450.9	32	300 – 2589	198
total cholesterol (mmol/L)	5.5	1.0	0.8	2.6 – 8.5	189
glucose (mmol/L)	4.7	0.8	0.1	2.8 – 7.0	172
creatinine (umol/L)	62.4	10.3	0.7	38 – 110	197
ionised Ca (mmol/L)	1.22	0.04	0	1.10 – 1.40	194
Total Ca (mmol/L)	2.37	0.09	0	2.089– 2.68	197
PO ₄ (mmol/L)	1.15	0.13	0.1	0.87 – 1.46	196
ALP (U/L)	80.7	23.4	1.7	27 – 175	201
CTX (ng/L)	439.1	170.2	12	70 – 1064	200
PTH (pmol/L)	4.5	1.8	0.1	1.6 – 15.4	199
25OHD (nmol/L)	71.6	24.9	1.8	21 – 167	201

The range of values obtained for each analyte is given in table 4.1. The number of subjects with values outside the laboratory reference interval for each biochemical analyte is listed in table 4.2.

The results outside the RI for total cholesterol, fasting glucose, creatinine, ionised and total Ca, PO₄ and ALP were not clinically significant. More than half the subjects had serum CTX values above the desirable limit set by the laboratory. The current Australian consensus for CTX reference interval for post-menopausal women is 50 – 800 ng/L (264), and when these limits are used, only 3% of the subjects are above the upper limit.

Table 4.2. The number (percentage) of subjects outside the reference interval (RI) for measured analytes.

analyte	RI	< lower limit	> upper limit
Total cholesterol	<5.5 mmol/L	NA	87 (46)
Fasting glucose	3.3 – 5.5 mmol/L	4 (2)	24 (14)
Creatinine	50 - 100 umol/L	15 (7)	1 (0.5)
Ionised Ca	1.10-1.15 mmol/L	0 (0)	3 (1.5)
Total Ca	2.10–2.55 mmol/L	1 (0.5)	6 (3)
PO4	0.65-1.45 mmol/L	0 (0)	2 (1)
ALP	30-110 U/L	1 (0.5)	19 (9)
CTX	<400 ng/L	NA	112 (56)
PTH	0.8-5.5 pmol/L	0 (0)	36 (18)
25OHD	50 - 150 nmol/L	71 (36)	6 (3)

Eighteen percent of the subjects had a serum PTH above the upper limit of RI, but they were included in the study because they all demonstrated normal total and ionised calcium levels. The elevated PTH therefore may reflect secondary hyperparathyroidism in these patients. More than a third of the subjects were vitamin D deficient or insufficient according to the laboratory RI. However vitamin D status as defined by the National Health Measurement Survey (NHMS) (39) places 84% of the subjects as adequate vitamin D (Table 4.3).

Table 4.3. Serum 25OHD of the subjects as per NHMS (39).

Serum 25OHD (nmol/L)	Number	percentage
Adequate 25OHD status (≥ 50)	169	84%
Total deficiency (< 50)	33	16%
Mild deficiency (30 – 49)	28	14%
Moderate deficiency (13 – 29)	5	2%
Severe deficiency (<13)	0	0

The majority of subjects were vitamin D sufficient with only 16% mild to moderately deficient. The tertiles of calcium intakes with mean serum 25OHD and PTH are presented in table 4.4

Table 4.4. Tertiles of calcium intakes with mean serum 25OHD and PTH.

Tertile	Ca intake mean (SD) mg/d	25OHD mean (SD) nmol/L	PTH mean (SD) pmol/L
1	635.5 (28.5)	75.5 (27.3)	5.2 (2.3)
2	1116.2 (20.2)	74.2 (28.3)	4.2 (1.3)
3	1817.9 (51.1)	75.2 (22.9)	4.2 (1.6)

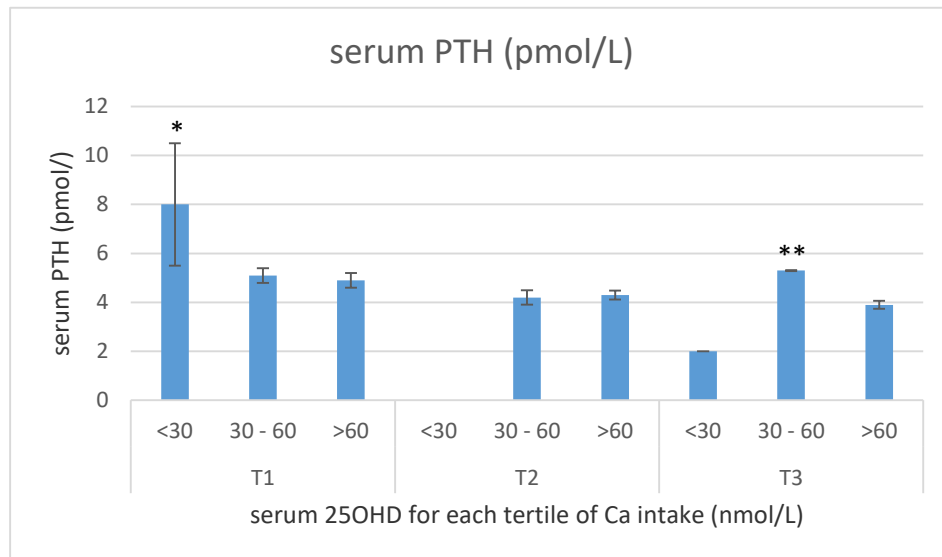
Cross sectional analysis demonstrates no significant difference in serum 25OHD or PTH between the tertiles of calcium intake. Within each tertile of calcium intake, subjects were further stratified into serum 25OHD levels of <30, 30 – 60 and >60 nmol/L. The number of subjects in each of these strata is listed in table 4.5.

Table 4.5. The number of subjects in each serum 25OHD category within tertiles of calcium intake.

tertile	Serum 25OHD (nmol/L)	number
T1	< 30	4
	30 – 60	24
	> 60	38
T2	< 30	0
	30 – 60	27
	> 60	39
T3	< 30	1
	30 – 60	15
	> 60	50

Women with the lowest Ca intake and serum 25OHD below 30 nmol/L demonstrated significantly higher PTH levels than those with 25OHD over 30 nmol/L within the same tertile (T1) ($P<0.01$) (Figure 4.1). Serum PTH for the women with 25OHD between 31 and 60 nmol/L in the highest Ca intake tertile (T3) was significantly

higher than PTH in those with 25OHD >60 nmol/L within the same tertile (P<0.01). Serum PTH for 25OHD 30-60 nmol/L in T3 was also significantly higher than serum PTH for 25OHD <30 nmol/L however there was only 1 subject with 25OHD <30 nmol/L in T3 making it difficult to conclude any physiological basis for this.



*= P < 0.05; ** = P < 0.01

Figure 4.1. Serum PTH level within each 25OHD bracket for each tertile of Ca intake.

Serum CTX for those in the lowest 25OHD bracket was significantly higher than for the other categories within the lowest Ca intake tertile (T1) (P<0.01) (Figure 4.2). This was not observed in tertiles of higher Ca intakes. There was no significant difference between ALP levels between the Ca intake tertiles or the 25OHD brackets.

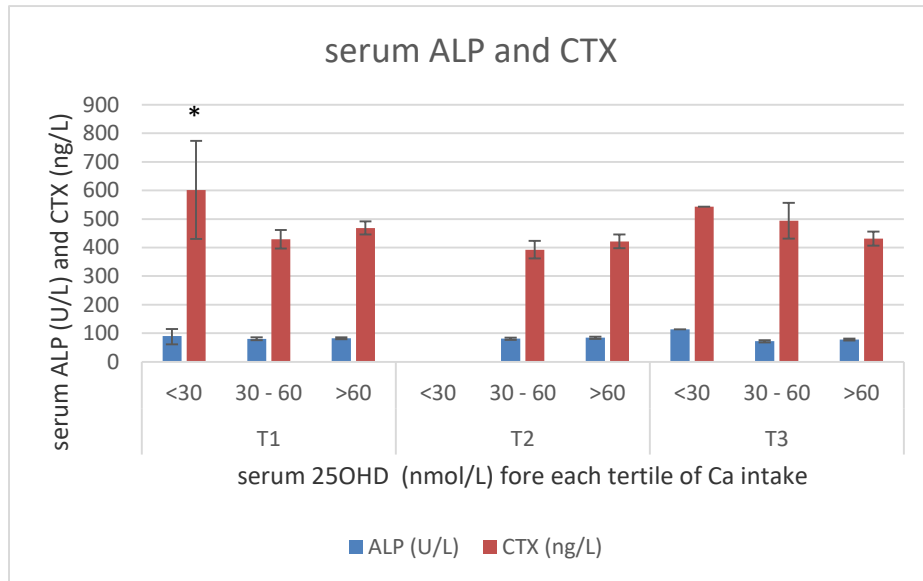


Figure 4.2. Serum CTX and ALP within each 25OHD bracket for each tertile of Ca intake.

Variables that showed a significant correlation between them on simple regression analyses are listed in table 4.5 together with the correlation coefficient. Serum PTH was positively related to age and BMI. Calcium intake declined with age in this group and lower BMI were observed with higher calcium intakes ($P=0.01$). The association between age and 25OHD was not significant. Calcium intake was positively related to serum total cholesterol, PO₄ level and serum 25OHD. Serum glucose was higher in heavier individuals as was expected as both greater body weight and higher fasting glucose are features of the metabolic syndrome. Fasting glucose also showed a significant negative relationship with serum 25OHD. Similarly, serum 25OHD was lower in women who were heavier. PTH was positively related to CTX and negatively related to 25OHD.

Table 4.5. Significant relationships as indicated by simple regression analyses (n = 202; r = correlation coefficient; 95 CI=95% confidence interval).

Pair of variables	r	95% CI	P
Age, Ca intake	-0.164	-0.327-0.009	0.05
Age, PTH	0.226	0.055-0.383	0.008
Ca intake, cholesterol	0.179	0.007-0.341	0.03
Ca intake, PO4	0.178	0.006-0.340	0.03
Ca intake, PTH	-0.19	-0.354- (-0.021)	0.02
Ca intake, 25OHD	0.238	0.068-0.394	0.005
Ca intake, BMI	-0.208	-0.368- (-0.037)	0.01
Glucose, 25OHD	-0.228	-0.385- (-0.057)	0.007
Glucose, weight	0.205	0.033-0.364	0.016
Glucose, BMI	0.260	0.091-0.414	0.002
CTX, PTH	0.182	0.010-0.344	0.03
PTH, 25OHD	-0.198	-0.359-(-0.027)	0.02
PTH, BMI	0.220	0.050-0.379	0.009
25OHD, weight	-0.213	-0.372- (-0.042)	0.01
25OHD, BMI	-0.235	-0.392- (-0.065)	0.005

Age, calcium intake, PTH, 25OHD, weight and BMI were not normally distributed (Kolmogorow-Smirnov test, P values 0.008, 0.0002, <0.0001, <0.0001, <0.0001 and <0.0001 respectively). Logarithmic transformation of these data was used to obtain Gaussian distributions. The adjusted correlation coefficients for the regression analyses between these values and their P values are listed in table 4.6.

Table 4.6. Adjusted correlation coefficient (r) between natural logarithm of variables and level of significance (P), ln - natural logarithm).

	ln age	ln PTH	ln 25OHD
ln Ca intake	0.003 (0.217)	-0.061 (0.0003)	0.004 (0.18)
ln PTH	0.058 (0.0004)		
ln 25OHD	-0.005 (0.941)	-0.02 (0.01)	
ln weight	0.020 (0.036)	0.015 (0.05)	-0.054 (0.001)
ln BMI	0.02 (0.01)	0.036 (0.007)	-0.054 (0.001)

Significant negative relationships were observed between Ca intake and PTH, and 25OHD and PTH. Weight and BMI rose significantly with age. PTH rose with weight and BMI and 25OHD level fell significantly with increasing weight. PTH increased with age.

CTX was found to be normally distributed. To explore the relationship between CTX and PTH, 25OHD, age, weight and BMI, multivariate Spearman's rank correlation was performed (table 4.7) which demonstrated a strong relationship between age and PTH, confirming logarithmic transformed data above. The inverse relationships between serum PTH and Ca intake, and the positive relationship between PTH and CTX were significant.

Table 4.7. Multivariate Spearman's rank correlation coefficient, r and significance (P)

	Age	Ca intake	CTX	PTH	25OHD
Age		-0.083 (0.246)	0.003 (0.967)	0.242 (0.0007)	0.041 (0.567)
Ca intake			-0.001 (0.987)	-0.197 (0.0059)	0.142 (0.0469)
CTX				0.203 (0.0043)	0.029 (0.684)

Multiple linear regression of serum PTH on age, BMI, calcium intake, CTX, 25OHD and calcium absorption (table 4.8) demonstrated significant positive relationships between serum PTH and age and BMI, when adjusted for calcium intake, 25OHD and calcium absorption. Age and BMI were the dominant determinants of PTH levels in this cohort although the relationship with calcium intake approached significance ($P=0.08$). Clearly the relationship between PTH and 25OHD was dependent on the effect of increasing weight but is lost when BMI is included in the regression presumably as a result of increased fat mass sequestering vitamin D (306).

Table 4.8. Determinants of serum parathyroid hormone (PTH)

		SE	<i>P</i>
PTH =	+0.0729 X age	0.029	0.01
	+0.0835 X BMI	0.034	0.01
	-0.0004 X Ca intake	0.000	0.08
	-0.003 X 25OHD	0.005	0.57
	+0.0157 X Ca absorption	0.654	0.81
	-1.505 pmol/L	-	-

$R^2 = 0.132$; adjusted $R^2 = 0.095$; $P < 0.005$; $n=125$.

Out of 202 subjects, only 48 (28%) met the recommended daily intake (RDI) of Ca of 1300 mg/d (9). There was no significant difference between the tertiles of Ca intake when the mean age, height, serum glucose, creatinine and ALP were compared. The analytes that showed a significant difference between at least 2 tertiles are listed with the difference between the means and the level of significance in table 4.9.

Table 4.9. Variables that showed a significant difference between at least 2 tertiles of Ca intake (difference between means and P values within brackets).

	T2 – T1	T3 – T1	T3 – T2
Weight (kg)	0.5 (0.43)	-4.7 (0.03)	-5.2 (0.05)
BMI (kg/m ²)	-0.41 (0.3)	-2.0 (0.01)	-1.7 (0.05)
Total cholesterol (mmol/L)	-0.39 (0.02)	0.24 (0.09)	-0.15 (0.19)
Ionised Ca (mmol/L)	0.01 (0.06)	-0.01 (0.01)	-0.0 (0.21)
Total Ca (mmol/L)	0.03 (0.02)	0.04 (0.01)	0.01 (0.37)
PO ₄ (mmol/L)	0.03 (0.12)	0.02 (0.04)	0.01 (0.29)
PTH (pmol/L)	-0.94 (0.002)	-0.96 (0.003)	-0.02 (0.47)
25OHD (nmol/L)	3.3 (0.22)	6.9 (0.04)	3.5 (0.19)
CTX (ng/L)	-53 (0.02)	-15 (0.31)	38 (0.10)

T1=lowest intake, T3=highest intake.

Total cholesterol was higher in the women in the middle tertile than the lowest. Serum total Ca tended to rise with increased dietary calcium intake, however ionised Ca appear to be greater in those with the lowest Ca intake. Likewise serum PO₄ was higher in women with the highest Ca intake. Serum CTX was highest in the lowest tertile of Ca intake. Serum 25OHD was highest in women with the highest intake of Ca.

Generally, women in the lowest and the middle tertile of Ca intake were heavier than those in the highest tertile. This is confirmed by correlation between variables for all subjects where there was a negative trend between calcium intake and weight ($r = -0.15$; $P=0.07$) and a significant negative relationship between calcium intake and BMI ($r=-0.2$; $P=0.01$) (Table 4.5).

4.4 Discussion

The majority of the women in this study did not meet the total calcium intake RDI of 1300mg/d for post-menopausal women. In contrast, the majority of the subjects were vitamin D sufficient, with levels above 50 nmol/L (84%). NHMS data demonstrated

that older adults were less likely to be vitamin D deficient than young adults (31% in those aged 18 – 34 years, and 15% in those aged 65 to 74 years) (39) likely due to more widespread use of supplements in the older age group. As depicted in figure 3.8 in Chapter 3, over 30% of women in this study were taking vitamin D with their calcium supplement. Some may have been taking vitamin D alone, and this may not be recorded because vitamin D intake was not specifically asked in the calcium intake questionnaire. The findings in our subjects with regards to vitamin D status are consistent with those of the NHMS (39).

Several statistical analyses were performed in the cross-sectional analysis to explore the relationship between biochemical variables associated with calcium metabolism and other parameters (weight, age, BMI). In order to reduce type II statistical errors, only relationships with a physiological basis or those with a P value <0.01 are discussed further.

The strong positive relationships between age and PTH and BMI and PTH are consistent with published data (125, 307,308). Lower than recommended calcium intakes appeared to be physiologically significant as indicated by the inverse relationship with PTH. However the major determinants of PTH in this group of women were age and BMI. Calcium intake was inversely related to PTH when the effects of age and BMI were adjusted for, but this relationship was not significant (P=0.8). PTH was not related to intestinal calcium absorption in this group of women, confirming previously published data on post-menopausal women (304).

While serum total Ca rose with increasing dietary calcium, ionised Ca appeared be significantly higher in the women with the lowest calcium intake compared to those with the highest intake. This may be because of other factors included in the calculation of ionised calcium (albumin, globulin, bicarbonate and anion gap) being different between these 2 groups. There is also evidence to suggest that ionised Ca is a more sensitive and earlier marker of hyperparathyroidism than total Ca (309). Therefore in women with lower calcium intakes, the high ionised Ca may be an early marker of hyperparathyroidism. Serum PTH in these women may be within the

laboratory reference interval, but may be physiologically higher than the optimal level for that individual.

Importantly however, within each tertile of Ca intake, PTH and CTX tended to be higher in those with lower vitamin D. The serum PTH level in women with vitamin D deficiency (<30 nmol/L) within the lowest tertile of Ca intake was significantly higher than in women with sufficient vitamin D status. Even within the highest Ca intake tertile, those with insufficient vitamin D status (30 – 60 nmol/L) had a significantly higher PTH level than those with sufficient vitamin D. This confirms the significant inverse relationship between PTH and 25OHD, as reported by other studies (304, 307,310). These findings support the concept that serum PTH is determined by both Ca intake and vitamin D status, and that, the effect of low 25OHD level exerted on PTH is more important than that of inadequate calcium intake. Serum CTX only rose in women with vitamin D deficiency if their Ca intakes were in the lowest tertile, suggesting that bone resorption is highest when both calcium intake and serum 25OHD are low. Serum 25OHD declined as body weight increased, confirming that 25OHD is sequestered in adipocytes (306).

