NUTRIENTS AND BONE MINERAL DENSITY IN POSTMENOPAUSAL WOMEN

by

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DEDICATION

To my family who has always supported me unconditionally and continually challenges me to achieve my best . . .

THANK YOU, I LOVE YOU!

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ABSTRACT

This dissertation's three studies investigated the short and long-term relationships of bone-related nutrient intakes with bone mineral density (BMD) in postmenopausal women. This dissertation compared the equivalency of dietary intakes assessed by eight days of diet records (DR) and the Arizona Food Frequency Questionnaire (AFFQ) at one year. It also determined the association of one year (DR) and the average of four-year (AFFQ) dietary intakes with cross-sectional BMD. The dietary intake associations with BMD were further investigated by hormone therapy (HT). Participant's BMD was measured at the lumbar spine (L2-L4), femur trochanter, femur neck, Ward's triangle and total body using dual energy X-ray absorptiometry. Separate multiple linear regression analysis ($p \leq 0.05$), controlled for various covariates, were used to examine the associations between dietary intakes and regional and total body BMD. In study number one (n=266), significant correlations (r=0.30-0.70, $p \leq 0.05$) between dietary assessment methods were found with all dietary intake variables. Iron, magnesium, zinc, dietary calcium, phosphorous, potassium, total calcium, and fiber intakes were positively associated with BMD at three or more of the same bone sites regardless of the dietary assessment method at one year. In study number two (n=266), femur trochanter, lumbar spine, and total body BMD had mostly significant inverse associations with dietary polyunsaturated fatty acid (PUFA) intake at one year. In the HT group (n=136), inverse associations with dietary PUFA intake were seen in the spine and total body BMD. In study number three (n=130), average dietary intake of selected bone-related nutrients, were significantly inversely associated with lumbar spine BMD and total body BMD at

year four. In the HT group (n=92), inverse associations with dietary PUFA intake were seen in the spine and total body BMD. The DR and AFFQ are acceptable dietary tools used to determine the associations of particular nutrients and BMD sites in healthy postmenopausal women at one year. At one and four year, dietary PUFA intakes had mostly inverse associations with lumbar spine and total body BMD. When categorized by HT use the associations remained significant only in the HT groups, suggesting that HT may influence dietary intake associations with BMD.

INTRODUCTION

Osteoporosis, or porous bone, is a disease characterized by low bone mass and strength and the deterioration of the integrity of the structure of bone tissue, resulting in fragile bones and an increased risk of fracture. Osteoporosis is a major public health concern. An estimated 34 million Americans with osteopenia (low bone) are at risk for osteoporosis, and another 10 million have osteoporosis. The prevalence of osteoporosis is expected to increase as the population ages. One in two women 50 years and older will experience an osteoporotic fracture in their lifetime (NOF, 2008).

Past research has shown that there is a link among non-modifiable risk factors such as gender, age, family history of osteoporosis or related fractures, frame size, race/ethnicity, low sex hormones (estrogen and testosterone) and modifiable lifestyle risk factors such as diet (low calcium and vitamin D intakes, excessive intake of protein, sodium and caffeine), inactive lifestyle, smoking, alcohol abuse, and certain medications (NOF 2008). Previous research has suggested that the most modifiable lifestyle risk factors include diet and exercise.

The specific purpose of this dissertation research was to investigate the associations between dietary nutrient intakes and bone mineral density (BMD) in postmenopausal women participating in the Bone Estrogen Strength (BEST) training study over four years. The BEST study investigated the effect of exercise on BMD in healthy, postmenopausal women (Going 2003). Participants were categorized by use of hormone therapy (HT) and then randomized to exercise or control conditions. They were provided with and requested to consume 800 mg of calcium citrate supplements each day

during the trial to minimize variability in calcium intake.

The three studies in this dissertation were completed using cross-sectional data that was collected from the first (Fall 1995-1997) through the fourth (Fall 1995-2002) years of the BEST study, a block- randomized, clinical trial. Year one dietary intake was assessed from eight randomly assigned days of diet records (DR) throughout the year. Dietary intake was assessed at the end of the first year and annually thereafter using the Arizona Food Frequency Questionnaire (AFFQ) to capture the overall pattern of food intake during the previous 12 months. The BMD was measured at the femur neck, femur trochanter, lumbar spine (L2-L4), and total body using dual energy X-ray absorptiometry (Going 2003, Cussler 2005). The University of Arizona Institutional Review Board (Human Subjects Committee) approved the study and the subjects provided written and informed consent.