Calcium intake appeared to be positively associated with serum 25OHD, which was also reported in previously with dietary calcium intake (306) and with calcium supplements (48). With increasing dietary calcium, a higher serum 25OHD has been attributed to a concomitant increase in vitamin D in the diet (306). With calcium supplementation, the rise in serum 25OHD was attributed to lower serum PTH and thus a lower rate of catabolism of 25OHD (48).

Women with lower Ca intakes tended to be heavier than those with higher Ca intakes, perhaps reflecting a poorer diet high in energy or a less healthy lifestyle. The major dietary component containing calcium amongst these women was dairy products and therefore these data do not provide any evidence that consuming dairy products increases the risk of increasing BMI (see Chapter 9 for relationship between visceral fat and calcium intake). It has been suggested that higher calcium intakes will lead to lipolysis (132) and higher dietary calcium may reduce the absorption of fat in the gut (311). The inverse relationship between serum 25OHD and fasting

glucose suggests that low 25OHD may be a risk factor for metabolic syndrome (312,313,314). This relationship has been reported previously in postmenopausal women, with fasting glucose rising steadily when serum 25OHD fell below 40 nmol/L (313). There is evidence that serum 25OHD may be inversely related to the prevalence of insulin resistance, hyperglycaemia (314,315) and diabetes (316). This phenomenon may be explained by beta cell dysfunction in hypovitaminosis D (317). In addition a higher BMI may be associated with a higher fasting glucose and a lower 25OHD.

Body weight, BMI and age were important determinants of PTH when adjusted for calcium intake, calcium absorption and 25OHD. Some report the rise in PTH with age to be independent of 25OHD economy or renal function (308) while others suggest that, when adjusted for body weight and 25OHD, there is little effect of age on PTH (304). Others suggest dissociation between the calcemic action and phosphaturic effect of PTH in older adults leading to a gradual rise in intact circulating PTH with age (318). Another hypothesis suggests a reduced calcemic response to PTH at the bone in older adults (319). These hypotheses have been suggested to explain the relatively normal Ca levels in the elderly subjects with higher basal and maximal PTH values in response to induced hypocalcaemia suggesting a 'PTH resistance' state.

The fall in 25OHD with increasing weight has been suggested as a cause of increasing PTH with weight (319) however later studies (307) and in the current study weight is a significant determinant of PTH after adjusting for serum 25OHD. There is evidence to suggest that higher levels of PTH may be associated with low grade inflammation and adipose tissue in patients with primary hyperparathyroidism demonstrated upregulation of inflammatory genes (320). Higher circulating proinflammatory cytokines were observed in rodents with induced secondary hyperparathyroidism (321) and in obese humans, serum PTH levels correlated with CRP levels and triglyceride:HDL ratio independent of serum 25OHD levels (322). NHANES data demonstrate a clear linear rise in CRP with rising PTH in US adults (323). These findings suggest a close relationship between adiposity, PTH and inflammation. In addition, PTH was shown to be an independent predictor of

metabolic syndrome, regardless of vitamin D status (324). It is likely that adipocytes play an inflammatory role due to their ability to produce proinflammatory cytokines, however the association between inflammation and PTH is, currently just that, with no clear explanation of the physiological or pathological role of PTH in the inflammatory process. Nevertheless, data from the current study and others as mentioned strongly suggest that PTH rises with age and with increasing weight.

Although calcium intake and absorption are physiologically important factors affecting PTH, in this group of post-menopausal women, age and body weight were more important determinants of PTH. The rise in 25OHD and lower body weight with higher calcium intake may reflect a healthier lifestyle in these women. The higher fasting glucose with lower 25OHD may reflect a less active lifestyle and suboptimal nutritional choices. Furthermore, total calcium intake may have a significant effect on 25OHD and energy metabolism.

The variable results in some of the studies in PTH may be due to the variability in assays and the fragments detected. Some second generation assays available now detect the intact PTH (iPTH, 1-84) using 2 antibodies that target epitopes in the NH₂-terminal (amino acids 1 to 7) and the mid-region (amino acids 44 to 65). Therefore it can be argued that they may detect fragments of PTH that have lost the –COOH terminal beyond the 65th amino acid, and may not only measure iPTH (318, 325). This may result in higher PTH measurements when these fragments are also added to the result. The physiological significance of these fragments that lack the –COOH terminal is yet to be determined.

Given the demonstrated associations between age and PTH and body weight and PTH, and the evidence for the positive linear relationship between PTH and proinflammatory markers, further investigations of the mechanism of PTH involvement in inflammation and the effect of suppression of PTH with vitamin D are needed. Modulating PTH may be a new method in the prevention of metabolic syndrome and obesity. Additionally, it may be concluded that the traditionally accepted simple relationship between calcium intake and PTH need to be revised to include age and body weight.

Chapter 5

Changes in bone densities over time and relationship to calcium intake

5.1 Introduction

Post-menopausal women are said to be in negative calcium balance (96) with a higher rate of bone resorption to maintain physiological levels of Ca (99) resulting in higher requirements for calcium in order to prevent bone loss. A slower rate of bone resorption has been demonstrated in post-menopausal women with high calcium intakes compared to those with lower intakes (100,101,102,103,104)). A review of clinical trials shows that, with calcium supplementation, postmenopausal bone loss can be abrogated (105).

Longitudinal observational studies have demonstrated that increased dietary calcium can lead to improvements in bone density (117,126) or prevention of age related bone loss as assessed by bone density (144).

The current study was designed to ascertain if changes in bone density in postmenopausal women over a 2 year period were determined by calcium intake.

5.2 Materials and Methods

Community dwelling post-menopausal women were recruited to the current study as described in Chapter 2. Aerial bone density measurements (BMD) were made using a Norland (Cooper Surgical Company) densitometer. Measurements were obtained at the lumbar spine (L1 – L4), femoral neck, total or standard hip, proximal forearm and ultradistal forearm (33%) at baseline and at 6 monthly intervals. The Ca intake questionnaire was completed again at 12 months to ensure that the subjects have not changed their diet significantly during the study. The subjects who were on Ca or vitamin D supplements were allowed to continue, and the amount of Ca from supplements were added to the total dietary intake.

Correlation between baseline bone density for each site and Ca intake was examined using a correlation matrix. Calcium intakes were divided to tertiles, and the mean change in BMD for each measured site within each tertile of Ca intake was calculated by subtracting the mean density at the final measurement from the mean baseline measurement. The change in mean BMD for each site within each tertile of Ca intake at each time point was calculated by subtracting the latter from the baseline values. All changes were compared between each site and between tertiles using Student's t tests.

5.3 Results

5.3.1 Completion of bone density scans by subjects

Table 5.1 shows the number of forearm and hip/spine bone density scans completed by subjects. Most subjects completed all 5 hip/spine scans. Forearm scanning was added to the protocol later, when some of the subjects had already had the hip and spine scans.

Table 5.1. The number of subjects completing various combinations of hip/spine and forearm scans.

Hip/spine	Forearm	number of subjects
5	5	33
5	4	45
4	4	6
5	3	10
3 or 4	3	8
1 or 2	1 or 2	24

5.3.2 Relationship between habitual calcium intake and baseline bone density

Baseline calcium intake was divided into tertiles as listed in table 5.2. Included in this table are also the age, weight and BMI data for the subjects within each tertile.

Table 5.2. Tertiles of calcium intakes, with mean (SD) of calcium intakes, age, weight and BMI for subjects in each tertile

tertile	Ca intake (mg)	Age (years)	Weight (kg)	BMI (kg/m ²)
1	653.5 (28.5)	61 (4.6)	68.2 (13.5)	25.9 (4.8)
2	1116.2 (20.0)	60.9 (5.1)	60.9 (5.1)	25.3 (3.8)
3	1817.9 (51.1)	60.6 (5.4)	60.6 (5.4)	24.4 (3.8)

There was no significant difference between the mean age of subjects between the tertiles. However subjects in the lowest tertile of Ca intake were heavier than those in the highest tertile of Ca intake. The mean (SD) of bone density for each measured site in each tertile are listed in table 5.3.

Table 5.3. Mean g/cm² (SD) bone density for each site in each tertile of calcium intake

tertile	LS	FN	TH	PFA	UDFA
1	1.0589 (0.2055)	0.8101 (0.1425)	0.8873 (0.1425)	0.6902 (0.1051)	0.3448 (0.0607)
2	1.0542 (0.2244)	0.8116 (0.1265)	0.8837 (0.1309)	0.6847 (0.0994)	0.3285 (0.0706)
3	1.0027 (0.1641)	0.8051 (0.1126)	0.8696 (0.1150)	0.6783 (0.0892)	0.3133 (0.0417)

Student t tests indicate that the only anatomical site that showed a significant difference between tertiles at baseline is the ultradistal forearm, where the subjects with the lowest tertile of calcium intake had a higher bone density than the highest tertile (P=0.05).

Cross-sectional analysis using a correlation matrix for baseline bone density at each site and baseline calcium intakes is presented in table 5.4. The ultradistal and proximal forearm bone densities were inversely related to age (P<0.0005 and P<0.005 respectively). Height was positively correlated to femoral neck and ultradistal forearm bone density (P<0.005). Weight and BMI were positively correlated to lumbar spine,

femoral neck, hip, proximal forearm and ultradistal forearm densities (all $P < 0.0005$ except BMI and femoral neck BMD, $P < 0.025$). Bone density in each site was also significantly correlated to other sites ($P < 0.0005$). Dietary calcium (but not total calcium) intake appeared to decline with age ($P < 0.01$). Only the femoral neck BMD correlated with calcium intake (dietary calcium, not total calcium, $P < 0.01$).

When corrected for age, calcium intake and serum 25OHD, BMI was significantly positively associated with the baseline bone density at all sites (lumbar spine $P = 0.0002$; femoral neck $P = 0.01$; total hip $P = 0.0003$; proximal forearm $P = 0.04$; ultradistal forearm $P = 0.004$). When corrected for BMI, calcium intake and serum 25OHD, the ultradistal forearm is the only site where baseline bone density was negatively associated with age ($P = 0.001$).

Table 5.4. Correlation matrix of measured anthropometric variables, calcium intake and bone density

	<i>age</i>	<i>height</i>	<i>weight</i>	<i>BMI</i>	<i>LS BMD</i>	<i>FN BMD</i>	<i>Hip BMD</i>	<i>PFA BMD</i>	<i>UDFA BMD</i>	<i>Ca intake</i>
<i>age</i>	1									
<i>height</i>	-0.12465	1								
<i>weight</i>	-0.08139	0.343543	1							
<i>BMI</i>	-0.03298	-0.05915	0.915033	1						
<i>LS BMD</i>	-0.09049	0.113009	0.352031	0.32135	1					
<i>FN BMD</i>	-0.13992	0.285006	0.31425	0.20761	0.503959	1				
<i>Hip BMD</i>	-0.14755	0.147364	0.375026	0.331238	0.56337	0.885372	1			
<i>PFA BMD</i>	-0.24447	0.149005	0.362634	0.340032	0.529071	0.516451	0.567288	1		
<i>UDFA BMD</i>	-0.42856	0.257557	0.44202	0.368392	0.639708	0.699723	0.739592	0.722873	1	
<i>Ca intake</i>	-0.02233	-0.03639	-0.13479	-0.12795	-0.06607	0.026116	-0.00472	-0.06606	-0.150306552	1

Figure 6.3. Correlation matrix representing the correlation between biometric measures, baseline bone densities and baseline calcium intake. Levels of significance:

P<0.05
P<0.025
P<0.01
P<0.005
P<0.0005

5.3.3 The effect of estimated baseline calcium intake on changes in bone density over 2 years

5.3.3.1 Changes in the lumbar spine bone density

The mean change in the lumbar spine density at 6 months, 12 months, 18 months and 24 months from baseline values were calculated for subjects in each of the tertiles of calcium intake. These data are graphed in figure 5.6. Lumbar spine densities were lower at every time point measured, but were not statistically significant from baseline values. The changes were also not statistically significant between tertiles of calcium intake within each time point.

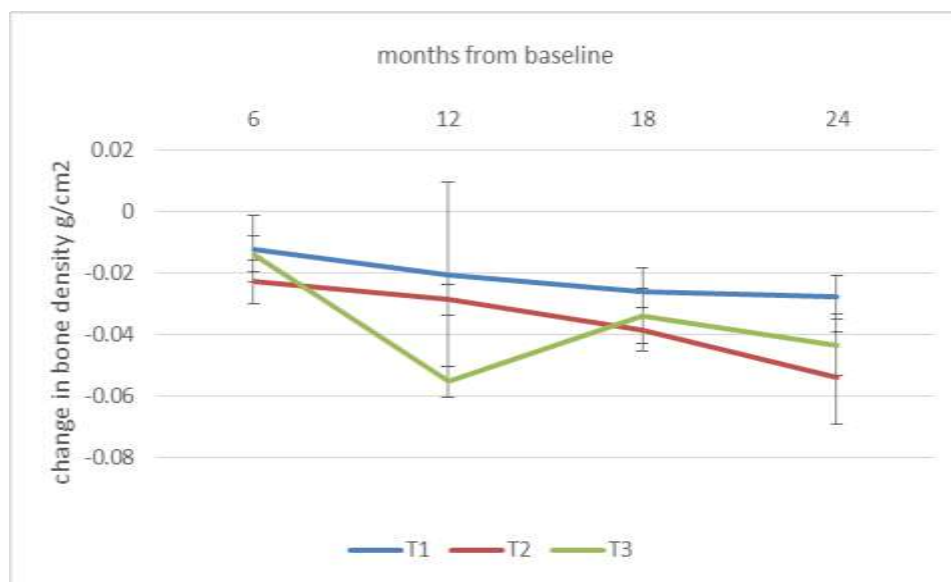


Figure 5.6. Change in lumbar spine bone density over time in each tertile of calcium intake. T1 = lowest tertile, T2 = middle tertile, T3 = highest tertile of calcium intake

5.3.3.2 Changes in the femoral neck bone density

The baseline femoral neck bone density between the 3 tertiles of Ca intake were not statistically different. The mean change in bone density at the femoral neck at each time point after baseline, for each tertile of calcium intake is shown in figure 5.7. Except at 6 months for tertile 1, femoral neck density decreased at each measured time point compared to baseline for all tertiles of calcium intake. There was no

significant difference between the tertiles when the changes from baseline were compared.

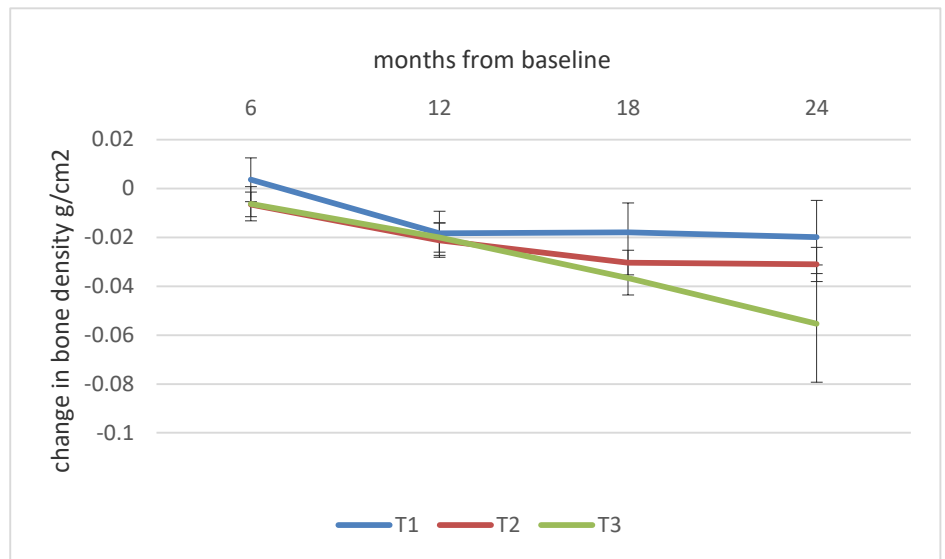


Figure 5.7. Changes in the femoral neck bone density over time within each tertile of calcium intake. T1 = lowest tertile, T2 = middle tertile, T3 = highest tertile of calcium intake

5.3.3.3 Changes in the total hip bone density

There was no significant difference in baseline total hip densities between tertiles. Total hip bone density decreased after baseline on all measurements in all 3 tertiles and there was no significant difference between the rates of decrease between the tertiles of calcium intakes (figure 5.8).

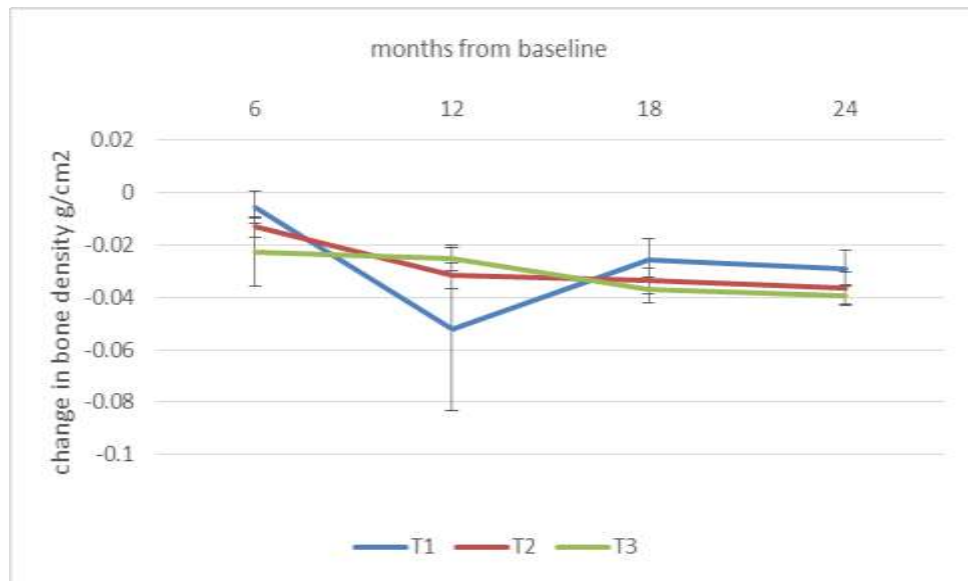


Figure 5.8. Changes in the total hip bone density over time within each tertile of calcium intake. T1 = lowest tertile, T2 = middle tertile, T3 = highest tertile of calcium intake

5.3.3.4 Changes in the proximal forearm bone density

The baseline proximal forearm densities were not different between the tertiles. Bone density declined in all tertiles in the proximal forearm and was lower than baseline at all time points. The rate of change was not significantly different between the tertiles (figure 5.9).