The original grant of the BEST study stated that the following list of nutrients would be studied because of their established relationship with BMD in previous research (Ilich 2000, Palacios 2006): energy, protein, carbohydrate, fat, alcohol, vitamin A, vitamin C, vitamin E, vitamin D, B-carotene, calcium, phosphorous, iron, magnesium, sodium, potassium, zinc, caffeine, fiber, cholesterol, and zinc. For study number one and three, the list of nutrients was truncated to include all of the above nutrients except for cholesterol, vitamin A, and beta-carotene. Vitamin A and beta-carotene, a precursor of Vitamin A, were eliminated from the list of nutrients studied because vitamin A is really a group of substances that are chemically known as carotenoids (retinoids, retinol, retinaldehyde, and retinoic acid). The activity of these caratinoids differs in the body

making it difficult to study each caratinoids activity (Bonnick 2000). Cholesterol was dropped from the analyses since it failed to show significance in any of the initial correlation analyses. For study number two, polyunsaturated fatty acids (PUFA) were selected because current research showed an association with BMD in postmenopausal women (Weiss 2005, Macdonald 2004). The following nutrients served as the independent variables: total polyunsaturated fatty acid (PUFA), total n-6 fatty acids (n-6 FA), linoleic acid (LA), arachidonic acid, total n-3 fatty acids (n-3 FA), alpha linolenic acid (ALA), and the ratios: LA:ALA, and n-6 FA:n-3 FA. In study number three, the same nutrients from study number one and two were used with the inclusion of vitamin B_{12}, docosahexaenoic acid (DHA), docosapentaenoic acid (DPA), eicosapentaenoic acid (EPA). These nutrients were included because of previous and current research showing an association with BMD (Palacios 2006, van Meurs 2004, Ilich 2000, Krugar 1998).

In all three studies, participants were not excluded from the total sample based on implausible energy intake as described by Harris *et al.* (2003) in order to maintain sample size. The following is a literature review of the nutrients studied in this dissertation and their relationship with bone (Frankel 2007).

MACRONUTRIENTS

Energy

While many Americans focus on reducing their energy intake in order to maintain their body weight, animal studies suggest that caloric restriction significantly reduces the rate of aging as well as increasing life span (Roberts 2001). It is well known that there is a positive association between body weight and BMD, and that increased energy intake

will cause an increase in body weight. It has been suggested that excess weight increases BMD because it acts in a similar manner as weight bearing exercises on bone (Felson 1993, Harris 1996). Furthermore, weight loss of 10% has been shown to decrease bone mass by 1 to 2% (Compston 1992, Hyldstrup 1993, Svendsen 1993). It is difficult to discern whether it is the weight loss, or other factors such as low macro and micronutrient intake, that cause the decrease in BMD (Haussler 1997). Specifically in people suffering from anorexia nervosa, there is an increase in incidence of bone loss, which is caused by an increase in bone resorption as well as impaired bone structure (Lennkh 1999). In people with anorexia, bone loss has been attributed to a number of features including amenorrhea, hypoestrogenemia, low body mass index (BMI), poor nutrient intake and low levels of lean and fat mass.

A number of studies have been conducted in animal models, which show the effect of calorie restriction on bone metabolism. These studies have found that initial body weight or weight loss may be a key to understanding this relationship (Black 2001, Cifuentes 2002). A study in rats examined the effect of energy restriction in both lean and obese rats, on calcium absorption and markers of bone metabolism (Cifuentes 2002). Regardless of whether the rats were initially lean or obese, a 40% reduction in energy intake, over a 10-week period, led to a decrease in calcium absorption. Only the lean rats given the energy-restricted diets saw a decrease in serum estradiol, which was associated with decreased calcium absorption in the lean rats. Despite the effect on bone markers, there was no effect on bone turnover in the rats given the energy-restricted diets. Due to these results, the authors suggested that the effect of energy restriction on calcium

metabolism may depend on initial body weight, and may be caused by a decrease in estradiol levels. One study examined the effect of calorie restriction on BMD, bone mineral content (BMC), body composition, markers of bone turnover, as well as hormones, in a group of male rhesus monkeys over an eleven-year period (Black 2001). The results of the study showed that a decrease in BMC and BMD in the calorie restricted monkeys was due to lower body weight, in particular, lean mass, since no change in osteocalcin or urinary pyridinium cross-link excretion was seen. In addition, no changes in calcium homeostasis were seen in the monkeys with the calorie restricted diet, as well as no difference in the concentration of 1,25 $(OH)D_2$.

While not much research has been conducted directly investigating the effect of calorie restriction on bone metabolism in humans, one study did review this relationship. Ihle and Loucks explored the dose-dependent relationships between energy availability and markers of bone turnover in a group of young women (Ihle 2004). Surprisingly the results of the study found that each of the bone markers, urinary N-telopeptide (NTX), serum type I procollagen carboxy-terminal propeptide (PICP) and plasma osteocalcin (OC), all depended on energy intake differently; when energy intake was extremely restricted, NTX, a measure of bone resorption, increased; while both PICP and OC were depressed at any level of energy restriction. These results suggested that even a little decrease in energy intake will cause impairment in bone formation, but a larger decrease in energy intake is necessary to affect bone resorption.

Dietary Fat

Lipids have a number of functions in the body including: barriers, sensors, energy source, electrical insulation, and biological detergents (Stipanuk 2000). While none of these functions appear to have a direct effect on bone, several mechanisms have been proposed, suggesting dietary fat's effect on bone. The first potential mechanism is that dietary fat affects intestinal calcium absorption; calcium absorption is increased when essential fatty acid deficiencies are corrected or diets are supplemented with dietary fats, such as primrose oil or fish oil (Corwin 2003). Another possible mechanism is due to growth hormone (GH), which is involved in bone formation but is inhibited by increased levels of free fatty acids. In addition, prostaglandin E1 (PGE1) and prostaglandin E2 (PGE2) are derived from omega-6 fatty acids and at high concentrations. The PGE2 is believed to inhibit bone formation while PGE1 has been shown to induce bone formation. Finally, dietary fat intake is believed to inhibit the formation of osteoblasts, which leads to uncoupling of resorption and formation of bone, causing a loss of bone tissue (Nuttall 1998).

Animal studies in a variety of species suggest that dietary fat intake may play a role in bone health (Mollard 2005, Watkins and Lippman 2000, Watkins 1997). A recent study by Mollard *et al*. (2005) looked at the effects of linoleic acid, α-linolenic acid, EPA and DHA on bone mass and markers of bone metabolism, while keeping the total PUFA intake constant, in obese and hyperinsulinimic rats and a control group (Mollard 2005). The obese and hyperinsulinemic rats had reduced femoral head and proximal epiphysis compared with the lean rats, leading the researchers to believe that the obese rats would

be more likely to experience fractures in these areas. In addition, omega-3 fatty acids were shown to reduce PGE_2 release; in other studies, the reduction in PGE_2 has been associated with bone formation (Watkins and Lippman 2000, Watkins 1997). While some animal studies have shown that omega-3 and omega-6 fatty acids are beneficial for bone health, other studies have shown that supplementation with decosahexonoic acid caused oxidative DNA damage in rats (Corwin 2003). Similarly, a study, which gave rats large doses of gamma-linolenic acid (GLA) and linoleic acid, caused a reduction in tibia biomechanical properties (Umegaka 2001).

A variety of studies have been conducted in humans to examine the effect of fatty acid intake, and more specifically type of fatty acid intake, on bone and have given conflicting results (Macdonald 2004, Kruger 1998). A longitudinal study by Macdonald *et al.* (2004) looked at the relationship between BMD, at the lumbar spine and femoral neck, and nutrient intake, as assessed by food frequency questionnaire (FFQ), in a population of 891 women over a 5-7 year period (Macdonald 2004). Increased intake of polyunsaturated and monounsaturated fatty acids was significantly associated with greater loss in BMD at the femoral neck. The same relationship was seen with retinol and vitamin E, both of which were highly correlated with polyunsaturated fatty acid intake. No relationship was seen between saturated fat intake and BMD, which the authors suggested was due to the high calcium intake in dairy foods, which are also high in saturated fat.

Epidemiological studies, such as data from NHANES III, suggest that diets high in fat, particularly saturated fat, reduced BMD and increased fracture risk (Corwin 2003).

A study by Kruger *et al.* (1998) examined the effect of supplementation with omega-3 and omega-6 fatty acids in elderly women already diagnosed with osteoporosis or osteopenia (Krugar 1998). The women in the study were either given a supplement containing both evening primrose oil and fish oil or as a control, coconut oil. Women who were given the supplement containing the evening primrose oil and fish oil had a decrease in bone loss in the lumbar spine, and increased bone density in the femur compared with the women given the coconut oil supplement. However, since the control group were given coconut oil, which is a saturated fat, it is difficult to determine if the unsaturated fatty acids were beneficial or if they just appeared beneficial when compared with the saturated fat. It is possible that the varying affects of fats on BMD and fracture risk may be due to differences in ages as well as the various controls used (Corwin 2003).

Protein

As part of the bone matrix for collagen structure, not surprisingly protein plays a role in bone function (Palacios 2006). In addition, protein is also necessary for the production of hormones and growth factors that are involved in bone synthesis. Since research has shown that a high protein intake causes hypercalciuria, increasing protein intake by 50 grams will cause an extra 60 mg of calcium to be excreted in the urine, it has been hypothesized that this would lead to bone resorption and eventually cause bone loss and osteoporosis (Melhus 1998). While it has been shown that a deficiency in dietary proteins will cause a decrease in bone mass, microarchitecture, and strength, there is still a belief that high dietary protein intake, particularly from animal sources, can also be a

risk factor for osteoporosis (Bonjour 2005). It is also hypothesized that a high intake in animal protein will lead to a metabolic acid load, which will cause dissolution of bone; this also explains the increased levels of calcium seen in the urine in people consuming high protein diets. In general, it has been suggested that low protein diets interfere with intestinal Ca absorption and IGF-1 levels and high protein diets induce excess urine Ca loss (Ilich 2000).