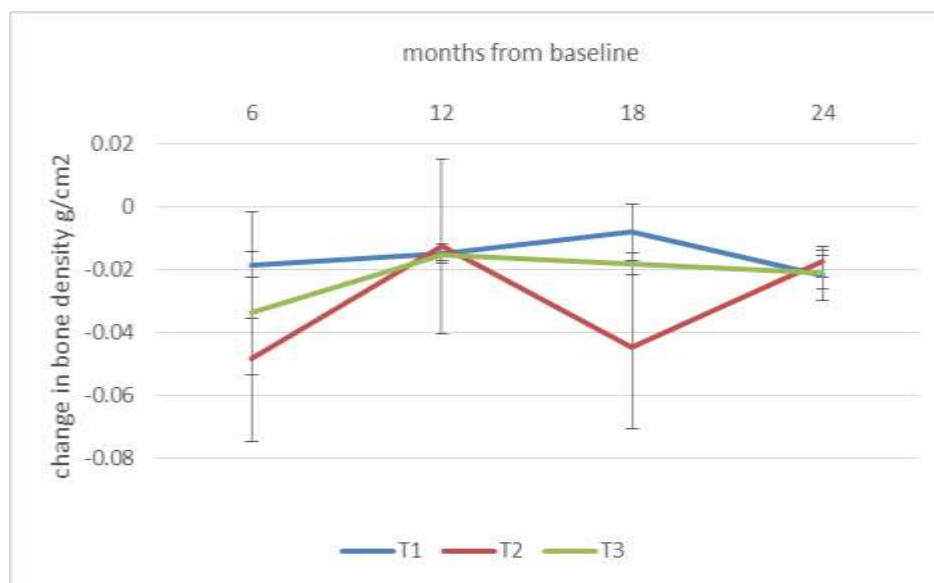


Figure 5.9. Change in bone density in the proximal forearm in the 3 tertiles of calcium intake at each time point. T1 – lowest tertile, T2 – middle tertile, T3 – highest tertile.

5.3.3.5 Changes in the ultradistal forearm bone density

The difference between the baseline ultradistal forearm (UDFA) densities between the tertiles was not significant. At 6 months, the mean UDFA density appear to rise in the middle tertile. All other measurements were lower than baseline. The rate of fall in UDFA bone density was significantly greater in the first tertile compared to the third tertile at 12 months and at 24 months.

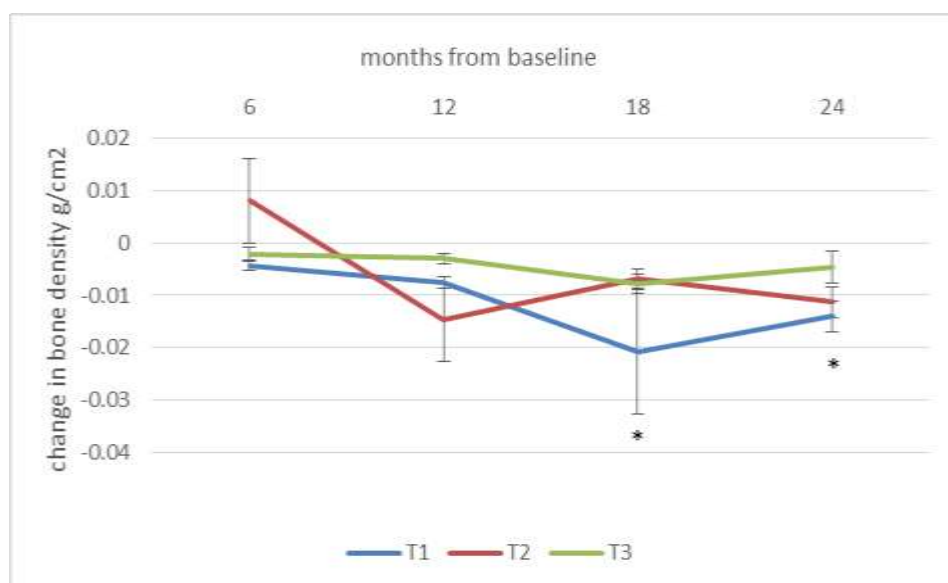


Figure 5.10. Changes in the ultradistal forearm bone density in each tertile of calcium intake at each time point. (T1 = lowest tertile, T2= middle tertile, T3 = highest tertile); * P<0.05

5.3.4 Calcium intakes and annual percentage loss of bone

Table 6.5 lists the annual percentage bone loss for each site for each tertile of calcium intake. The only significant difference between the rates of losses was between T1 and T2 in the ultradistal forearm where the rate of loss was greater in the lowest calcium intake group (P=0.01).

Table 5.5. Mean (SD) and SE annual percent loss of bone at each site within each tertile

site	T1	T2	T3
	mean (SD), SE	mean (SD), SE	mean (SD), SE
LS	-1.7 (3.8), 0.6	-2.1 (3.5), 0.5	-1.8 (4.0), 0.6
FN	-1.7 (4.0), 0.6	-1.9 (3.1), 0.4	-1.7 (2.6), 0.4
TH	-1.5 (2.8), 0.4	-2.2 (2.5), 0.4	-2.2 (1.5), 0.3
PFA	-1.9 (1.8), 0.3	-1.7 (1.9), 0.3	-1.9 (1.9), 0.3
UDFA	-2.3 (2.5), 0.4	-1.1 (2.6), 0.4	-1.7 (2.6), 0.4

LS=lumbar spine, FN=femoral neck, TH= total hip, PFA= proximal forearm, UDFA=ultradistal forearm.

There was no significant difference in 25OHD status between tertiles of calcium intake. Vitamin D status in the subjects is discussed in detail Chapter 4. In tertiles 1 and 3, there was at least one subject with vitamin D deficiency. Vitamin D deficiency was most prevalent in the lowest tertile of calcium intake. When annual percentage loss of bone density for each site was regressed against serum 25OHD within each tertile of calcium intake, only the rate of loss at the femoral neck and total hip in the middle tertile of calcium intake were significantly related to 25OHD (P=0.03 and P=0.02 respectively).

There were 53 women who met the RDI for calcium intake with levels >1200mg/d and completed bone density measurements. Ten of these women had a serum 25OHD of <60 nmol/L. The effect of vitamin D on the rate of loss was most pronounced at the lumbar spine (P=0.04) and femoral neck (P=0.009) in this group. Within this group the percentage of women who gained bone (had a positive rate of annual bone ‘loss’) for each site during the 2 years of observation are shown in table 6.5.

Table 5.6. Percentage of women who gained bone when RDI for calcium intake was met, according to 25OHD status

Site /25OHD status	<60 nmol/L	> 60 nmol/L
LS	2	28
FN	1	30
TH	1	23
PFA	0	19
UDFA	2	21

LS=lumbar spine, FN=femoral neck, TH=total hip, PFA= proximal forearm, UDFA=ultradistal forearm

More women gained bone when calcium intake met RDI, and serum 25OHD level was >60 nmol/L, than when RDI for calcium was met but serum 25OHD was insufficient (<60 nmol/L). A level of 60nmol/L was chosen for serum 25OHD used as a categorical variable because at the time of analysis the laboratory reference

interval was 60 to 160 nmol/L. Other cut offs were not compared prior to this subgroup analysis. The rate of loss was significant at the femoral neck and the total hip in the women who met RDI for calcium but were vitamin D insufficient (P=0.05 and 0.03 respectively).

When corrected for age, calcium intake and serum 25OHD, BMI demonstrated a strong positive relationship with all measured sites (lumbar spine P = 0.0002, femoral neck P = 0.01, total hip P = 0.0003, proximal forearm P = 0.04 and ultradistal forearm P = 0.004). Only the ultradistal forearm showed a positive relationship to age when corrected for BMI, calcium intake and 25OHD (P = 0.001).

5.4 Discussion and Conclusions

There was no significant difference in mean baseline bone densities between the tertiles at any site. The rate of fall in the lumbar spine, femoral neck, total hip and proximal forearm were not significantly different between the tertiles at any of the time points measured. UDFA density fell significantly at the 6, 12 and 24 month measurements in the lowest tertile of calcium intake compared to the highest tertile of intake. However the strongest relationship between calcium intake and loss of bone was observed in the highest tertile of calcium intake at this site.

The baseline bone density at any of the sites measured did not show a relationship to baseline habitual calcium intake. This may be because there were factors other than calcium intake affecting bone density such as vitamin D status, exercise, body weight and genetic factors. In fact, the women with the lowest calcium intake were heavier as discussed in Chapter 4, and strong positive relationships were observed between BMI and bone density at all sites. Therefore the positive impact of body weight or BMI on bone density in women with lower calcium intakes may have mitigated the negative effects of low calcium intakes to some extent. It is also possible that the estimated calcium intakes may not accurately reflect the actual usual calcium intake in these women.

The mean serum 25OHD level did not differ significantly between tertiles of calcium intake however vitamin D deficiency was most prevalent in the lowest tertile of calcium intake. This may be due to the fact that those in the higher tertiles of calcium intake were taking calcium supplements that included vitamin D. The women in the

lowest tertile of intake were also heavier, suggesting that greater amounts of adipose tissue may have sequestered 25OHD and reduced the circulating 25OHD levels (306). Although there were more subjects with vitamin D deficiency in the lowest tertile, their rate of bone loss at the lumbar spine and hip did not differ significantly from those in the other tertiles. Those in the middle tertile of calcium intake demonstrated the strongest relationship between rate of bone loss at the hip and serum 25OHD. It may be that a higher intake of calcium (tertile 3, levels above 1800mg/d) protects against effects of low vitamin D. These data suggest that, in addition to meeting the RDI for calcium, vitamin D sufficiency (serum levels >60 nmol/L) is required to gain bone at the lumbar spine, hip and forearm, and to significantly reduce the rate of bone loss (at least at the femoral neck and total hip).

Only the rate of bone loss at the ultradistal forearm was significantly greater in the lowest tertile of calcium intake compared to the highest tertile. This may be due to the greater proportion of trabecular bone at this site (326) and the resultant higher metabolic rate, responding to calcium intake and vitamin D status during the measurement intervals. However the bone density at this site was not lower in the subjects taking the lowest amount of calcium at baseline. This may be because, after completing the calcium intake questionnaire, the subjects did not change their diet significantly during the study, whereas the diet prior to recruitment may have not been reflected accurately in the estimate of calcium intake. When calcium intake was adequate then the rate of loss at the UDFA was strongly related to the amount of calcium ingested. The International Society of Clinical Densitometrists Adult Official Position Statement does not recommend using forearm measurements other than the 33% radius (1/3 radius, proximal forearm) (327), possibly due to the rapid response to changes in more distal parts of the radius.

Chapter 6

The influence of bone densitometry result feedback on habitual calcium intakes

6.1 Introduction

Osteoporosis is the most prevalent metabolic bone disease in the developed world, placing considerable health, economic and social burden on society. In addition to the impact of morbidity of fragility fractures, the risk of re-fracturing following an osteoporotic fracture rises, also increasing mortality (8). Therefore both primary and secondary prevention of osteoporosis are important aspects of public health. Increasing calcium intake and maintaining adequate vitamin D have been proven to prevent bone loss and reduce fracture risk. There are several community based campaigns that promote increased dietary calcium.

Despite public education campaigns drawing attention to the increased requirement of Ca (38), studies show that the Ca intake in postmenopausal women falls well short of the recommended daily intake (RDI) (103,127,328) and adherence to Ca supplements is poor (165). There is evidence that, when given feedback on their calcium intake (295) or fracture risk and bone density result (329,330) women increased their Ca intake.

The following study aims to investigate the influence of DXA results on the self-reported calcium intake in community dwelling postmenopausal women in South Australia.

6.2 Materials and Methods

Community dwelling healthy postmenopausal women with no history of bone disease or other chronic disease were invited to participate as discussed in detail in Chapter 2. Bone density measurements at the lumbar spine (L1 to L4), total hip, femoral neck, proximal and ultradistal forearm were obtained using a Norland (USA) scanner. Copies of results were provided to the subjects and a verbal explanation of results, including the diagnosis (osteoporosis, osteopenia or normal bone density for each site) (208) and the fracture risk were given to each subject by a certified clinical

densitometrist. The calcium questionnaire was administered again 12 months following recruitment. Feedback on calcium intakes were not given to the subjects. Daily calcium intakes were calculated from the baseline and 12 month questionnaires. A change in calcium intake of 50mg or more a day was considered to be significant.

6.3 Results:

Out of 175 women eligible, 129 women completed the calcium intake questionnaires and the bone density measurements. The dietary questionnaire was completed by 112 subjects 12 months later. The mean age, BMI and years since menopause were 61 years, 25 and 10 years respectively. Demographic data is given in table 6.1.

Table 6.1 Demographic data

Variable	mean	SD	range
Age (y)	61	5	51 – 73
Height (m)	1.62	0.06	1.48 – 1.81
Weight (kg)	66	12	45 – 108
BMI kg/m ²	25.2	4.2	17.8 – 42.2

The baseline total Ca intake was 1193mg/day and 40% were on Ca supplements. Only 45% met their daily recommended Ca intake of 1300mg. These data are presented in figure 6.1. Ca intakes at baseline and after 12 months are recorded in table 6.2. The number and percentage of women meeting the RDI for calcium at baseline and at 12 months, with dietary sources alone and with supplementation are depicted in figure 6.1. The mean total Ca intakes do not equal the mean dietary and the mean supplemental intakes because the mean values for these 3 categories were calculated separately.

Table 6.2. Calcium intakes at baseline and at 12 months

Ca mg/d	mean	SD	range
Baseline total	1193	508	300 – 2581
Baseline dietary	666	305	300 – 1790
Baseline supplemental	584	313	17 – 1200
12 months total intake	1102	500	300 – 2845
12 months dietary Ca	860	295	300 – 1719
12 months supplemental Ca	609	307	70 – 1200

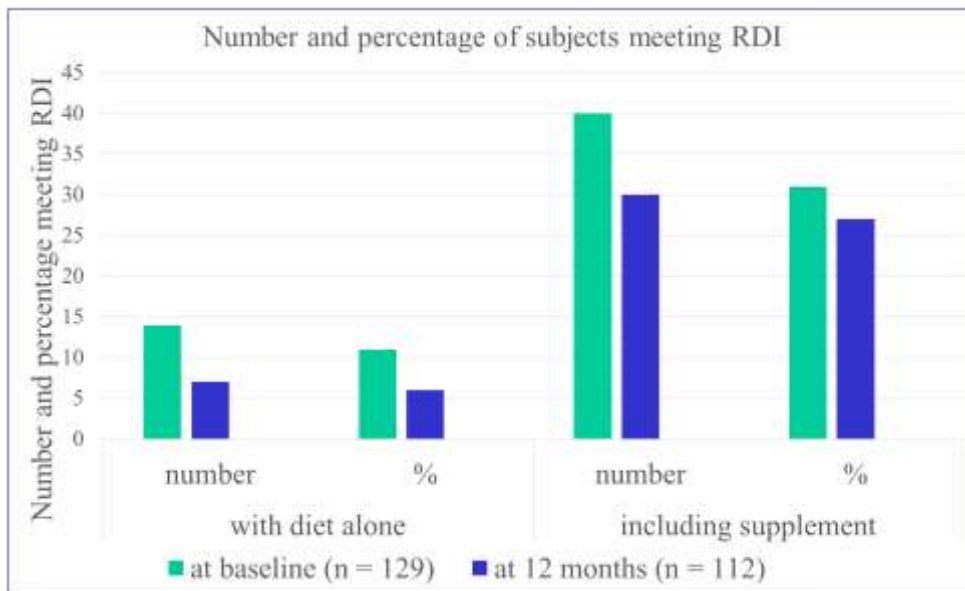


Figure 6.1. Number and percentage of women meeting the recommended daily intake (RDI) of calcium with and without supplements, at baseline and 12 months after bone density scanning.

The supplement types taken by subjects and baseline and 12 months are listed in table 6.3.

Table 6.3. Types of supplements taken by subjects at baseline and 12 months.

Supplement type	baseline (%)	12 months (%)
Carbonate	31 (61)	30 (67)
Citrate	8 (16)	8 (18)
Citrate + hydroxyapatite	4 (8)	2 (5)
Hydroxyapatite	2 (4)	1 (2)
PO4 + citrate	1 (2)	2 (5)
Oxide + hydroxide	1 (2)	1 (2)
PO4	1 (2)	1 (2)
Unknown	3 (6)	0 (0)
Total	51 (100)	45 (100)

Fifteen women had osteoporosis in at least one measured site (11.6%), while 60 (46.5%) women had osteoporosis or osteopenia in at least one site. Normal bone density for all measured sites were reported in 69 (53.5%) of women. The prevalence of osteoporosis and osteopenia in this group was lower than that reported in our community (see table 6.4) (127).

Table 6.4. Prevalence of osteoporosis and osteopenia at each measured site

Number (%)	LS	FN	TH	PFA	UDFA
Osteoporosis	11 (8.5)	7 (5.4)	4 (3)	1 (2)	0 (0)
Osteopenia	18 (14)	40 (31)	19 (15)	8 (17)	4 (8)
Normal	100 (77.5)	82 (63.5)	106 (82)	39 (81)	44 (92)
Total	129	129	129	48	48
Osteoporosis (GOS)	13%	13%	13%		
Osteopenia (GOS)	49%	49%	49%		

LS = lumbar spine (L1 – L4), FN = femoral neck; TH = total hip; PFA = proximal forearm, UDFA = ultradistal forearm. Reported prevalence quoted here are from the Geelong Osteoporosis Study (127).

The changes in calcium intakes between the baseline estimates and the estimates at the end of 12 months were calculated for each subject in terms of total intakes, dietary sources and supplements in order to ascertain if subjects increased their Ca

intake overall and if they did, whether it was by a higher intake of dietary sources or by increasing Ca supplement intake. Figure 6.2 shows the changes in supplements, dietary sources and total intakes in subjects.

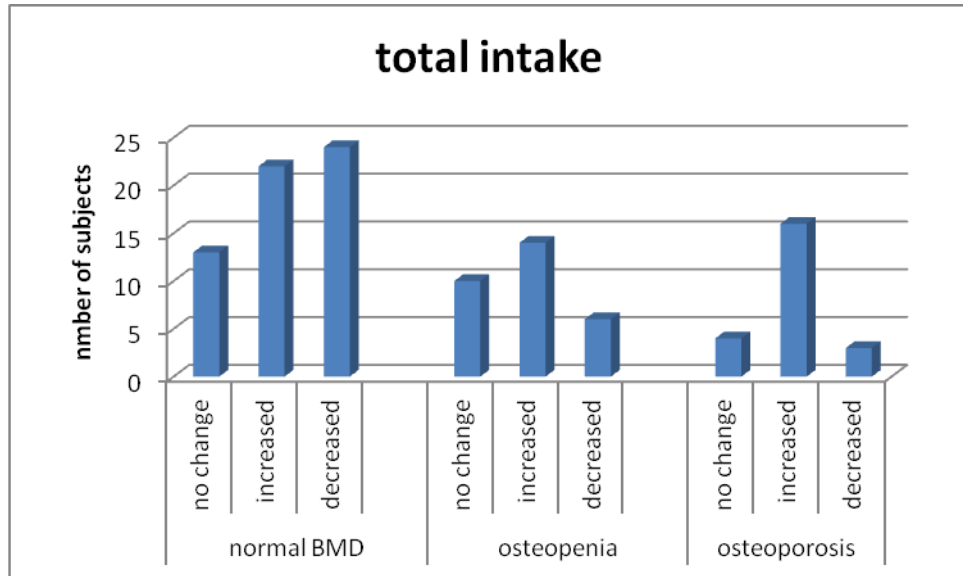


Figure 6.2. Change in total calcium intake 12 months after feedback on baseline bone density scan and fracture risk (change of at least 50mg/day)

Overall there was no significant difference between the baseline and 12 month total Ca intake. Total Ca intake increased by at least 50mg a day in 46% and 35% of women diagnosed with osteoporosis and osteopenia respectively. However 23% and 40% diagnosed with osteoporosis and osteopenia decreased their total Ca intake.