Studies in people of a variety of ages have examined the effect of dietary protein intake on BMD and risk for hip fracture. A recent study investigated six months of protein supplementation on bone and various bone markers, in a population of 52 healthy people ages 18-25 years, involved in a strength and condition exercise program (Ballard 2005). The subjects in the study received either a protein supplement containing 42 grams of protein, 21 grams of carbohydrate, 1.5 grams of fat and 280 kcal, or a carbohydrate supplement containing 70 grams of carbohydrate and 280 kcal, twice a day for 6 months. Regardless of their supplementation, the subjects continued to consume their normal diet, which led to a total protein intake of 1.1 grams of protein/kg of body weight in the group provided the carbohydrate supplement, and 2.2 grams of protein/kg of body weight. Subjects given the protein supplement had higher plasma IGF-1 and BAP (B-activator protein) concentrations than those given the carbohydrate supplement. There were no significant differences between the two supplemental groups in bone geometry and volumetric and areal BMD (Ballard 2006).

A case-control study in Utah looked at the effect of dietary protein intake on osteoporotic hip fracture in 2,501 people (1,167 with hip fracture and 1,334 control

subjects) ages 50-89 years old (Wengreen 2004). The results of the study showed that dietary protein intake was significantly associated with a reduced risk of osteoporotic hip fracture, but only in men and women ages 50-69; in the subjects ages 70-89, there was no association seen between dietary protein intake and risk for hip fracture. Therefore, it was concluded that the effect of dietary protein intake on risk for hip fracture is dependent on age. However, a study by Devine *et al*. (2005), examined the effect of protein consumption, as determined by a semiquantitative FFQ developed by the Anti Cancer Council of Victoria, on qualitative ultrasound of the heel and BMD of the hip in a population of 1,077 Australian women over the age of 70 (Devine 2005). A significant positive association was seen between dietary protein intake and both qualitative ultrasound of the heel and BMD. After creating tertiles of protein intake, it was observed that the women with the lowest protein intake (<66 g/day) had significantly lower qualitative ultrasound of the heel as well as BMD when compared with the women with the highest protein intake (>87 g/day). However, it is important to note that high protein intake was associated with a high intake in calcium due to the consumption of dairy foods.

Since it has been suggested that a high intake of animal protein will cause an acid load (Bonjour 2005), several studies investigated the effect of animal protein versus plant protein on bone health (Weikert 2005, Yoon 2006). An animal study compared the soy protein to animal protein ratio on bone metabolism in rats fed high soy, middle soy, low soy or no soy diet and found that the rats fed either the low soy or no soy diet had significantly higher urinary calcium excretion, while the rats fed the high soy or medium

soy diet had a significantly higher retention of calcium in their bodies (Yoon 2006). In addition, both the ash level and BMD were highest in the medium soy group. This suggests that optimal soy to animal protein ratio was seen in the medium soy group, who had a ratio of 1:1.

Table 1. Summary of macronutrient relationships with bone.

MACRONUTRIENTS DRI[1]/NHANES[2]	Function Related to Bone
Energy (calories) DRI: based on age/gender/Physical activity level NHANES: 1718	Increase in weight gain increases BMD, excess weight may act as weight bearing exercise. A 10% weight loss decreases bone mass by 1-2%. Malnutrition (disordered eating) decreases bone as a result of inadequate intake of macro and micro-nutrients.
Carbohydrate (% of total calories from) DRI: 45-65 NHANES: 48	
Fat (% of total calories) DRI: 25-35 NHANES: 36%	Fat inhibits the formation of osteoblasts, which leads to uncoupling of resorption and formation of bone, causing loss of bone tissue. Growth hormone is inhibited by high levels of free fatty acids. Prostaglandin E1 (PGE1) and prostaglandin E2 (PGE2) are derived from omega 6 fatty acids. PGE2 may inhibit bone formation, while PGE1 induces bone formation.
Linoleic Acid (n-6) (g) DRI: 11 NHANES: 13	
Linolenic Acid (n-3) (g) DRI: 1.1 NHANES: 1.3	
Protein (% of total calories) DRI: 10-15 NHANES: 16	Protein is part of the bone matrix for collagen structure. Protein produces hormones and growth factors (e.g. IGF-1) involved in bone synthesis. Low protein diets interfere with intestinal calcium absorption and IGF-1 levels and high protein intakes induce excess urine calcium loss.

[1]Dietary reference Intake (DRI) = the average daily dietary nutrient intake level for healthy women >51 years.

[2] National health and nutrition examination survey (NHANES) = Nutrient Intakes from food: mean amounts consumed per female 50-59 years, one day, 2005-2006 (USDA 2008).

VITAMINS

Vitamin B_{12}

Vitamins B_6, B_{12}, and folic acid also affect homocysteine levels. Deficiencies in folate, vitamin B_6 and vitamin B_{12} cause elevated homocysteine levels in adults (Herrmann 2005). Homocysteine is an amino acid, which is formed during the metabolism of methionine and high levels have been associated with increased risk of cardiovascular disease (Refsum 1998). Recent studies suggest an association between homocysteine levels and osteoporotic risk as well as risk for fractures (Herrmann 2005, Cashman 2005, McLean and Jacques 2004, McLean and Karasik 2004, van Meurs 2004). While studies only recently have begun to directly investigate the effect of homocysteine levels on bone, this association is not surprising. Homocystinuria is a genetic disorder, caused by a mutation in the cystathionine beta-synthase gene leading to high levels of homocysteine (Kraus 1999). Patients with homocystinuria suffer from skeletal deformities as well as osteopenia, which suggests that high homocysteine may lead to low BMD (Herrmann 2005). There are several ways in which homocysteine may alter bone metabolism: homocysteine is believed to inhibit the formation of osteoblast and reduce their activity; high homocysteine levels could increase osteoclast formation and affect bone resorption, or homocysteine may interact with the extracellular matrix proteins directly causing structural changes (Herrmann 2005). In addition, homocysteine has been shown to interfere with the formation of collagen cross-links and fibrils in solution.

Recently, a number of studies have been conducted which directly examined the relationship between homocysteine levels and BMD or fracture risk. A study by van Meurs *et al*. (2004) examined the relationship between circulating homocysteine levels and the risk of fracture in 2,406 men and women over the age of 55 years. The results of the study suggested that high homocysteine levels were associated with an increased risk of fracture regardless of BMD or any other risk factors including nutritional deficiencies. After adjustment for age and sex, an increase in homocysteine levels by 1 SD led to an increase in fracture risk of 30 percent. Similar results were seen in a study by McLean *et al.* (2004), where high homocysteine levels were again associated with greater risk for fracture.

While studies suggest that homocysteine levels are associated with bone and risk for fracture, it is difficult to know if it is the homocysteine levels or the levels of folate, vitamin B_6 and vitamin B_{12}. Freudenheim *et al.* (1986) conducted a 4-year longitudinal study to assess the relationship between nutrient intake and bone in a group of 99 women ages 35-65 years; half of the women were given a calcium supplement of 500 mg of calcium carbonate while the other women were provided with a placebo (Freudenheim 1986). In the postmenopausal women given the placebo, folate intake correlated significantly with a change in radius BMD, showing that a higher folate intake would slow bone loss at this site. Currently, the DRI for folate is 400 μg/day with an upper limit (UL) of 1,000 μg/day (Trumbo 2001, Yates 1998). Vitamin B_{12} has several mechanisms by which it may be involved in bone formation: vitamin B_{12} is important for osteoblast formation and it is involved in iron metabolism. Low vitamin B_{12} levels, high

homocysteine concentrations or a combination of both was shown to increase risk for fractures by three times in a population of 1,267 men and women, ages 55-85 years (Dhonukse-Rutten 2005). However, this study could not determine whether the high levels of homocysteine or the deficiency in vitamin B_{12} caused an increased fracture risk since elevated homocysteine levels are highly correlated with low levels of vitamin B_{12}. The current DRI for vitamin B_{12} is 2.4 μg/day while no UL has been determined (Trumbo 2001, Yates 1998).

Vitamin B_6 has been suggested to have a role in bone due to its function as a cofactor to build collagen cross-linkages, which help stabilize the bone matrix (Masse 1995). A study by Lumbers *et al.* (2001), explored the relationship between nutrient intake and fracture in a group of elderly females, half of who had suffered fractures and the other women were age-matched (Lumbers 2001). The women who had suffered from hip fractures had lower vitamin B_6 intake compared with the women without fractures. However, these women also had lower intakes of other nutrients including energy, fat, carbohydrate, protein and calcium. Currently the DRI for vitamin B_6 in women ages 50 to 70 years is 1.5 mg/day with a UL of 100 mg/day (Trumbo 2001, Yates 1998).

Vitamin C

Ascorbic Acid, or vitamin C, is known to play a role in bone due to its function in collagen cross linkages as well as its antioxidant properties (Ilich 2000, Nieves 2005). Vitamin C stimulates the osteoblasts to produce osteocalcin and alkaline phosphatase, and helps lay down bone matrix (Schaafsma 2001). Ascorbic acid has been shown to

induce embryonic stem cells to differentiate into osteoblasts (Carinci 2005). Therefore, vitamin C is important in bone for a number of reasons; it enhances collagen formation, is a precursor to bone matrix mineralization, stimulates osteoblast differentiation and enhances the rate of formation as well as the life span of osteoclasts (Morton 2001).