There was no significant change in supplement intake after bone density feedback, regardless of the bone density result. However 10 women decreased their supplemental Ca when normal bone density was reported. Most women who were given the diagnosis of osteoporosis and increased their calcium intake did so by increasing dietary sources of calcium rather than taking a calcium supplement (14 out of the 16 women that increased calcium intake).

6.4 Discussion

The prevalence of osteoporosis in this group is consistent with that reported for healthy Australian postmenopausal women (299, 331) but lower than that reported by the Geelong Osteoporosis Study, which did not exclude subjects with chronic disease (127). The women in the current study are active, healthy women with no history of chronic disease that may impact on bone, therefore are representative of the healthy Australian community.

Calcium intakes in this group of women fell short of the RDI, also consistent with reported data for Australian women (127). It is noteworthy that when given the diagnosis of osteoporosis, about half of women increased their calcium intake and this was done mainly by increasing dietary sources of calcium rather than taking a supplement. Despite commercial campaigns encouraging women to take calcium supplements, the preference for dietary sources of calcium over supplementation may be because of the media attention on the negative effects of calcium, namely cardiovascular effects attributed to supplemental calcium intake.

Overall, in this group the mean Ca intake fell following bone density result feedback. Although some women increased their intake, an equal number of women decreased their Ca intake. These results suggest that at least strong feedback on Ca intakes is needed, including educational material on dietary Ca sources and Ca supplements, for subjects to increase their intakes considerably (299). Feedback on DXA result is a potential tool in educating women in prevention and management of osteoporosis and may be further improved if combined with feedback on calcium intake.

Chapter 7

Acute effects of calcium with or without vitamin D supplementation on bone resorption in vitamin D deficient post-menopausal women

7.1 Introduction

Vitamin D deficiency, as defined by serum levels <60 nmol/L is common among the elderly. Hip fractures in this group have been attributed to vitamin D deficiency, likely due to low bone mineral density and mass, low muscle mass and propensity to fall. In addition, secondary hyperparathyroidism is a consequence of low vitamin D which leads to an increased rate of bone turnover and low bone mass.

Low vitamin D is generally treated with oral vitamin D supplementation. This study was designed to investigate the effects of short term supplementation of calcium with or without vitamin D on the rate of bone resorption in vitamin D deficient postmenopausal women. Twenty two postmenopausal women (59 to 75 years, mean 66) were randomly allocated to receive either 7 days of 1000mg of Calcium (as carbonate) and then 1000 IU of vitamin D3 for a further 7 weeks or to received 1000 IU vitamin D3 only for 7 weeks and then 1000mg added Ca (as carbonate for another week. All subjects were instructed to continue with their usual diet and activity, and to take the doses of Ca and vitamin D at 9 pm at night daily. Fasting blood samples for routine biochemistry, C terminal telopeptides (CTX), PTH and vitamin D were collected at 9 am before the commencement of the regimens, and after 7 days of Ca alone and after 7 weeks of Ca and vitamin D (for group 1) and in reverse order for group 2. There was no significant difference in any biochemical parameter measured between the 2 groups at the start of the study. The effect of calcium and vitamin D on PTH was more significant and consistent than with either supplement alone. However calcium alone was more effective in causing a significant reduction in CTX than vitamin D alone. When combined, the fall in CTX was even greater and more significant.

An interesting observation was a significant positive effect of calcium alone on serum 25OHD. A significant rise in serum 25OHD in deficient women was noted after 7 days of 1000mg Ca/d. We propose that adequate calcium supplementation may inhibit the metabolism of 25OHD, effectively prolonging the half-life of circulating 25OHD.

Statement of Authorship

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Contribution to the Paper	Contributed to the design of the study, recruitment of subjects, analysis of samples, interpretation of data, wrote the manuscript, corresponding author.		
Overall percentage (%)	85%		
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Contribution to the Paper	Contributed to the design of the study, supervised the project, contributed to data interpretation and edited the manuscript.		
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Suppression of C-Terminal Telopeptide in Hypovitaminosis D Requires Calcium as Well as Vitamin D

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Abstract We compared the effects of oral calcium and vitamin D separately and together on relevant variables in 22 postmenopausal volunteers with initial serum 25OHD levels below 60 nmol/L. Subjects were allocated randomly to two regimens: group 1 received 1 week of calcium 1,000 mg, followed by 7 weeks with additional vitamin D₃ 1,000 i.u. daily; group 2 received 7 weeks of D₃ 1,000 i.u. daily, followed by 1 week with additional calcium 1,000 mg. We measured serum calcium, phosphate, PTH, 25OHD, CTX, and ALP at baseline and after 1 and 8 weeks in group 1 and after 7 and 8 weeks in group 2. There were no significant changes in ALP from either vitamin D or calcium. Calcium caused significant elevation of serum 25OHD as well as major suppression of serum CTX, which could not easily be accounted for by suppression of PTH. Vitamin D caused no significant change in any variable except elevation of serum 25OHD. The suppressive effect of calcium (whether given first or second) on serum CTX was threefold greater than that of vitamin D (whether given first or second) ($P < 0.001$), although their suppressive effects on serum PTH were the same. Calcium and vitamin D yielded greater and more significant effects on all variables (except ALP) than either

treatment alone. We suggest that calcium may elevate serum 25OHD by prolonging its half-life and that it may have an inhibitory effect on bone resorption independent of, or in addition to, its suppression of PTH.

Keywords Vitamin D₃ supplementation · Calcium supplementation · CTX · Parathyroid hormone · Postmenopausal women

There are at least three reasons for the association between hypovitaminosis D and hip fracture [1]: the first is that D deficiency can cause muscle weakness and, thus, increases the risk of falling [2]; the second is that it can lower the quality of bone by reducing its mineral content [3]; and the third (and probably most important) is that it leads to secondary hyperparathyroidism [4–7], which not only increases bone turnover (a risk factor for fracture in its own right [8–11]) but also reduces bone mass and, thus, accelerates the osteoporotic process. The authors of the seminal trial of vitamin D with calcium in hip fracture prevention [12] attributed their success to the reversal of secondary hyperparathyroidism [13], but improved muscle strength and the consequent reduction in falls probably made a significant contribution as well [2].

Despite the confirmed dose-dependent power of vitamin D with calcium to prevent osteoporotic fractures [14, 15], there is still uncertainty about the intervention level of serum 25-hydroxyvitamin D (25OHD) [16–18], about the target serum concentration of 25OHD [19], and about the optimal dosage to achieve the target [20, 21]. In this debate, surprisingly little attention has been paid to the role of calcium supplementation, despite the general recognition that the most convincing evidence of fracture prevention with vitamin D comes from studies in which it was

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combined with calcium [14, 15]. We have therefore carried out a prospective study designed to establish the suppressive effects of calcium and vitamin D, alone and in combination, on serum parathyroid hormone (PTH) and on the bone resorption marker C-terminal telopeptide (CTX) in postmenopausal volunteers with vitamin D insufficiency as defined by serum 25OHD concentrations below 60 nmol/L, which is the threshold below which we see a rise in bone turnover markers [22].

Subjects and Methods

Subjects

One hundred and one women aged between 59 and 75 years who had a serum 25OHD level below 60 nmol/L on routine screening ordered by their general practitioners were invited (with their doctors' consent) to participate in the study. Fifty-four declined and 23 were excluded due to impaired renal function, malignancy, hypercalcemia, diabetes, or bisphosphonate or estrogen therapy. Twenty-four subjects who were living independently with no chronic illness gave informed consent and commenced the study, but two dropped out, leaving 22 who completed the trial. One of these, with an initial serum 25OHD of 59 nmol/L, had a value of 64 nmol/L on entering the actual trial; but the remainder were still below 60 nmol/L on entry.

The study was approved by the Royal Adelaide Hospital Research Ethics Committee.

Methods

The study design was an intra- and intersubject comparison. Subjects were randomized into two groups using a computer-based random number generator. Group 1 received 1 week of 1,000 mg of calcium as the carbonate (CalSup; 3 M Pharmaceuticals, Pymble, NSW, Australia), followed by 7 weeks of continuing calcium with added vitamin D₃ 1,000 i.u. (Ostelin; Reckitt Benckiser, West Ryde, NSW, Australia). Group 2 received the same in reverse order, i.e., 1,000 i.u. vitamin D₃ daily alone for 7 weeks, followed by 1 week of continuing vitamin D₃ with added calcium 1,000 mg daily. Subjects were instructed to take the supplements at 9:00 p.m. Serum samples were collected at 9:00 a.m. after an overnight fast after 1 and 8 weeks in group 1 and after 7 and 8 weeks in group 2. The measured variables were serum calcium, phosphate, 25OHD, PTH, CTX, and alkaline phosphatase (ALP).

Total serum calcium and phosphate were measured spectrophotometrically using the metallochromogen arsenazo III and ammonium molybdate reagents, respectively, on an Olympus (Tokyo, Japan) AU 5400 instrument.

Serum 25OHD was measured by an enzyme immunoassay (ImmunoDiagnostic Systems, Fountain Hills, AZ) and intact PTH, by an automated enzyme-labeled chemiluminescent assay (Immulite 2000; Diagnostic Products, Los Angeles, CA). C-terminal telopeptides were assayed by an automated electrochemiluminescent immunoassay (Elecys E170; Modular Analytics, Indianapolis, IN). Serum ALP was measured in the Olympus instrument using pNPP as the substrate.

Results

Demographics and Baseline Values

The median baseline values and ranges of the relevant measured variables are shown in Table 1. All values were within the reference ranges except for the low 25OHD levels (due to selection) and the raised PTH in four subjects, presumed to be due to hypovitaminosis D. There was one subject with an unexplained high ALP (144 U/L); but there were no significant changes in ALP during the study, and this variable will not be considered further.

Effects of Calcium and Vitamin D

The mean values of the measured variables at the first, second, and third visits in the two groups are shown in Table 2. At the second visit, group 1 (calcium first) showed significant elevation of serum calcium, phosphate, and 25OHD. There was a small, nonsignificant fall in PTH but a major, significant fall in CTX. In group 2 (vitamin D first), there was no significant change in any variable except for a rise in serum 25OHD. At the third visit, the only major change was a significant fall in serum CTX in group 2 after the addition of calcium to vitamin D. Comparing absolute values in the two groups at each visit, the only significant differences were that the CTX at visit two was significantly lower in group 1 than in group 2 and the

Table 1 Mean values and ranges of relevant measured variables at baseline in 22 postmenopausal volunteers

Variable	Mean	Range
Age (years)	66.0	59–75
Weight (kg)	81.8	56–114
Height (cm)	164.6	145–184
Calcium (mmol/L)	2.36	2.17–2.52
Phosphate (mmol/L)	1.11	0.87–1.30
PTH (pmol/L)	6.09	3.10–15.4
25OHD (nmol/L)	48.8	29.0–64.0
CTX (ng/L)	425	154–762
ALP (U/L)	80.2	27.0–144

serum phosphate at visit three was significantly higher in group 2 than in group 1.

The mean changes in the measured variables after 1 week of calcium supplementation (whether given first or second) and 7 weeks of vitamin D supplementation (whether given first or second) in all 22 subjects are shown and compared in Table 3. Seven days of calcium caused a small but significant rise in total serum calcium and phosphate, a significant rise in 25OHD, and a very significant fall in CTX; the fall in PTH was not significant. Seven weeks of vitamin D had no significant effect on any variable except for the rise in 25OHD.

The initial and final values of the measured variables, with the significance of the changes within the subjects, are shown in Table 4; they represent the total effect of 1 week of calcium combined with 7–8 weeks of vitamin D. The combined treatment produced significant elevation of serum calcium, and 25OHD and significant suppression of PTH and CTX.

The changes described in the tables are illustrated in Figs. 1–3. Figure 1 shows that the effect of calcium plus vitamin D on serum PTH was more consistent and

significant than either alone. Figure 2 tells the same story for 25OHD. Figure 3 shows how much more effective was calcium than vitamin D at suppressing serum CTX.

Discussion

Our study is, as far as we know, the first one in which calcium and vitamin D have been compared separately and together in the same individuals. The logic of our protocol was that calcium is known to exert its effect on PTH and bone resorption within 12 hours [23], whereas it is known that vitamin D in conventional dosage takes several weeks to produce an equilibrium 25OHD level [24]. The design of our trial was such that it allowed 1 week of calcium to be compared directly with 7 or 8 weeks of vitamin D in the same individuals—we did not anticipate any difference between the effects of 7 and 8 weeks of vitamin D on serum 25OHD.

The two points of particular interest are the significant positive effect of calcium alone on serum 25OHD and the significantly greater negative effect of calcium than

Table 2 Mean values (SD) of all measured variables at first, second, and third visits by group

Variable	Visit 1	Regimen	Visit 2	Regimen	Visit 3
Total calcium (mmol/L)					
Group 1 (9)	2.39 (0.093)	Ca 1 week	2.47 (0.099)**	Ca + D 7 weeks	2.41 (0.064)
Group 2 (13)	2.34 (0.11)	D 7 weeks	2.38 (0.082)	Ca + D 1 week	2.43 (0.13)
<i>t</i>	1.03		2.18		-0.4
<i>P</i>	0.32		0.046		0.69
PO₄ (mmol/L)					
Group 1 (9)	1.11 (0.061)	Ca 1 week	1.26 (0.11)***	Ca + D 7 weeks	1.09 (0.12)**
Group 2 (13)	1.11 (0.13)	D 7 weeks	1.18 (0.15)	Ca + D 1 week	1.22 (0.12)
<i>t</i>	0.07		1.54		2.56
<i>P</i>	0.94		0.14		0.020
PTH (pmol/L)					
Group 1 (9)	5.64 (2.28)	Ca 1 week	5.19 (2.57)	Ca + D 7 weeks	4.90 (1.41)
Group 2 (13)	6.40 (3.38)	D 7 weeks	5.62 (2.95)	Ca + D 1 week	4.76 (2.82)
<i>t</i>	0.63		0.37		0.15
<i>P</i>	0.54		0.72		0.88
25OHD (nmol/L)					
Group 1 (9)	49.11 (9.4)	Ca 1 week	70.8 (14.6)***	Ca + D 7 weeks	76.6 (17.8)
Group 2 (13)	48.5 (10.7)	D 7 weeks	90.5 (20.7)***	Ca + D 1 week	90.4 (25.3)
<i>t</i>	0.13		2.6		1.5
<i>P</i>	0.90		0.17		0.15
CTX (ng/L)					
Group 1 (9)	412 (193)	Ca 1 week	279 (149)***	Ca + D 7 weeks	231 (123)
Group 2 (13)	434 (137)	D 7 weeks	412 (129)	Ca + D 1 week	312 (112)***
<i>t</i>	0.29		2.18		1.57
<i>P</i>	0.75		0.046		0.14

Significance of difference from preceding sample: *** $P < 0.001$, ** $P < 0.01$, * $P < 0.05$

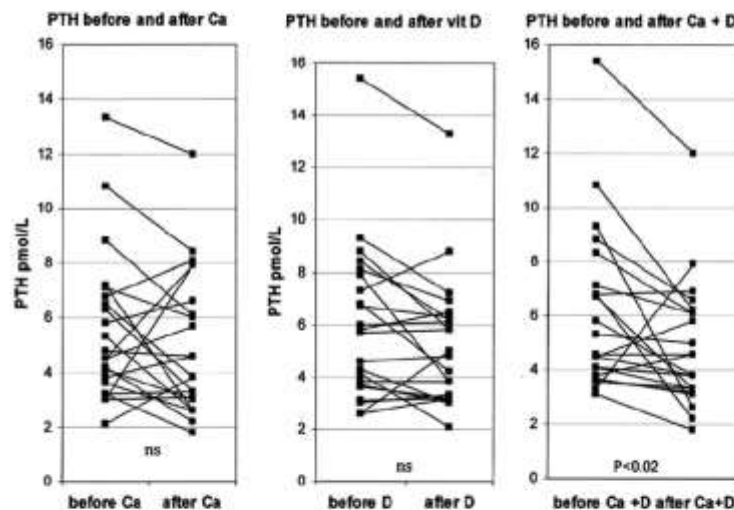
Table 3 Changes in measured variables after treatment with calcium (first or second) for 1 week or with vitamin D (first or second) for 7 weeks (means SE) (n = 22)

Variable	Change after 1 week of calcium	Change after 7 weeks of vitamin D	Difference between calcium and vitamin D	
			t	P
Total calcium (mmol/L)	0.061 (0.020)**	0.00 (0.023)	2.0	<0.10
PO ₄ (mmol/L)	0.088 (0.027)**	-0.032 (0.037)	2.7	<0.02
PTH (pmol/L)	-0.70 (0.49)	-0.63 (2.26)	0.03	0.98
25OHD (nmol/L)	9.57 (4.7)*	24.4 (5.8)***	2.03	<0.10
CTX (ng/L)	-114 (18.1)***	-32.5 (18.4)	4.1	<0.001

** $P < 0.01$, *** $P < 0.001$

Table 4 Mean values of measured variables at beginning (SD) and mean change (SE) at end of study with paired *t*-test and significance

Variable	Basal (SD)	Change on Ca + D (SE)	t	P
Total calcium (mmol/L)	2.36 (0.10)	0.061 (0.020)	3.1	<0.01
PO ₄ (mmol/L)	1.11 (0.11)	0.056 (0.033)	1.7	NS
PTH (pmol/L)	6.1 (2.9)	-1.27 (0.48)	2.7	<0.02
25OHD (nmol/L)	46.2 (12.8)	11.5 (1.38)	8.3	<0.001
CTX (ng/L)	425 (158)	-146 (27.3)	5.3	<0.001

Fig. 1 Effects of calcium and vitamin D₃ separately and together on serum PTH

vitamin D on serum CTX. The data also show that the combination of calcium and vitamin D is generally more powerful than either alone, particularly in respect of PTH suppression. None of these conclusions can easily be attributed to the small power of the study.