The majority of current research suggests that vitamin C intake, through both diet and supplementation, has a beneficial effect on BMD and may even lower hip fracture risk (Nieves 2005, Schaafsma 2001, Morton 2001, Illich 2003, New 1997). In a study by Morton *et al.* (2001), researchers looked at the relationship between vitamin C supplement use and BMD in 994 postmenopausal women, ages 50-98, at five skeletal sites: ultradistal and midshaft radii, femoral neck, total hip and lumbar spring. Supplement use ranged from 100 to 5000 mg, with a mean dose of 745 mg. The current DRI for vitamin C is 75 mg/day with a UL of 2,000 mg/day (Trumbo 2001, Yates 1998), suggesting that the supplement dose may be rather high in some cases. Supplement users had BMD levels 3% higher than non-supplement users at three of the five sites. Furthermore, women who were on estrogen and taking vitamin C supplements had higher BMD at all five sites. Hall and Greendale (Hall 1998), examined the relationship between dietary vitamin C intake and BMD in a cross-sectional group of 775 postmenopausal women, ages 45-64 years. For every 100 gram increase of dietary vitamin C intake, there was a 0.017 g/cm^2 association with BMD at the femoral neck and total hip. In addition, women with a higher calcium intake ($\geq$500 grams), showed a stronger association between vitamin C and BMD. This positive association was not seen in women with calcium intake under 500 grams.

Several studies have shown that vitamin C's effect on BMD depends on a variety of other factors (Kaptoge 2003, Simon 2001). A study by Kaptoge *et al.* (2003), examined nutrient intake and BMD in 470 men and 474 women ages 67-79, over a 3-year period. In the male subjects, vitamin C intake did not affect BMD. However, the women in the lowest tertile of vitamin C intake (7-57 mg/day) had significantly faster rates of BMD loss when compared with women in the middle (58-98 mg/day) and upper (99-363 mg/day) tertiles of intake. In the third NHANES study, researchers looked for a relationship between dietary ascorbic acid intake and BMD in 13,080 men and women (Simon 2001). Dietary ascorbic acid intake, which averaged 102 mg/day, was associated non-linearly with self-reported fracture in men; a similar relationship was seen between serum ascorbic acid and BMD in men. In premenopausal women, a linear relationship was observed between dietary ascorbic acid and greater BMD. However, in postmenopausal women, those with a history of smoking and HT use showed an inverse association between serum ascorbic acid and a decreased prevalence of self-reported fractures; postmenopausal women with no history of smoking or HT use showed an positive association between serum ascorbic acid and lower BMD.

Vitamin D

Vitamin D plays a critical role in bone health (Stipanuk 2000, Ilich 2000, Grant 2005, Larsen 2004, Palacios 2006, Smith 2004, Trivedi 2003). The active form of vitamin D, $1,25(OH)_2$ vitamin D_3, also known as calcitriol, is important in facilitating the absorption of calcium in the intestine because it stimulates the synthesis of calbindin, a

calcium binding protein (Ilich 2000). In addition, vitamin D is involved in bone turnover. Deficiencies result in rickets in children and osteomalacia in adults; both diseases are characterized by defects in bone mineralization. Reduced levels of vitamin D also stimulate parathyroid hormone (PTH), which causes the resorption of calcium, suggesting multiple mechanisms by which vitamin D interacts with bone (Stipanuk 2000). Aging reduced the capacity of the skin to produce vitamin D_3 by approximately 75% due to less efficient synthesis in the skin as well as reduced sun exposure, making the elderly particularly at risk for deficiency (Stipanuk 2000, Palacios 2006).

A number of studies support the beneficial effect of vitamin D intake on bone, as suggested above (Larsen 2004, Trivedia 2003). When one considers previous studies that investigated vitamin D intake and bone health, it is important to remember that while the adequate intake (AI) for vitamin D is 5 μg/day, much of vitamin D is generated from sunlight (Trumbo 2001, Yates1998). A study conducted in Denmark in a population of 9,605 elderly people, ages 65-103 years, assessed the effect of a combined treatment of calcium and vitamin D supplementation on fracture risk over a three year period (Larsen 2004). Four thousand nine hundred and fifty-seven of the subjects were offered a prevention program consisting of 1,000 mg elemental calcium, as calcium carbonate, and 400 IU vitamin D_3, while the other 5,063 subjects were on an environment and health program. The patients provided with the supplementation saw a 16% decrease in fracture risk during the 3 year period, which was significantly lower than the subjects not provided with the supplementation. Specifically, the women had a 19% reduction in fracture risk, also significantly lower than the control subjects. A study by Trivedi *et al*.

(2003), examined the effect of vitamin D supplementation on risk for fracture (Trivedi 2003). The study was a randomized, double blind, controlled trial which provided the 2,686 male and female subjects ages 65-85 years, with 100,000 IU of vitamin D, or a placebo, every 4 months for a 5 year period. The results of the study showed a reduction in fracture risk in the hip, wrist/forearm, and vertebrae by 33% in the group receiving the vitamin D supplementation.