To take this last point first, previous studies of the effect of vitamin D with or without calcium on serum PTH are summarized in Table 5 [25–35]. In six studies

in which vitamin D was given without calcium, the mean fall in PTH was 12% and significant in three. In seven studies in which the subjects were given vitamin D with calcium, the mean fall in PTH was 30% and significant in six. These results are not only qualitatively but also quantitatively very similar to ours, in which the combined treatment caused twice the fall in PTH than either treatment alone.

Fig. 2 Effects of calcium and vitamin D₃ separately and together on serum 25OHD

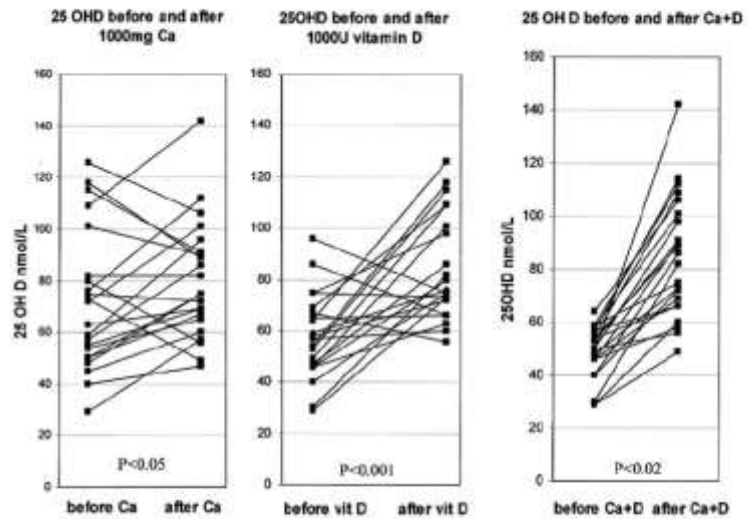
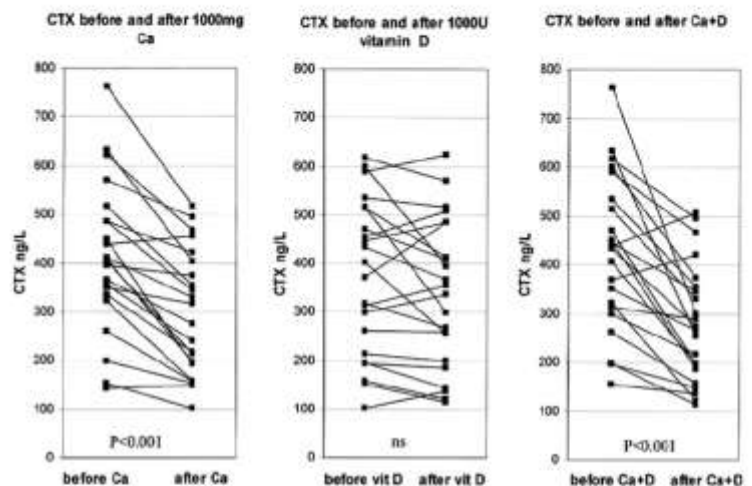


Fig. 3 Effects of calcium and vitamin D₃ separately and together on serum CTX



The second point of interest which, as far as we know, has not been noted before is the rise in 25OHD from 49 to 71 nmol/L ($P < 0.001$) after 7 days of calcium alone (group 1). Although this was not seen when calcium was added to vitamin D (group 2), we think it is potentially important and offers an alternative explanation for low serum 25OHD levels, which are currently universally attributed to low input of vitamin D. We note that calcium deficiency and hyperparathyroidism lower serum 25OHD by shortening its half-life of about 25 days [36–38]. We

suggest that the converse may also hold; i.e., calcium supplementation may inhibit the metabolism of 25OHD, to account for our observation. Could it be that some of the widely reported hypovitaminosis D in Western countries is accounted for by its shortened half-life, not just reduced input?

The other intriguing aspect of this small trial is that the suppression of serum CTX by calcium was not only much greater than its suppression by vitamin D but appeared to be out of all proportion to its suppression of PTH. This

Table 5 Reported effects of vitamin D₃ and/or calcium on serum PTH

References	n	Vitamin D (IU/day)	Calcium (mg/day)	Effect on serum PTH (%)	P
Meyer et al. [25]	36	400	0	0	ns
Paaci et al. [26]	177	400	0	-6	0.005
Grant et al. [27]	?	400	0	-13	?
Barger-Lux et al. [28]	10	1,000	0	-19	<0.001
Trivedi et al. [29]	268	800 ^a	0	-6	ns
Lyons et al. [30]	101	800 ^b	0	-25	ns
Chapuy et al. [12]	381	800	1,200	-45	<0.001
Chapuy et al. [31]	73	800	1,200	-44	<0.05
Grant et al. [27]	?	400	1,000	-35	?
Krieg et al. [32]	50	440	500	-18	<0.001
Dawson-Hughes et al. [33]	148	500	700	-15	<0.001
Grados et al. [34]	95	400	500	-38	<0.001
Larsen et al. [35]	67	400	1,000	-15	<0.001

^a 100,000 IU cholecalciferol every 4 months, ^b 100,000 IU ergocalciferol every 4 months

suggests that calcium may have a direct suppressive effect on bone resorption—at least in postmenopausal women—either by suppressing osteoclasts [39] or by acting directly through a physicochemical equilibrium between the calcium phosphate in the tissue fluids and the bone mineral [40]. This latter is implied by Parfitt [41] in particular, who has drawn attention to the very large interface between tissue fluid and bone mineral at resting surfaces and has shown that the regulation of bone formation and resorption cannot adequately explain the tight regulation of calcium homeostasis [42].

It may be asked why we confined our measurements to fasting blood samples despite the known diurnal variation in serum PTH [43] and bone markers [44–46]. The fact is that these variables tend to peak in the night and early morning after the overnight fast and fall after feeding during the day unless the fast is continued [45]. The effect of extra calcium is to lower serum PTH to much the same degree at all time points [43], so the change in fasting level is representative of the rest. The main effect of an evening calcium load on bone markers is to lower the overnight and fasting markers with little change in the daytime levels [46]. Clinical evidence also suggests the need for both calcium and vitamin D. The only osteoporotic fractures directly linked to hypovitaminosis D are hip fractures [1], which were not prevented by vitamin D alone in two studies [29, 30]. Most of the fracture prevention trials included in recent meta-analyses have used a combination of vitamin D and calcium [14, 15], but there is one that specifically claims that calcium is required in addition to vitamin D for maximal benefit [47].

We conclude that the combination of calcium with vitamin D is more effective at suppressing bone resorption

than either treatment alone and that the calcium effect does not necessarily operate only or mainly through PTH suppression. We readily admit that nonsignificant changes and differences could be due to the small power of the study, but the main points of interest do not look like Type II errors. The lack of significant changes in ALP might be due to lack of power but is probably due to the well-known time lag between the effect of antiresorptive therapy on bone resorption and bone formation [48].

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Chapter 8

Influence of supplement type – carbonate or citrate on bone resorption in post-menopausal women

Introduction

With the majority of postmenopausal women not meeting their RDI of calcium through dietary sources alone, there is a public health interest in promoting calcium supplements to prevent osteoporosis. Commonly marketed calcium supplements are carbonate, citrate and hydroxyapatite. Absorption of carbonate preparations require stomach acid while citrates do not. Therefore in achlorhydria citrate is preferred. In addition, if the patient is at risk of renal calculi, calcium citrate is preferred because citrate is an inhibitory factor in urine calcium oxalate precipitation. Calcium carbonate contains 40% elemental calcium, while citrate contains 21% by weight. However calcium citrate has a bioavailability 25% greater than that of calcium carbonate.

Calcium supplements are commonly prescribed to postmenopausal women in order to prevent osteoporosis and also as adjunctive therapy for those on anti-resorptive treatment. Bone turnover markers CTX and PTH can be measured to monitor the effectiveness of calcium given. The following study is a double blind crossover study designed to assess the effects of calcium carbonate and calcium citrate in suppressing CTX and PTH in healthy postmenopausal women. Briefly, 22 subjects were randomly assigned to receive either 1000mg of calcium carbonate or 500mg calcium citrate once at night. The alternate dose was given 1 week later. Fasting samples were collected for biochemistry each morning after the calcium dose. ANOVA suggested no difference between the variables measured after each calcium salt. Bioequivalence studies suggested that calcium citrate was superior to calcium carbonate in raising serum total and ionised Ca, and at least as effective as the carbonate in suppressing PTH and CTX. Therefore calcium citrate appears to be as effective as calcium carbonate in suppressing bone resorption, at half the dose.

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Suppression of Parathyroid Hormone and Bone Resorption by Calcium Carbonate and Calcium Citrate in Postmenopausal Women

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Abstract This study was conducted to compare the suppressive effects of calcium carbonate and calcium citrate on bone resorption in early postmenopause. Calcium citrate is thought to be better absorbed. We therefore tested the hypothesis that calcium as citrate is more effective than calcium as carbonate in suppressing parathyroid hormone (PTH) and C-terminal telopeptide. Twenty-five healthy postmenopausal women were recruited in this double blind crossover study. The subjects were randomly allocated to receive either 1,000 mg of elemental calcium as carbonate or 500 mg of calcium as citrate. They were given the alternate calcium dose 1 week later. Serum measurements of total and ionized calcium, phosphate, PTH, and CrossLaps were repeated 12 hours after each dose. Analysis of variance found no significant difference between measures for the two salts.

Tests for equivalence indicated that 500 mg of calcium citrate may be superior to 1,000 mg of calcium carbonate in raising serum total and ionized calcium ($P = 0.04$ and 0.05 , respectively). For all parameters measured, 500 mg of calcium citrate was at least as beneficial as 1,000 mg of calcium carbonate. Calcium citrate is at least as effective as calcium carbonate in suppressing PTH and C-terminal telopeptide cross-links, at half the dose. This may be because calcium as citrate is better absorbed than calcium as carbonate. If calcium citrate can be used in lower doses, it may be better tolerated than calcium carbonate.

Keywords Calcium citrate · Calcium carbonate · Parathyroid hormone · C-terminal telopeptide cross-link

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There is a rise in calcium requirement at the menopause due to a fall in calcium absorption and a rise in obligatory calcium loss, which may account for the rise in bone resorption that occurs at this time [1]. We and others have shown that calcium supplements reduce bone resorption in these women and reduce the rate of bone loss, whether given as calcium carbonate [2] or as fortified milk [3]. Although calcium supplements are generally considered safe, they are not acceptable to some women because of abdominal bloating and constipation. This has led to poor compliance in some clinical trials [4].

It has been reported that calcium as citrate is better absorbed than calcium as carbonate, causing a greater rise in serum calcium and a greater fall in serum parathyroid hormone (PTH) [5, 6]. If that is correct, it should be possible to use smaller doses of calcium to prevent bone loss in postmenopausal women and perhaps reduce the gastrointestinal side effects; but the antiresorptive effects of calcium as citrate and carbonate have not been directly compared. We therefore

compared the effects on the resorption marker serum C-terminal telopeptide (CrossLaps) and other related measures of a dose of 500 mg of calcium citrate and 1,000 mg of calcium carbonate in early postmenopausal women.

Methods

Postmenopausal women within 6 years of the menopause were recruited by advertising in a local newspaper. Respondents were sent a brief health questionnaire. Those with bone or calcium disorders such as Paget disease, hyperparathyroidism, or renal failure were excluded, as were those on medications known to affect bone or calcium metabolism. We also excluded volunteers who had received any hormone replacement therapy within the last 6 months and those who had ever received bisphosphonate therapy. The study was approved by the Research Ethics Committee of the Royal Adelaide Hospital. Informed consent was obtained from all subjects after they received written information detailing the study and its purpose.

The subjects attended our laboratory at about 9 a.m. after an overnight fast and, after giving written informed consent, completed an in-house questionnaire to estimate their habitual calcium intake. Blood was then taken for measurement of routine biochemistry, serum 25 hydroxyvitamin D (25(OH)D), CrossLaps, and PTH. Total calcium was measured on the Olympus AU 5400 (Tokyo, Japan), and ionized calcium was calculated from total calcium, albumin, globulins, bicarbonate, and anion gap as previously described [7]. Serum 25(OH)D was measured by radioimmunoassay (IDS, Boulden and Tyne, UK) and PTH by Immulite 2000 (DPC, Los Angeles, CA). C-terminal telopeptide (CrossLaps) was measured by electrochemiluminescence immunoassay (ECLIA; Roche Diagnostics, Mannheim, Germany). The serum CrossLaps measurement is specific for a β -isomerized fragment from the C-terminal region of type I (bone) collagen (EKAHD- β -GGR). It is formed in collagen as the α isomer and converted over time to the beta form. Its presence in serum therefore reflects the breakdown of mature bone collagen. The fragment is measured in a "sandwich" assay using two different monoclonal antibodies to the eight-amino acid fragment in an automated system (Elecsys, Roche Diagnostics).

The subjects were then randomized to receive either 500 mg of calcium citrate (Citracal; Key Pharmaceuticals, Macquarie Pk, Australia) or 1,000 mg of calcium as the carbonate (Calsup; 3M, Pymble, Australia) at 9 p.m. and then returned to the laboratory the following morning for a repeat blood test. They were then given the alternate calcium preparation to take at 9 p.m. 1 week later and asked to attend the laboratory again on the morning after for a further blood test.

Changes in the measured variables on the two salts were compared using repeated measures analysis of variance (ANOVA) models using Stata, version 9 (StataCorp, College Station, TX), pharmacokinetic commands (pkcross and pkequiv). $P < 0.05$ was deemed to be significant. Equivalence tests were conducted at 90% confidence for a 20% difference between treatments using the classic confidence interval for bioequivalence, Schuirman's one-sided test, and Anderson and Hauck's two-sided test.

Results

The subjects are described in Table 1. Their ages ranged 50–60 years, weight 50–91 kg, height 154–172 cm, number of years since menopause 2–6, and habitual calcium intake 610–1,607 mg/day. Of note, 19 of the subjects (76%) had a habitual calcium intake less than the recommended 1,300 mg/day [8]. Thirteen of the subjects received calcium citrate first and the other 12 received calcium carbonate first. Changes in the measured variables on each treatment are shown in Table 2. Total and ionized calcium and phosphate rose, while PTH and CrossLaps fell on both treatments. All the changes were numerically superior after 500 mg of calcium citrate than after 1,000 mg of calcium carbonate, though none of the differences in the responses between the two calcium salts was significant.

Table 3 shows the correlations between the changes in the measured variables. The rise in serum phosphate was significantly related to the rise in ionized calcium after both the citrate and carbonate salts. The fall in serum C-terminal telopeptide was related to the rise in serum ionized calcium after the citrate salt ($P = 0.01$) but not after the carbonate

Table 1 Demographic and biochemical variables, mean (standard deviation) ($n = 25$)

	Mean (SD)	Range
Demographic variables		
Age (years)	56.08 (0.98)	50–60
Weight (kg)	68.68 (10.82)	50–91
Height (cm)	163.44 (4.59)	154–172
Years since menopause	4.2 (1.15)	2–6
Habitual Ca intake (mg/day)	1,059 (289)	600–1,600
Serum variables		
Ca (mmol/L)	2.31 (0.089)	2.20–2.55
Ionized Ca (mmol/L)	1.19 (0.04)	1.17–1.27
Phosphate (mmol/L)	1.13 (0.12)	0.75–1.35
Creatinine (μ mol/L)	62 (8.2)	51–77
PTH (pmol/L)	4.0 (1.6)	0.8–5.5
25(OH)D (nmol/L)	66 (13)	60–150
CrossLaps (ng/L)	405 (149)	Desirable <400

Table 2 Changes in measured variables after 500 mg of Ca as citrate and 1,000 mg of Ca as carbonate, mean (standard deviation), ANOVA ($n = 25$)

Serum	Citrate	Carbonate	
Δ Ca (mmol/L)	0.063 (0.089)**	0.027 (0.069)*	ns
Δ ionized Ca (mmol/L)	0.030 (0.029)***	0.012 (0.037)	ns
Δ Phosphate (mmol/L)	0.101 (0.175)**	0.076 (0.157)*	ns
Δ Creatinine (mmol/L)	-0.93 (6.8)	-3.24 (5.72)	ns
Δ PTH (pmol/L)	-0.76 (1.3)**	-0.30 (1.2)	ns
Δ CrossLaps (ng/L)	-74 (85)***	-69 (85)**	ns

Significance of change of variables from baseline after each treatment: * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$; ns, no significant difference between treatments

salt. All other correlations were nonsignificant. The changes in serum phosphate were related to the changes in ionized calcium after calcium citrate ($P < 0.05$) and calcium carbonate ($P < 0.01$).

No significant differences between the treatments were found by ANOVA, although the tests had low power. Statistical tests for equivalence indicated that 500 mg of calcium citrate may be superior to 1,000 mg of calcium carbonate in raising the serum total ($P = 0.04$) and ionized calcium ($P = 0.05$) and at least as good as 1,000 mg of calcium carbonate for all other measured variables. The P values for tests of equivalence are given in Table 4.

Discussion

Our study has shown that 500 mg of calcium as the citrate salt causes at least as great a rise in serum calcium and

as great a fall in serum PTH and C-terminal telopeptide cross-links as 1,000 mg of calcium as the carbonate salt, supporting the higher absorption of the former than the latter salt [5, 6]. If the chance of the citrate salt being superior to the carbonate salt was 0.5 for serum total and ionized calcium, phosphate, PTH, and CrossLaps, then the likelihood of it being superior for all four comparisons is 0.5^5 or 0.03125, which means it is unlikely that our findings are due to chance.

Calcium supplementation has an inhibitory effect on bone resorption through the suppression of PTH. In the current study, an evening calcium supplement raised total and ionized calcium for at least 12 hours, with a resultant drop in PTH and CrossLaps. Serum PTH fell 8% with 1 g of calcium carbonate and 17% with 500 mg of calcium citrate. It may, of course, have fallen more than this during the night and been on the rise at 12 hours as Zikan et al. [9] reported. An inverse steep sigmoidal relationship exists between fasting PTH and ionized calcium [10], and we cannot know when the PTH nadir was reached. Suppression of PTH would be expected to raise serum phosphate due to increased renal tubular reabsorption, which probably explains why the rise in PO_4 significantly correlated with the rise in ionized calcium. Calcium was administered in the evening as there is evidence that an evening dose of calcium has a significant effect on bone resorption markers and PTH compared to a morning dose [11].