Some recent studies have shown that the relationship between vitamin D and bone may be dependent on other factors (Grant 2005, Smith 2004). A randomized, double blind trial investigated the effect of vitamin D supplementation on risk for fracture (Smith 2004). The subjects in this study were 10,000 men and women over the age of 75 years. The subjects were injected with an annual dose of vitamin D of 300,000 IU; the vitamin D supplementation caused a decrease in PTH by 17% and increased serum levels of vitamin D initially, but the serum levels of vitamin D were no longer elevated at 8 months. The results of the study showed no reduction in risk of fractures even though the vitamin D injection showed an increase in serum vitamin D levels for the first 8 months. The RECORD (Randomized Evaluation of Calcium and/or vitamin D) trial compared the effects of 1000 mg calcium carbonate, 800 IU vitamin D_3, combined 800 IU vitamin D_3 and 1000 mg calcium carbonate to a placebo on 5,292 men and women over the age of 70 years with previous low trauma fracture, in a randomized, double-blind trial during a 2 year period (Grant 2005). Overall, 13% of the subjects developed a new low trauma fracture. Of the subjects given the combined supplementation of calcium and vitamin D, 12.6% had a new low-trauma fracture, and 13.4% of the placebo group had a low-trauma

fracture. In addition, no differences were detected in all fractures or hip fractures. These results suggest that no significant differences exist between vitamin D supplementation and a placebo, even when calcium is supplemented.

Vitamin E

Vitamin E refers to all tocopherols and tocotrienols and their derivatives that exhibit the activity of RRR-α-tocopherol (Stipauk 2000). As an antioxidant, vitamin E works to protect lipid membranes from oxidative damage. Because vitamin E plays a role in protecting lipid peroxidation in cartilage, it has been suggested that vitamin E may be critical for bone remodeling (Xu 1995). While little research has been done in humans to test the effect on vitamin E intake on BMD, animal studies suggest an association may exist.

Research in a population of white female smokers, over the age of 40, suggests that high vitamin E intake will reduce the odds ratio for hip fractures. Currently, the DRI for vitamin E is 15 mg/day with an UL of 1,000 mg/day (Trumbo 2001, Yates 1998). In a study by Melhus *et al.* (1999), 66,651 Swedish women, ages 40-76 years, were involved in a prospective study to test the effect of antioxidant intake on hip fracture in smokers. Female smokers with a high intake of vitamin E (>6.2 mg/day) had an odds ratio for hip fracture of 1.1 compared with an odds ratio of 3.0 in the female smokers with a low intake ($\leq$6.2 mg/day). This effect was less pronounced in the women who were no longer smoking. While this study only showed significant results in smokers, it is possible that vitamin E's antioxidant properties may have beneficial effects in other

populations. Research by Maggio *et al.* (2003), examined plasma levels of vitamin E in 150 postmenopausal women over the age of 60, half of whom were osteoporotic, to examine the relationship between osteoporosis and vitamin E levels. Plasma vitamin E levels were significantly lower in the osteoporotic women (mean = 46.7 μmol/liter) compared with the control subjects (mean = 62.8 μmol/liter). The results of this study suggest that low levels of vitamin E may have an adverse affect on BMD.

A number of animal studies have suggested that vitamin E intake will have a beneficial effect on BMD (Ima-Nirwana 1999, Norazlina 2002, Smith 2005, Turan 2003). Ninety-six rats were treated with either hindlimb unloading or normal loading, and low dose, adequate dose, or high dose of vitamin E to test the effect of vitamin E intake in conditions where bone was diminished (Smith 2005). Supplementation with vitamin E showed a positive influence on bone metabolism under hindlimb unloading conditions; suggesting an advantageous effect under these circumstances. However, since these rats were under hindlimb unloading conditions the beneficial effects of vitamin E on bone may only translate to particular populations such as those on long-term bed rest. In a study by Norazlina *et al*. (2002), the effects of vitamin E deficiency and supplementation on bone calcification were studied in a population of rats. The rats on a vitamin E deficient diet had significantly lower bone calcium content than the rats on the control diet. When the rats on the vitamin E deficient diet were supplemented with palm oil, the loss of bone calcium was prevented.

Table 2. Summary of vitamin relationships with bone.

VITAMINS DRI[1]/UL[2]/ NHANES[3]	Function Related to Bone
Vitamin B_{12} (mcg/d) DRI: 2.4 UL: ND[4] NHANES: 4	Low levels of vitamin B_{12} correlate with elevated homocysteine levels. Homocysteine may inhibit the formation of osteoblasts, decrease bone resorption, cause structural changed with the extracellular matrix protein, and interfere with the formation of collagen cross-links and fibrils.
Vitamin C (mg/d) DRI: 90 UL: 2000 NHANES: 75	Vitamin C has a role in the enhancement of collagen formation, precursor to bone matrix mineralization, stimulation of osteoblast differentiation, and enhancement of the rate of formation and life span of the osteoclasts.
Vitamin D (IU/d) DRI: 400 NOF:1000 UL: 2000 NHANES: --	Vitamin D_3, calcitriol, the active metabolite of vitamin D functions to stimulate synthesis of the calcium binding protein, calbindin, and facilitates active calcium absorption in the intestine.
Vitamin E (mg/d) DRI:15 UL: 1000 NHANES: 6	As an antioxidant, vitamin E plays a role in protecting lipid peroxidation in cartilage and, therefore; may be an important intermediary for sustaining normal bone growth and remodeling.

[1]Dietary reference Intake (DRI) = the average daily dietary nutrient intake level for healthy women >51 years.
[2]Tolerable Upper Limits (UL)= The highest level of daily nutrient intake that is likely to pose no risk of adverse health effects to almost all individuals in the general population.
[3] National health and nutrition examination survey (NHANES) = Nutrient Intakes from food: mean amounts consumed per female 50-59 years, one day, 2005-2006 (USDA 2008).
[4]Not determinable = ND

MINERALS

Calcium

Calcium's role in bone health is not surprising because 99% of the body's calcium is found in bone (Stipanuk 2000). In order to have normal growth and development of bone, calcium intake is necessary. Dietary calcium intake has a threshold effect, when bone is provided with adequate calcium levels, higher calcium intake will not cause an increase bone mass (Ilich 2000). Serum calcium levels are tightly regulated by

parathyroid hormone, 1,25-dihydroxyvitamin D and calcitonin (Flynn 2003). When serum calcium levels drop, PTH is secreted and causes resorption of calcium from the bone and causes a reduction in urinary calcium excretion by acting on the kidney. There is also an increase in 1,25-dihydroxyvitamin D, which acts on the intestine to increase calcium resorption, and it causes resorption of calcium from bone.

Although calcium is certainly important for bone health, some studies suggest that calcium's effect may only be seen at certain sites. A study by Macdonald *et al.* (2004), examined the effect of various nutrients on BMD in a population 891 women over the age of 45 years for a 5-year period (Macdonald 2004). After adjusting for confounders (age, weight, annual percentage change in weight, height, smoking status, physical activity levels, baseline BMD measurement at appropriate site, menopausal status, HT use and energy intake), high dietary calcium intake significantly reduced bone loss at the femoral neck. Calcium intake, in this population averaged over 1,000 mg/day, however, the average intake (AI) for women age 50 to 70 years is 1,200 mg/day (Trumbo 2001, Yates 1998). When examining absolute BMD, no relationship was seen with calcium intake.

It is possible that the effect of calcium on bone is dependent on age and calcium intake. In one study, women of different ages received various amounts of calcium and the effect on mean serum 24-hour PTH levels was assessed (McKane 1996). Elderly women with a calcium intake of 1,600 mg/day and younger women with an intake of 900 mg/day showed the same levels of parathyroid hormone. However, elderly women with an intake of 800 mg/day of calcium had elevated PTH levels, such as those seen by

people with hyperparathyroidism. Cumming performed a meta-analysis in early postmenopausal women, using 49 separate cross-sectional studies (Cumming 1990). A positive correlation was found between dietary calcium intake and bone mass, where a 500 mg increase in dietary calcium was related to a 0.5% to 1% cortical bone loss. This relationship was not seen in trabecular bone. Not surprisingly, the greatest benefit to bone was seen in subjects with low baseline calcium intake. It is important to note that the majority of studies included in this analysis were cross-sectional, so a causal relationship cannot be drawn from the conclusions.

In addition, to its effect on bone, calcium supplementation has also been shown to reduce the risk of hip fractures, particularly in people with low dietary calcium intake (Palacios 2006). Meta-analyses of randomized controlled trials in women with mean ages between 58 and 84 years, have shown that calcium supplementation, of doses between 800 and 1,200 mg/day, reduces the relative risk of hip fracture by 25-70%, vertebral fractures by 23% and nonvertebral fractures by 14% between 1.5 and 4 year period (Cumming 1997, Shea 2002).

Iron

The primary role of iron, when coupled with a variety of proteins, is the transport and metabolism of oxygen (Stipanuk 2000). Iron may play a role in bone due to its action as a cofactor for enzymes, such as prolyl and lysyl hydroxylases, involved in collagen bone matrix formation (Palacios 2006). In addition, iron is a cofactor for 25-hydroxycholcalciferol hydroxylase, which transforms vitamin D into its active form.

Studies conducted in both animals and humans, suggest that iron intake is beneficial to bone and deficiency will cause weakness and decreased BMD (