In another study, the effect of 1,000 mg of calcium citrate was reported to be superior to calcium carbonate in lowering free urine deoxypyridinoline [12]. In that study, 1,000 mg of calcium carbonate did not have a significant effect on free urine deoxypyridinoline, perhaps because it

Table 3 Correlations and P values (in parentheses) between changes in serum ionized Ca, phosphate, PTH, and C-terminal telopeptides after either 500 mg of Ca citrate or 1,000 mg of Ca carbonate ($n = 25$)

	Δ Ca	Δ Ca ²⁺	Δ PO ₄	Δ PTH
Δ Ca ²⁺	0.754 (0.000)			
Δ PO ₄	0.534 (0.000)	0.470 (0.001)		
Δ PTH	-0.211 (0.142)	-0.303 (0.029)	-0.002 (0.991)	
Δ CTX	-0.095 (0.510)	-0.248 (0.082)	0.117 (0.420)	0.131 (0.365)

CTX, C-terminal telopeptide

Table 4 P values for tests of equivalence

Variable	Classic confidence interval for bioequivalence	Schirman's two-sided test P	Anderson and Haucks one-sided test P
Total Ca	0.0000	0.139, 0.046	0.0461
Ionized Ca	0.0000	0.171, 0.071	0.0501
PO ₄	0.0000	0.789, 0.303	0.2429
PTH	0.0000	0.220, 0.0115	0.0525
CTX	0.0000	0.870, 0.198	-0.3364

CTX, C-terminal telopeptide

is a less sensitive marker of bone resorption than serum CrossLaps.

Although most evidence suggests that calcium is better absorbed as the citrate than the carbonate salt, Heaney et al. [13] reported that equal amounts of calcium given orally as the citrate or the carbonate gave similar rises in serum calcium and concluded that the calcium was equally well absorbed in either form. Our results are in keeping with the majority of reports which state that the citrate salt is better absorbed.

The reduction in bone resorption by calcium explains how it can prevent bone loss in postmenopausal women [14]; but dropout rates in clinical trials of calcium have been substantial, and its effect on fracture prevention is uncertain [4, 15]. Since the citrate molecule does not yield CO₂ in contact with acid and calcium as citrate is effective at half the dose of calcium as carbonate, adverse gastrointestinal events may be less after calcium citrate, although this has not been tested in the long term to our knowledge.

The current study does not prove that calcium citrate is better absorbed than calcium carbonate, merely that it is at least as effective in raising serum ionized calcium and lowering serum PTH and CrossLaps. The most reasonable explanation is that the citrate salt is better absorbed, as suggested by pharmacokinetic studies [6]. In addition, calcium citrate is better absorbed than calcium carbonate in achlorohydric patients and in those on proton pump inhibitors [16]. More studies will be required to establish whether calcium citrate is better tolerated and gives better outcomes in the long term than calcium carbonate. However, a study by Dawson-Hughes et al. [17] has already shown that lumbar spine bone mineral density was preserved better with calcium citrate malate than with calcium carbonate. Another limitation of the study is that the rise in calcium and fall in PTH and CrossLaps were only measured at one time point, namely, 12 hours after the calcium doses, and the nature of the changes is not known.

We conclude that the use of calcium citrate may reduce bone resorption in postmenopausal women at lower doses than calcium carbonate. Therefore, calcium citrate may lead to less adverse effects and better tolerance with improved long-term compliance.

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Chapter 9

Effects of obesity and metabolic syndrome on bone

Introduction

Osteoporosis is a multifactorial complex condition with up to 80% of the bone strength (quality and density) determined by genetics (332) with other factors such as nutrition, lifestyle and physiology (age, hormonal status) playing a significant role. The effects of energy metabolism, as expressed by weight, body mass index, visceral fat and the metabolic syndrome (insulin resistance, glucose intolerance) on bone is an evolving field.

Traditionally, nutrients such as protein, calcium and vitamin D as well as other minerals have been the most studied nutritional factors in the context of bone metabolism. However there is a growing body of evidence linking energy metabolism and glucose and fat with bone metabolism, with the hypothalamus centrally mediating adipose tissue and bone metabolism (333). It has been demonstrated that leptin secreted by adipose tissue influence bone formation by osteoblasts. Higher circulating levels of leptin inhibit bone formation while removing leptin from the circulation using a soluble leptin receptor led to increased bone mass in a mouse model (190). This suggests that the amount of adipose tissue may influence bone turnover in an individual. Several studies have shown that obesity or overweight is associated with higher bone density and afford some protection against osteoporosis.

There have been several publications that suggest an inverse relationship between calcium intake and body weight (132, 334, 144). A strong inverse relationship between relative risk of obesity and calcium intake was reported for the participants of NHANES III (132). The proposed mechanism is accelerated lipogenesis in adipocytes with rising intracellular calcium under the influence of PTH and 1,25(OH)₂D (132, 335). If this was the case, then habitual low calcium intakes will lead to higher circulating PTH and in turn, the rate of lipogenesis will rise

leading to triglyceride deposition. In fact, PTH and 1,25(OH)₂D were reported to be positively associated with BMI (336). Conversely, higher calcium intakes will suppress PTH, resulting in a lower rate of lipogenesis. Indeed, low calcium intake has been recognised as a non-traditional risk factor for overweight and obesity (337,338).

The association between body weight and BMI and BMD in older women has been shown to explain a large proportion of variance in BMD (8.9 – 20% of total variance) (339,340). Given the evidence supporting higher BMD associated with higher body weight, it may follow that fracture incidence, at least to some extent, may depend on body weight or composition. In fact, epidemiological evidence from large studies such as Study of Osteoporotic Fractures EPIDOS and European Vertebral Osteoporosis Study show that a higher incidence of hip and vertebral fractures in women with lower body weight than those with higher weights (341,342,343,344). Similar results have been obtained for forearm fractures (345). A study in 3500 Australian men and women showed that the observed association between BMI and fracture risk is mediated by femoral neck BMD (346).

The majority of these studies assessed the contribution of body weight, BMI or weight gain on BMD and some assessed the whole body composition and percentage whole body fat and their association with BMD. The following study was undertaken to determine if BMD was influenced by the overweight or obese state in postmenopausal women. In addition, this study aims to investigate if waist circumference has the same associations with BMD as visceral fat and BMI, and if the rate of bone loss is influenced by visceral fat.

Materials and Methods

Postmenopausal women (n=117) in the observational study of the relationship between calcium intakes and bone density had the visceral adipose tissue measured by DXA in the lumbar region, using manufacturer's instructions (Norland XR 36, Swissray) at a scan speed of

25cm/s. The instrument was calibrated daily with the manufacturer's phantom for bone. The software provided by the manufacturer assumes a weighted linear fat distribution model. Truncal obesity, a predominant feature of metabolic syndrome was measured as the visceral fat mass in the lumbar region (L2 to L4). In addition, other anthropometric measures such as height, weight and BMI, biochemical variables in serum and calcium intake were collated as described in chapter 2. Associations between BMI, Ca intake, VF (visceral fat mass) and waist circumference (WC) were explored using linear regression analysis. Subjects were categorised into overweight and obese (OW/O) ($BMI \geq 25$) or non-obese (NO) ($BMI < 25$) groups. Measured variables for the 2 groups were compared using a Student's T test. The effects of age, weight, BMI and visceral fat mass on baseline BMD at each measured site was assessed by linear regression analysis. Bone loss at each site was calculated using the difference between the final BMD and baseline BMD. This measurement was then used to estimate the annual loss by dividing it by the number of months between measurements and multiplying by 12. Pearson's product moment correlation coefficient was used to assess the strength of the relationship between annual bone loss (g/cm^2) at each site and other measured variables. The variables that showed a significant relationship with annual bone loss for each site were used in multivariate regression analysis (SPSS Statistical Package, IBM, USA) to correct for these factors so that the effect of BMI on bone loss can be assessed.

Results

In this group of women, the estimated daily Ca intake was not related to BMI or VF. BMI, VF and WC were significantly correlated to each other ($P=0.000$). Variables significantly related to baseline BMD for each site are listed in table 9.1. Age was associated only with forearm BMD while BMI was strongly related to all sites.

Table 9.2 lists the mean (SD) for measured variables for OW/O and NO groups with the level of significance (P) for differences between the groups. Bone density was significantly higher at all measured sites in the

OW/O individuals while calcium intakes were not significantly different. Predictably OW/O individuals also had significantly higher fasting glucose levels. In addition, higher levels of PTH were found in OW/O subjects but lower levels of 25OHD compared to NO subjects.

Table 9.1. Variables correlated with baseline BMD at each site and level of significance (P)

Measured site	variable	P
Lumbar spine	BMI	0.000
	WC	0.007
	VF	0.004
Femoral neck	BMI	0.03
	WC	0.006
	VF	0.006
Standard hip	BMI	0.001
	WC	0.003
	VF	0.004
Proximal forearm	Age	0.04
	BMI	0.01
	WC	0.03
Ultradistal forearm	Age	0.001
	BMI	0.003

BMI = body mass index, WC = waist circumference, VF = visceral fat mass.

The Pearson's r correlation coefficient between the annual bone loss for each site and other variables suggested those listed in table 9.3 to have a significant correlation with the rate of bone loss. In addition, fasting glucose and total cholesterol were strongly related to visceral fat mass (P = 0.01 and 0.05 respectively). CTX and PTH were correlated with a P of 0.05.

Table 9.2. Mean (SD) for measured parameters for the overweight or obese vs non-obese subjects

Variable	Non-obese (n = 66)	OW/Obese(n=63)	P
Age (yr)	60.1 (4.6)	61.1 (5.3)	0.12
Weight (kg)	57.6 (9.1)	74.8 (9.9)	0.000
Height (m)	1.67 (0.43)	1.62 (0.06)	0.15
WC (cm)	77.4 (6.9)	90.9 (10.9)	0.000
Visceral fat (g)	669.1 (237.4)	1141.4 (298.5)	0.000
Lean mass (g)	1521.8 (181.9)	1484.2 (191.0)	0.13
BMD (g/cm ²)	0.9249 (0.1709)	1.0189 (.1675)	0.001
BMC (g)	40.198 (8.025)	46.572 (9.424)	0.000
LS BMD (g/cm ²)	0.9865 (0.1963)	1.0941 (0.1904)	0.001
FN BMD (g/cm ²)	0.7789 (0.1078)	0.8406 (0.1353)	0.002
Hip BMD (g/cm ²)	0.8383 (0.1176)	0.9243 (0.1211)	0.000
PFA BMD (g/cm ²)	0.6406 (0.0962)	0.7320 (0.0756)	0.000
UDFA BMD (g/cm ²)	0.3095 (0.060)	0.3542 (0.0514)	0.003
Ca intake (mg/d)	1142.8 (523.4)	1058.3 (485.2)	0.17
Creatinine (µmol/L)	59.5 (10.7)	61.6 (11.5)	0.42
Fasting glucose (mmol/L)	4.4 (0.6)	4.8 (0.9)	0.002
Total cholesterol (mmol/L)	5.4 (0.9)	5.5 (1.0)	0.38
iCa (mmol/L)	1.22 (0.04)	1.23 (0.03)	0.08
total Ca (mmol/L)	2.37 (0.09)	2.39 (0.08)	0.35
PO4 (mmol/L)	1.15 (0.12)	1.14 (0.13)	0.25
ALP (U/L)	79 (22)	87 (25)	0.03
CTX (ng/L)	465.5 (179.5)	429.2 (187.1)	0.13
PTH (pmol/L)	4.08 (1.09)	4.68 (1.99)	0.01
25OHD (nmol/L)	78.7 (27.6)	69.2 (21.6)	0.01
UCa/Cr (mmol/mol)	0.25 (0.13)	0.23 (0.15)	0.27
Ca absorption	0.59 (0.26)	0.59 (0.17)	0.49

OW = overweight; WC = waist circumference; BMC = bone mineral content (L2 – L4), LS = lumbar spine (L1 – L4), FN = femoral neck, PFA = proximal forearm, UDFA = ultradistal forearm, BMD = bone mineral density, iCa = calculated ionised calcium; UCa/Cr = urine calcium to creatinine ratio on fasting spot urine sample.

Table 9.3. Variables correlated with rate of bone loss and level of significance (n=98)

Site	variable	P (<)
Lumbar spine	Age	0.05
Femoral neck	Ca absorption	0.05
Proximal forearm	Ca intake	0.001
	PTH	0.05
	VF	0.01
Ultradistal forearm	Ca intake	0.05

Multiple linear regression analysis between the rate of loss and BMI was performed for each site, taking into consideration those variables that showed a significant relationship to the rate of bone loss at that specific site. Tables 9.4, 9.5, 9.6 and 9.7 show the regression analysis for lumbar spine, proximal forearm and ultradistal forearm respectively.

Table 9.4. Multiple linear regression of rate of bone loss (g/cm²/year) at the lumbar spine (ALLS) on age and BMI (n=117)

	SE	P
ALLS = 0.0015 x age	0.001	0.03
-0.0032 x BMI	0.001	0.67
-0.0109 g/cm ² /year		
R ² = 0.040; adjusted R ² = 0.024; P = 0.09		

Table 9.5. Multiple linear regression of rate of bone loss (g/cm²/year) at the femoral neck (ALFN) on intestinal calcium absorption and BMI (n=117)

	SE	P
ALFN = 0.0200 x abs	0.014	0.05
-0.0007 x BMI	0.000	0.18
-0.0096 g/cm ² /year		
R ² = 0.043; adjusted R ² = 0.027; P = 0.07		

Table 9.6. Multiple linear regression of rate of bone loss (g/cm²/year) at the proximal forearm (ALPFA) on age, baseline PTH, calcium intake and BMI (n=113)

	SE	P
ALPFA = -0.0014 x PTH	0.000	0.09
+1.728x10 ⁻⁶ x Ca intake	2.69x10 ⁻⁶	0.52
+5.166x10 ⁻⁶ x visceral fat	4.67x10 ⁻⁶	0.27
-0.0002 x BMI	0.000	0.48
-0.0072 g/cm ² /year		

R² = 0.049; adjusted R² = 0.007; P = 0.33

Table 9.7. Multiple linear regression of rate of bone loss (g/cm²/year) at the ultradistal forearm (ALUDFA) on calcium intake and BMI (n=116)

	SE	P
ALUDFA = +4.5442x10 ⁻⁶ x Ca intake	1.84x10 ⁻⁶	0.01
-0.0002 x BMI	0.000	0.47
-0.0107 g/cm ² /year		

R² = 0.076; adjusted R² = 0.046; P = 0.062

When corrected for variables that were significantly related to the rate of bone loss at each site, BMI was not associated with the rate of loss at any site. VF was not related to age or calcium intake when corrected for fasting glucose and total cholesterol. These results are shown in table 9.8.

Table 9.8. Multiple linear regression of visceral fat mass (g) on age, fasting glucose, total cholesterol and calcium intake (n=114)

	SE	P
Visceral fat mass = +2.117 X age	6.88	0.75
+145.5 X glucose	43.39	0.001
+56.71 cholesterol	34.46	0.10
-0.1099 X Ca intake	0.068	0.11
-68.8 g		

R² = 0.135; adjusted R² = 0.103; P = 0.889

Discussion

Consistent with published data BMI was positively associated with BMD at all measured sites at baseline. An association between BMD and age was only observed at the forearm sites, suggesting that the effect of weight or BMI is stronger than that of age on BMD at the hip and spine. Data from the Framingham study group support this with their finding of greater impact of weight on weight bearing sites such as the hip and spine (340). VF and WC were also significantly related to BMD at weight bearing sites, suggesting that WC, an easily obtained measurement to diagnose metabolic syndrome, can be used as a surrogate marker for factors such as BMI and VF in predicting the effects on BMD.

When the subjects were divided into OW/O and NO groups based on BMI, BMD at all measured sites were significantly higher in the OW/O group, while there was no difference in daily Ca intake, intestinal Ca absorption or urinary Ca excretion, suggesting that the effect of adiposity may be more significant than calcium intake in this group. There was no difference in CTX between the groups, suggesting a similar rate of bone turnover in the 2 groups. An explanation is that, the skeletal response of OW/O women to the same amount of dietary Ca is more significant than the response of NO women, as demonstrated in chapter 10 (347). Consistent with published data, OW/O women had higher PTH and lower 25OHD. It appears that OW/O women maintained a higher BMD despite higher levels of serum PTH and lower 25OHD compared to NO women. In addition, ALP was higher in OW/O women suggesting some element of fatty liver or a contribution from bone formation.

The rate of bone loss was not associated with BMI. The rate of loss at the forearm was associated with Ca intake as described in chapter 5. PTH was strongly associated with the proximal forearm bone loss consistent with the widely accepted position that forearm bone (cortical bone) losses are more pronounced in primary hyperparathyroidism. However VF was also associated with bone loss at this site.

When factors significantly associated with bone loss at each site were corrected for, there was no significant relationship between the rate of bone loss and BMI. Although not significant, the rate of loss for all sites was negatively associated with BMI suggesting that a higher BMI may be related to a lower rate of bone loss.

In contrast to published data, this study failed to show a significant association between Ca intake and BMI or VF. Notable is the analysis of NHANES III data set (1988 – 1994, 380 women and 7114 men) that found an inverse relationship between dietary Ca intake and the risk of greater body fat (348, 349). The weak association between Ca intake and VF in this study was however negative suggesting that a higher Ca intake tends to reduce VF. VF was strongly associated with fasting glucose.

These findings suggest that there is an interaction between fat cells and bone metabolism, and this may be more pronounced in overweight or obese women, who maintain a higher BMD than non-obese women at similar intakes of Ca. This chapter and the following chapter strongly suggest that women with a greater amount of visceral fat utilise dietary Ca more efficiently. The physiological process involved in this ‘Calcium thrift’ in women with more fat cells may involve incretins, leptin and other adipocyte factors and central mediation via the hypothalamus.

Chapter 10

Acute effect of a supplemented milk drink on bone metabolism in healthy post-menopausal women is influenced by the metabolic syndrome

Introduction

Ingested calcium exerts a suppressive effect on bone resorption as demonstrated by a suppression of markers such as PTH and CTX following a dose of calcium. When a mixed meal including calcium is ingested, other nutrients in the meal may exert effects on skeletal metabolism. In addition, a dysregulation of metabolism of other nutrients, as in the metabolic syndrome (Met S), may impact on calcium metabolism and bone metabolism. In Met S, energy metabolism is thought to be dysregulated with impaired glucose metabolism as a key feature. High energy nutrient intake or caloric intake plays a role in the development of Met S. The concept of gut-brain-bone axis postulates that nutrient intake has feedback and feed forward effects on the hypothalamus that then exerts effects on skeletal metabolism. Osteocalcin secreted by osteoblasts has been shown to exert effects on pancreatic beta cells and regulate insulin secretion, which has a feed forward action on osteoblast metabolism.

The following study was designed to investigate whether Met S had an impact on the suppressive effects of bone turnover exerted by calcium in postmenopausal women. Briefly, 25 postmenopausal women were given 200 mL of milk supplemented with 560mg additional calcium (Milo) once prior to bedtime. Fasting blood was collected for biochemistry 12 hours after the drink. The baseline CTX level declined with rising abdominal fat mass as measured by DXA ($P=0.04$). While there was no significant drop in CTX after the drink in the group as a whole. When the women were divided to lean women and women with Met S (waist circumference ≥ 88 cm) there was a significant fall in CTX in women with Met S after the supplemented milk drink. This suggests that bone turnover in overweight or obese women with Met S is more sensitive to

dietary calcium intake. These findings are consistent with previous proposed theories of obesity protecting against osteoporosis, with close associations with energy metabolism.

Statement of Authorship

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Name of Principal Author (candidate)	Sunethra Devika Chin Thomas		
Contribution to the Paper	Contributed to the design of the study, recruitment of subjects, analysis of samples, interpretation of data, wrote the manuscript, corresponding author.		
Overall percentage (%)	85%		
Signature		Date	

Co-Author Contributions

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Contribution to the Paper	Supervised the project, contributed to data interpretation and manuscript evaluation; edited the manuscript.		
Signature		Date	

Name of Co-Author	B.E.C. Nordin		
Contribution to the Paper	Contributed to the design of the study, supervised the project, contributed to data evaluation.		
Signature	Deceased	Date	

RESEARCH

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Acute effect of a supplemented milk drink on bone metabolism in healthy postmenopausal women is influenced by the metabolic syndrome

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Abstract

Background: Dietary factors acutely influence the rate of bone resorption, as demonstrated by changes in serum bone resorption markers. Dietary calcium exerts its effect by reducing parathyroid hormone levels while other components induce gut incretin hormones both of which reduce bone resorption markers. The impact of dietary calcium on bone turnover when energy metabolism is modulated such as in metabolic syndrome has not been explored. This study was designed to investigate whether metabolic syndrome or a greater amount of visceral fat influences the impact of dietary calcium on bone turnover.

Methods: The influence of the metabolic syndrome on effects of dietary calcium on bone turnover in community dwelling postmenopausal women was studied. Twenty five volunteers consumed 200 mL of low fat milk with additional 560 mg calcium (one serve of Milo®) in the evening on one occasion. Fasting morning serum biochemistry before and after the milk drink with lumbar spine bone density, bone mineral content, fat and lean mass using dual energy X-ray absorptiometry (DXA) and waist circumference were measured. The women were divided into 2 groups using the waist measurement of 88 cm, as a criterion of metabolic syndrome. Student's *t* tests were used to determine significant differences between the 2 groups.

Results: The lumbar spine mineral content was higher in women with metabolic syndrome. After consuming the milk drink, serum bone resorption marker C terminal telopeptide (CTX) was suppressed to a significant extent in women with metabolic syndrome compared to those without.

Conclusions: The results suggest that dietary calcium may exert a greater suppression of bone resorption in post-menopausal women with metabolic syndrome than healthy women. Despite substantial evidence for close links between energy metabolism and bone metabolism this is the first report suggesting visceral fat or metabolic syndrome may influence the effects of dietary calcium on bone metabolism.

Keywords: Dietary calcium, Postmenopausal, Metabolic syndrome, Osteoporosis

Introduction

Osteoporosis is common in postmenopausal women, in which estrogen deficiency plays an important role. Low dietary calcium intake and vitamin D deficiency also contribute to the high prevalence of osteoporosis amongst these women. Post-menopausal bone loss is driven by

increased rate of bone resorption relative to bone formation which is demonstrated biochemically by an increase in serum levels of metabolites of type I collagen.

Dietary calcium and vitamin D are essential for skeletal health throughout life and are recognised as bone sparing nutrients [1, 2]. The calcium requirement rises after the menopause due to increased losses and reduced intestinal absorption [3, 4].

The main dietary source of calcium is dairy food with high calcium bioavailability and optimal ratio of calcium to phosphate optimal for intestinal absorption [5].

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Regular intake of vitamin D-fortified, calcium rich food, such as dairy inhibits markers of bone resorption in post-menopausal women—within 4 to 8 weeks [6–8]. An inadequate dietary calcium intake in post-menopausal women leads to increased risk of osteoporosis and fragility fractures [9]. However a longitudinal prospective cohort study did not show a reduction in fracture risk or osteoporosis rates with an increased dietary calcium intake [10].

The variable results in terms of bone mineral density and fracture reduction may arise from factors other than dietary calcium and vitamin D influencing bone metabolism. Among these factors, energy nutrients as well as dysregulation of metabolic pathways as occurs in metabolic syndrome may be important factors.

To our knowledge, the acute effects of dietary calcium on bone metabolism in the context of metabolic syndrome in postmenopausal women have not been evaluated. The present study determined the interaction between a single evening supplemented milk drink and anthropometric and biochemical measures of metabolic syndrome on bone resorption in post-menopausal women. The aim is to investigate if metabolic syndrome or a greater amount of visceral fat influences the impact of dietary calcium on bone resorption in post-menopausal women.

Method

Participant recruitment

Ethics approval as granted by the Royal Adelaide Human Research Ethics Committee (Protocol number 09071; 29th July 2009). Community dwelling postmenopausal women who participated in a non interventional observational study were invited to participate in the current study. They completed a general health, a dietary calcium intake questionnaire and a consent form. Subjects who were taking calcium supplements were instructed to cease the supplements for 7 days prior to participation.

Intervention

On the day of the study each subject had morning fasting blood samples collected for routine electrolytes, liver function, creatinine, urea, total cholesterol, glucose, calcium, phosphate (analysed by Olympus AU 5700, Olympus Japan), parathyroid hormone (PTH,—Immulate 2000, Siemens Healthcare Australia), 25OH vitamin D (25OHD) (IDS-iSYS, Immunodiagnostic Systems, -UK) and C-terminal telopeptide of type I collagen (CTX,—Roche E170, Roche Diagnostics, Australia). Subjects were provided with Pura Tone milk (Pura, Victoria, Australia) (1 L) and a sachet of Milo (Nestle, Australia) (20 g) with a 200 mL plastic measuring cup. They were instructed to measure out 200 mL milk (containing 400 mg Ca, 10 g protein, 13.5 g carbohydrate) and add the sachet of Milo (containing 160 mg Ca, 2.6 g protein, 13.3 g carbohydrate). The drink (containing a total of 560 mg Ca) either warm or cold, was

taken at 9 pm the same night as the blood test. The next morning another set of fasting blood samples were collected from each subject to repeat the assays of the biochemical variables, except for 25OHD. All subjects had a radiocalcium absorption study performed using ^{45}Ca [11] within 12 months of the study and a DXA bone mineral density measurement (Norland XR 36, Swissray International Switzerland) within 6 months of the study. An abdominal fat and lean mass obtained from the lumbar spine DXA, height, weight and waist circumference (WC) were recorded. Subjects—completed a validated food frequency questionnaire to ascertain their habitual dietary calcium intake.

Subjects were divided into 2 groups according to their WC using 88 cm as a cut off with WC > 88 cm indicating metabolic syndrome (ATP III criteria) [12]. WC was chosen to categorise subjects into metabolic syndrome because it compares closely with the BMI and visceral fat. Waist circumference is also the theme of a public education campaign conducted by the Heart Foundation of Australia [13]. The Heart Foundation recommends measuring the waist circumference as an inexpensive and non-invasive method to indicate visceral fat and a predictor of risk for chronic disease such as diabetes and heart disease.

Statistical analysis

A Student's *t* test was performed to determine significant difference between measured variables before and after the supplemented milk drink, and also differences between the metabolic syndrome women and lean women. Linear regression analysis and a correlation matrix were used to determine correlations between variables.

Results

Twenty five postmenopausal women volunteered to participate in the study. Demographic data and anthropometric measurements are given in Table 1.

Their mean fractional calcium absorption was 0.67 (range 0.33 to 1.24; normal >0.5) and mean urine calcium/creatinine ratio was 0.28 (range 0.03 to 0.73; reference interval 0.03–0.4). There was no significant difference in

Table 1 Demographic and anthropometric data for all subjects

Variable	Mean	SD	Range
Age (years)	63	5	51–71
Years since menopause	12	7	1–28
Height (m)	1.62	0.05	1.55–1.71
Weight (kg)	66	9	50–81
Waist circumference (cm)	84	9	67–99
BMI (kg/m ²)	24.9	3	20–31
Habitual daily Ca intake (dietary, mg)	1012	260	642–1605

the fractional calcium absorption, urinary calcium excretion or habitual daily calcium intake between the women with and without metabolic syndrome, as defined by a WC > 88 cm.

Serum CTX levels, a marker of bone resorption, was negatively related to abdominal fat mass (Fig. 1) and serum 25OHD was negatively related to WC ($P < 0.01$) amongst all the subjects at baseline. Biochemical variables before and after the supplemented milk drink for all women are presented in Table 2. Only ionised calcium changed significantly after the supplemented milk.

WC, fasting blood glucose and lipid profiles were available as determinants of metabolic syndrome in these women. Fasting blood glucose of >5.6 mmol/L, triglyceride >1.7 mmol/L and HDL <1.29 mmol/L (as suggested by the American Heart Foundation) [14] were not used because these biochemical criteria did not distinguish between the 2 groups. In support of the use of WC as a marker of metabolic syndrome the change in blood glucose after the supplemented milk drink correlated with WC, abdominal fat mass and body weight ($P < 0.01$).

Table 3 represents the anthropomorphic measures on the two groups based on WC (<88 cm compared with >88 cm). There was no significant difference in the fractional calcium absorption, urinary calcium excretion or habitual daily calcium intake between them. Lumbar spine bone mineral content was increased and bone mineral density tended to be higher in those with a greater WC (≥ 88 cm). These women also had a significantly greater abdominal fat mass, body weight and BMI than those with WC <88 cm. There was no significant difference in height or lean mass between the groups. Table 3 also presents the measured biochemical variables in women with WC <88 cm and with WC ≥ 88 cm.

The changes in measured biochemical variables after the supplemented milk drink in women with and without metabolic syndrome are presented in Table 4. The

changes in bone turnover markers ALP and CTX and the calcitropic hormone PTH in each group after the milk drink are depicted in Fig. 2. Women with a larger WC responded to the supplemented milk drink with a significant fall in CTX levels with the fall in ALP tending to significance. There is no evidence that these changes in bone turnover in women with metabolic syndrome are mediated by changes in PTH.

Discussion

Weight, abdominal fat mass, BMI and lumbar spine bone mineral content were significantly greater in women whose WC greater than or equal to 88 cm. The lumbar bone density was also higher in these women, but the difference did not achieve statistical significance. There were no significant differences in the biochemical variables or daily dietary calcium intake between the 2 groups of women. Serum 25OHD level was negatively correlated with WC implying that overweight or obese states or metabolic syndrome may be associated with lower vitamin D status.

Following the supplemented milk drink, only ionized Ca changed significantly from baseline. After this drink, the fall in CTX was only significant in women with a WC ≥ 88 cm and not in those with a WC <88 cm.

Dietary calcium when ingested with a meal including a dairy drink, is influenced by other co-ingested nutrients likely to influence bioavailability and metabolism of calcium. The effect of carbohydrate on bone metabolism is marked and acts through the increase in plasma levels of incretin hormones, particularly glucagon-like peptide 2 (GLP-2) [15]. Osteocalcin, a protein produced by the bone forming osteoblasts is considered to provide a link between energy metabolism and bone [16]. The absence of osteocalcin reduces beta cell proliferation and leads to glucose intolerance and insulin resistance. Osteocalcin is thought to sensitize adipocytes to insulin via adiponectin,

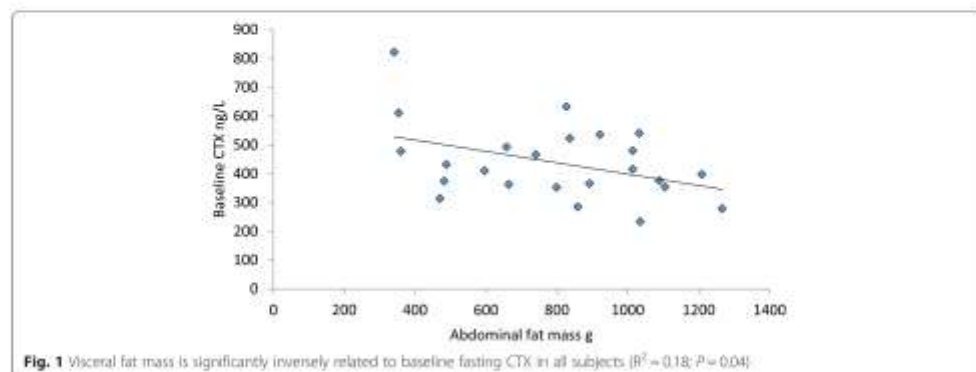


Table 2 Biochemical variables before and after the supplemented milk drink

Variable	Before drink [mean (SD)]	After drink [mean (SD)]	Level of significance
Glucose mmol/L	4.9 (1)	4.8 (1)	0.4
Total cholesterol mmol/L	5.6 (1)	5.6 (1)	0.4
Total Ca mmol/L	2.37 (0.08)	2.4 (0.09)	0.12
Ionised Ca mmol/L	1.21 (0.04)	1.23 (0.04)	0.05*
Phosphate mmol/L	1.18 (0.13)	1.22 (0.13)	0.15
ALP IU/L	75 (19)	71 (20)	0.24
CTX ng/L	432 (132)	416 (143)	0.3
PTH pmol/L	43 (1.1)	4 (1)	0.13
25 OH D mmol/L	88 (26)	NA	NA

*P = 0.05

thereby improving glucose tolerance. These findings suggest that bone turnover is intimately associated with carbohydrate metabolism and insulin sensitivity or resistance.

In addition protein plays a major role in calcium homeostasis, presumably via IGF-1 [17]. Indeed, medium term studies on dietary calcium intakes show a rise in IGF-1 due to protein in cheese with a concurrent fall in bone turnover markers. Very low protein diets result in hypocalcaemia, low fractional calcium absorption and secondary hyperparathyroidism while a high protein diet enhances fractional calcium absorption and consequent urinary calcium excretion [18].

Nutrient intake and caloric intake, among other factors, plays a major role in the development of metabolic syndrome. Metabolic syndrome, due to derangement in various metabolic processes such as insulin resistance, may influence the handling of ingested nutrients.

Obesity may protect mammals from osteoporosis, and bone metabolism may indeed be linked to energy metabolism. This link may be the hormones exerting their effects on both bone and energy metabolism under the influence of the hypothalamus [19]. This concept of a gut-brain-bone axis has led to investigations on the effects of nutrients on enteroendocrine cells, and the effect of their secretions (incretins or gut derived hormones) on bone and other tissue. The proposed link between incretins and bone is an evolving concept in nutrient-dependent regulation of bone turnover. The postprandial fall in bone resorption markers and the rise in bone formation markers (albeit to a lesser extent) has been attributed to the effects of incretins GLP-1 mainly, with GLP-2 playing a minor role [20, 21].

The adipocyte derived hormone, leptin is a major regulator of osteoblast function, and inhibit bone

Table 3 Anthropometric and biochemical measures for the 2 groups of subjects

Anthropometric measurement	Subjects with waist circumference <88 cm; mean (SD) N=16	Subjects with waist circumference ≥88 cm; mean (SD) N=8	P
Lumbar spine BMD g/cm ²	1.039 (0.212)	1.167 (0.115)	0.06
Lumbar spine BMC g	45.35 (9.95)	55.46 (8.75)	0.011*
Lean mass g	1567 (190)	1540 (253)	0.38
Fat mass g	655 (222)	1071 (132)	0.000**
Height m	1.63 (0.05)	1.63 (0.04)	0.47
Weight kg	62.5 (8.9)	73 (5.6)	0.003*
Waist circumference cm	79 (7)	94 (3)	0.000**
BMI kg/m ²	23.57 (2.52)	27.59 (2.38)	0.000**
Fasting glucose mmol/L	4.65 (0.49)	5.34 (1.8)	0.08
Total cholesterol mmol/L	5.48 (0.8)	5.98 (0.8)	0.13
Triglycerides mmol/L	0.99 (0.37)	1.24 (0.43)	0.11
HDL mmol/L	1.74 (0.37)	1.6 (0.14)	0.21
LDL mmol/L	3.23 (0.69)	3.6 (1.23)	0.31

*P < 0.01

**P < 0.001

Table 4 Changes in biochemical variables after the supplemented milk drink in the 2 groups of subjects

Variable	Change after the supplemented milk drink in women with WC < 88 cm; mean (SD)	Change after the supplemented milk drink in women with WC ≥/ > 88 cm; mean (SD)	P
Glucose mmol/L	-0.11 (0.15)	-0.1 (0.36)	0.08
Total cholesterol mmol/L	-0.05 (0.33)	0.09 (0.19)	0.1
PC4 mmol/L	0.03 (0.12)	0.06 (0.04)	0.24
Total Ca mmol/L	0.03 (0.07)	0.02 (0.04)	0.35
Ionised Ca mmol/L	0.02 (0.04)	0.02 (0.03)	0.35
ALP IU/L	-0.9 (5.7)	-10.6 (24.3)	0.07
CTX ng/L	-0.33 (37.46)	-53 (45.2)	0.003*
PTH pmol/L	-0.4 (0.60)	-0.57 (0.66)	0.27

*P < 0.005

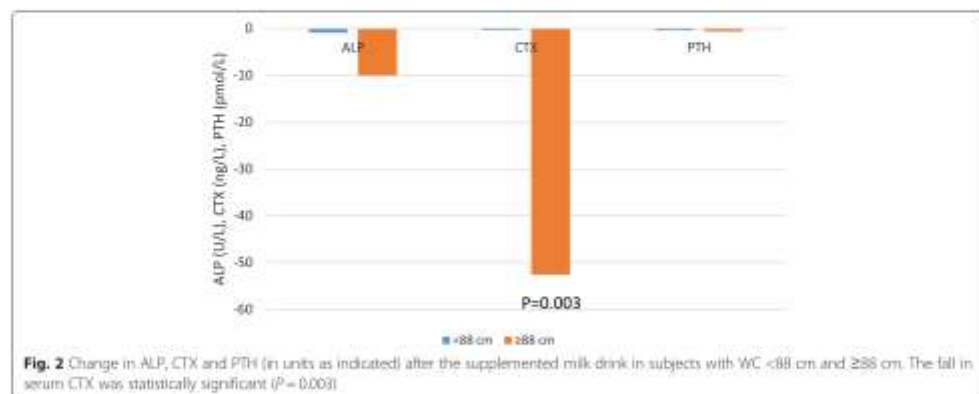
formation in vivo [22, 23]. Leptin deficient or leptin receptor deficient mice display an osteopetrotic skeletal phenotype at an early age with high trabecular bone volume [24], however there was a demonstrable decline in the rate of bone formation (BFR) and resorption (BRR). Interestingly, peripheral administration of leptin corrected BFR and BRR demonstrating that leptin was directly involved in regulating bone turnover. In contrast, in humans, low circulating leptin was associated with low bone density [25].

As in any other endocrine pathway, it can be postulated that adipocytes and leptin production in turn, may be under a feedback regulatory control of bone metabolism. Osteocalcin, an osteoblast specific protein, seems a suitable candidate. Indeed, glucose intolerance and greater amounts of visceral fat were noted in Osteocalcin knockout mice (*Osteocalcin*^{-/-}) [16]. Furthermore, deletion of other genes in osteoblasts in mice led to pancreatic β cell proliferation, a rise in insulin secretion and sensitivity that protected against obesity and diabetes, demonstrating at least the capacity of osteoblasts to contribute to energy

metabolism. It follows then that deranged energy metabolism, as in the metabolic syndrome with glucose intolerance and greater visceral fat, may influence bone metabolism. We hypothesise that responses of bone metabolism to dietary calcium may differ between women with and without metabolic syndrome.

Conclusions

Given that there were no differences in calcium absorption or the rate of urinary calcium excretion between the 2 groups of women, the findings reported here suggest that metabolic syndrome may have influenced the fall of CTX in response to dietary Ca. There was a trend for baseline CTX to be lower in women with higher visceral fat. These findings are consistent with previous proposed theories of obesity protecting against osteoporosis presumably via leptin and hypothalamic feedback, closely associated with energy metabolism [19]. Our data also imply that bone turnover in overweight women or women with metabolic syndrome was more sensitive to dietary calcium intake.



Competing interests

The authors declare that they have no competing interests.

Authors' contributions

BECH designed the experiment, SDCT conducted the experiment, analysed the data and drafted the manuscript, HAM helped analyse the data and contributed to the manuscript. SDCT and HAM read and approved the final manuscript.

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Chapter 11

Conclusions

Osteoporosis remains one of the most important public health concerns in the world. The burden of osteoporosis on society and economy is likely to rise with an aging population. Post-menopausal women are affected by osteoporosis due to oestrogen deficiency and other factors including genetic inheritance. Prevention of osteoporosis in this group is likely to increase their quality of life and productivity.

The most inexpensive and accessible preventative measures of osteoporosis are adequate calcium, vitamin D and exercise. There is strong evidence that calcium supplementation with or without vitamin D is effective in the prevention of fractures albeit more effective with vitamin D. The evidence for dietary calcium preventing fractures is not as strong. Additionally shorter term studies investigating the effects of calcium supplementation in increasing bone density or preventing loss of bone report variable results.

The data obtained from this study suggest that, despite public health campaigns promoting higher calcium intakes for post-menopausal women, most community dwelling women did not meet their daily requirement of calcium, consistent with published data for Australian women. However, the majority of them were vitamin D sufficient, consistent with reports for Australians over the age of 50 years.

The major finding from this study suggest that higher intakes of calcium (dietary sources with or without supplemental calcium) in the short term (ie: in 2 years) does not increase or prevent the loss of bone at the lumbar spine and the hip. The ultradistal forearm, with a greater proportion of trabecular bone may respond to higher calcium intakes and the changes may be detectable by bone density.

Two of the main findings of this study are that women with higher BMI and more visceral fat had higher bone densities compared to those with lower

BMI, and women with metabolic syndrome (higher waist circumference and more visceral fat) demonstrated a greater impact on bone resorption from a dietary calcium load. This is despite similar calcium intakes, lower 25OHD and higher PTH than women with lower BMI. They suggest that women with more visceral fat may require less calcium than lean women to maintain bone. Overall these results suggest that there is an important interaction between fat, bone metabolism and dietary calcium which may have implications on the calcium requirement. The biochemical processes involved in the gut-brain-bone axis require further investigation with PTH as a key hormone. The effect on weight gain and weight loss on PTH, and the effect on and response of PTH in anorexia and other catabolic states that lead to loss of fat as well as bone requires further investigation.

The findings of this study suggest links between bone and energy metabolism, consistent with a growing body of evidence. Firstly, from a biochemical point of view, hormones affecting bone also affect fat cells. PTH has consistently been shown to be higher in men and women with greater body weight or BMI. Likewise hormones such as leptin and adiponectin traditionally assigned to adipocyte metabolism are now believed to be involved in bone metabolism. Secondly, women with higher BMI consistently demonstrate a greater bone density independent of their reported calcium intakes. Thirdly, as shown in Chapter 10, women with metabolic syndrome appeared to respond to a dietary calcium load more favourably than those without, in that, the rate of bone turnover showed a greater degree of suppression as a response to a calcium load in these women compared to lean women. This may be due to the link between energy and bone metabolism, and also suggests that adipocytes may play a role in the response to dietary calcium. The theoretical gut-brain-bone axis suggests that incretins released in response to certain nutrients may have an impact on bone turnover. Thus dietary loads of calcium when given together with carbohydrate, protein and fat, may lead to the release of incretins that have an influence on calcium homeostasis.

The women recruited to this study were healthy, independently living individuals. They enrolled in the study because they were 'health conscious' and wanted to be informed of their bone density. Although no feedback was given to them about their dietary calcium intakes, the baseline bone density was reported to them with an explanation of their fracture risk. Their calcium intake did not change significantly. Less than half of the women diagnosed with osteoporosis increased their calcium intake by increasing dietary intake rather than adding a supplement. This may be due to the negative publicity calcium supplements received at the time, due to the publication of cardiovascular effects of calcium.

There are several limitations of this study. Firstly, at baseline, there was no significant difference between bone densities of women with adequate or lower calcium intakes. This suggests either the dietary calcium estimation may not be adequate or does not reflect the long term diet, or that dietary calcium may not have played a major role. However, when the effects of obesity and metabolic syndrome on bone are considered the role of dietary calcium may become clear. Secondly, only the bone density at the ultradistal forearm showed any difference between high and low calcium intake women after 2 years. Technical issues such as positioning of the patient affects the femoral neck and total hip density measurements and may have introduced variability to the serial measurements, and osteoarthritis and other factors may have affected the lumbar spine measurement. The forearm is the least affected by positioning and may have been the most consistent measurement taken. In addition, 2 years may not be a sufficiently long time to detect major changes in bone density, and had the study continued for a longer period changes may have been detected at other sites.

Thirdly the dietary calcium estimate may not be accurate due to the exclusion of a number of common food items. The online questionnaire was very extensive and many of the participants reported that it was difficult to complete in one sitting. There may have been instances when they 'glossed over' some questions which may have led to an over- or under-estimation of

their intakes, which also introduced inconsistency between the 2 questionnaires.

In conclusion, the ultradistal forearm density is more likely to show shorter-term changes in response to calcium intakes, however this site is not recommended for the diagnosis of osteoporosis by the International Society of Clinical Densitometrists (ISCD). The response to dietary calcium by bone is modulated by energy metabolism, those with metabolic syndrome demonstrating more benefit in terms of reduced bone turnover. Women with lower calcium in the diet appeared to be more likely to be overweight or obese, suggesting an overall poorer diet, lifestyle or suggesting a role for calcium in the maintenance of healthy weight.

The prevalence of osteoporosis in the study subjects was consistent with that published by a self-reported survey but much lower than the prevalence reported for an unselected Australian population. The average calcium intake in the women in the current study is higher than reported for the unselected Australian population therefore may not reflect the average calcium intake of Australian post-menopausal women. This suggests that the conclusions drawn from this study may be relevant to the community dwelling post-menopausal women who are health conscious and free of chronic disease.

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APPENDIX 1 - Information to Volunteers



Government of South Australia

Central Northern Adelaide
Health Service

ROYAL ADELAIDE
HOSPITAL

North Terrace,
Adelaide, SA 5000

Protocol Name: **Study of the relationship between dietary calcium and the rate of bone loss in postmenopausal women**

Investigators:

Dr S. Devika Thomas

Trainee Medical Officer

Division of Chemical Pathology, SA Pathology

Prof B.E. Christopher Nordin

Consultant Physician, Endocrine and Metabolic Unit

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Dr Penelope S. Coates

Clinical Director, Consultant Endocrinologist

Division of Chemical Pathology, SA Pathology

1. **Voluntary Study**

You have been invited to join in a study of the relationship between dietary calcium and bone loss after the menopause. This is a research

project and you do not have to be involved. If you do not wish to participate, your medical care will not be affected in any way.

2. **Introduction**

The evidence for a relation between calcium in the diet and the rate of bone loss after menopause is inconsistent. In this study, we will attempt to establish the relation between dietary calcium and the rate of bone loss in women like you.

We will estimate your dietary calcium from a questionnaire which is enclosed. If you decide to participate in this study we will ask you to come back one morning without breakfast for a full blood and urine test and measurement of your calcium absorption with radiocalcium. We will then measure your bone density either on the same day or another day. If the bone density is too low you can withdraw from the study to have treatment. Otherwise we will ask you to stay on your usual diet and your bone density will be checked every 6 months for 2 years. Another blood test (without the calcium absorption or the urine test) will be performed between 12 months and 24 months from the start of the study.

3. **Possible benefits from the study**

This study will be of benefit to you because it provides you with a bone density assessment and a lot of information about yourself. Copies of the report can be sent to your General Practitioner if you wish. If the initial bone density measurement shows that you have osteoporosis then you will be withdrawn from the study and given the option of attending the

Osteoporosis Clinic in the Royal Adelaide Hospital if you and your doctor wish it. Any abnormalities in the blood tests will also be communicated to you and your doctor so that you can be treated. If your bone density below expected values during the course of this trial this will be communicated to your General Practitioner.

4. **What you will be asked to do**

(1) If, after reading the Information sheet you decide to take part in the study you will be asked to complete a food questionnaire either online or by mail. After this you will be given an appointment to attend the Endocrine Test Unit at the Royal Adelaide Hospital for an information session (45 minutes) during which you can ask questions and also sign a consent form. You will then be given an appointment to attend the test unit after an overnight fast, blood and urine specimens will then be taken. You will then be given a drink (about 1 cup) containing radiolabelled calcium. Another blood sample will be taken 1 hour later to assess the amount of calcium you absorb. The bone density measurement may be done on the same day (or on another day convenient to you).

(2) After this initial visit, you will be contacted by telephone to arrange the next visit, which will only a bone density measurement and a food questionnaire (6 months).

The following table explains what is involved in each visit after you have consented to take part in the study.

	1 st visit	2 nd visit	3 rd visit	4 th visit	Final visit
Timing:	start	6 months	12 months	18 months	2 years
Blood test:	Yes	No	Yes	No	No
Urine test:	Yes	No	No	No	No
Calcium drink:	Yes	No	No	No	No
Bone density:	Yes	Yes	Yes	Yes	Yes
Food	Yes	Yes	Yes	Yes	Yes

questionnaire:

(3) It is important that you do not change your diet during this study. However, you can continue medication if it does not affect your bones. If you start any new medication please let us know as soon as possible.

5. Payment

We will cover your travel and parking expenses. No further payment is available for the participation in this study but you will be provided with information gained from the tests.

6. Possible risks from the Study

i. Blood samples are drawn from the vein on the inside of your elbow. Some bruising and swelling can occasionally occur and will be treated at the test unit.

ii. This research study involves exposure to a very small amount of radiation. As part of every day living everyone is exposed to naturally occurring background radiation and receives a dose of about 2 millisieverts (mSv) each year. The effective dose from this study is

about 0.33 mSv. At this dose level no harmful effects of radiation have been demonstrated as any effect is too small to measure. The risks are believed to be very small. You should not be pregnant at the time of any radiation test (absorption study and the bone density measurements). If you are pregnant or planning to become pregnant please let us know as soon as possible.

iii. You will receive dietary advice and other information regarding prevention of osteoporosis at the end of the study.

7. Confidentiality

The data gathered in the trial will be securely stored for 15 years. All data points will be deidentified (ie given a number or symbol rather than your name). If the results are published in a scientific journal all data will be similarly deidentified and your identities will be kept confidential.

8. Further information

If you have any concerns or need further information about this study, please contact Dr Thomas (telephone 8222 3611 or 0401120720).

If you wish to speak to someone not directly involved in the study about your rights as a volunteer, or about the conduct of the study, you may also contact the Chairman of the Human Ethics Committee, Royal Adelaide Hospital, on 8222 4149.

APPENDIX 2 – letter to volunteers

Dr Devika Thomas
Chemical Pathology
IMVS, level 2
Frome road
Adelaide
08 82223611
0401120720

28/06/2010

Dear Mrs Smith

Re: Diet and Bone Density Study

Thank you for your interest in our study looking at the link between your diet and your bone density. I have enclosed an information sheet and 2 questionnaires. Please read the information carefully and if you would like to participate in our study, fill out the diet and general health questionnaires. Please return these 2 questionnaires in the stamped envelope.

Upon receipt of the questionnaires I will contact you to make appointments for your bone density scans and blood test.

If you have any queries please do not hesitate to contact me.

Thank you

Yours sincerely

SDC Thomas

APPENDIX 3 – Brief Health Questionnaire

ROYAL ADELAIDE HOSPITAL

Comparison of the effects of calcium tablets alone with effects of calcium and vitamin D or vitamin D alone on bone resorption in postmenopausal women

Name.....

Address.....

Telephone number.....

Date of birth.....

Date of last menstrual period.....

Was your menopause surgical or natural?.....

If surgical, were the ovaries removed?.....

Current medications.....

Have you had any treatment for bone loss?.....

If yes, when did it stop?.....

Have you any current illnesses?.....

Have you had any hormone replacement therapy?.....

If yes, when did it stop?.....

Please place this completed questionnaire in the envelope supplied together with the completed “Dietary Calcium and Physical Activity Questionnaire” and mail to Dr Thomas as indicated on the envelope.

APPENDIX 4 Short Questionnaire for estimating daily calcium intake

DIETARY CALCIUM QUESTIONNAIRE

Name:.....

Date:..... Date of Birth:..... Sex:F/M

This questionnaire has been designed to assist in estimating your dietary calcium intake. While your food intake may vary from time to time, try to give answers that reflect your normal, ‘yearly average (summer/winter)’ intake to the questions asked. If the ‘standard serve’ size given in some of the questions does not reflect your usual normal serve size, adjust your normal serve size to reflect the standard serve size (eg. Two of your ‘normal’ large (300mL) glasses of milk per week would become three ‘standard’ serve sizes (200mL) of milk), or please make a comment near the question.

Please read carefully before answering the questions. Then be as accurate as you can be and give an answer to **every** question on both sides of the paper.

For office use only

<p>1. Do you have an intolerance or allergy to any foods? Yes / No (Please circle the correct answer) If ‘Yes’, what foods?</p>	
<p>2. What type of milk do you usually buy? (eg: wholemilk, skimmer, tone, Goats’ milk, soy milk)</p>	
<p>3. How many of the following beverages do you have during One Day? a) Tea _____ c) Herbal Tea _____ b) Coffee _____ d) Coffee substitute _</p>	
<p>4. Do you have milk in the following beverages? (please circle the correct answer) a) Tea Yes / No c) Herbal Tea Yes / No b) Coffee Yes / No d) Coffee substitute Yes / No</p>	
<p>5. How many days of the week do you have breakfast cereal, porridge or muesli? _____ days per week</p>	
<p>6. How many cups of milk do you usually add to your breakfast cereal, porridge or muesli? (please circle the correct answer) None ½ cup 1 cup 1 ½ cup 2 cups</p>	
<p>7. How many of the following drinks do you have in One</p>	

<p>Week?</p> <p>a) Glass of milk (200 mL) _____</p> <p>b) Glass of milk based drinks (200 mL) _____ (eg: flavoured milk, milkshake, hot cocoa, Milo)</p>	
<p>8. How many serves of the following foods do you have in One Week?</p> <p>a) Cheese (all types) - 1 slice (30 gram) _____</p> <p>b) Yoghurt – 1 carton (200 gram) _____</p> <p>c) Custard or milk based puddings – ½ cup (eg: Blancmange, junket, mousse, cheese cake) _____</p> <p>d) salmon, mackerel, sardine – ‘1 serve’ _____</p>	
<p>9. Do you add salt:</p> <p>a) when cooking and preparing meal ? Yes / No</p> <p>b) to your meal at the table ? Yes / No</p>	
<p>10. Do you take any calcium supplements ? Yes / No If ‘Yes’ what type, how much, how often and when?</p>	

For Office Use

#. Baseline for regular, balanced diet = 300mg calcium

Q3./Q4. – Milk in each beverage = 35mg Ca.

Q6. ½ cup = 150mg Ca. 1 cup = 300mg Ca.

Q8. a) 30gram = 200mg Ca

b) 200gram = 350mg Ca

c) ½ cup = 125mg Ca

d) 1 serve = 200mg Ca

Q7. 200mL = 240 mg Ca.

[Ca = calcium]

**APPENDIX 5 – letter to volunteers after completion of Brief
Health Questionnaire**

Study of the relationship between bone density and diet.

Thank you for completing the surveys for the above project. Your personal details will be stored for the purposes of contacting you for future appointments. All results obtained will be numbered so your identity will remain confidential.

The blood tests (includes a **urine test**) take 1 hour and are done at the RAH. They can be done any day of the week (except Thursdays), They are done first thing in the morning, starting anytime from 0800. You need to fast from the night before from about 10 pm. You are allowed water until about 7am on the day of the test. If you take multivitamins or calcium tablets please hold off on them the day before and the day of the test. All other medication is OK.

The tests are done on level 9. You need to come in via the Main Entrance on North Terrace, and walk past the escalators and take the yellow Out Patient Lift (located behind and to the left of the escalators) to level 9. As you exit the lift turn left and you will see a small sign to the Endocrine Test Unit. The tests are done there and I will meet you there.

You can directly book the blood test at the Royal Adelaide hospital by ringing **82225505**. Alternately if you wish to have your tests done at your local IMVS collection centre, let me know via email and I can post you a blood test request form and contact information for them. Please tell them you are coming for the **calcium absorption** as well as part of the research study, so that they can have the drink prepared for you. (they will need a few days' notice). Let me know when you have made the appointment for (if coming to RAH).

If you prefer, you can have your blood tests done at a local IMVS centre. You need to ring them and make the appointment quoting

calcium absorption test, with 2 days notice. There is also a urine test, and a container will be provided to you when you arrive for the test. I have enclosed the phone numbers for IMVS collection centres.

For Bone density scans you need to ring the bone density unit directly on **82225520** and speak to Jody or Fiona so that you can make that appointment at a time convenient for you. It takes about half an hour and they are done on level 7 in the RAH. They are located in the Emergency block and you will be given direction. When you have done your bone density scan please make sure that you make another appointment to come back in 6 months for your second appointment. There are a total of 5 bone densities, one every 6 months.

You may be able to book the blood tests and the bone density on the same day.

When you have made the bookings, please let me know when you are coming in for them.

If you have any difficulties with the appointments, let me know and I will try and sort them out.

Alternately, I can book these for you. This may involve phone calls and emails back and forth because I will be liaising between you and the respective departments.

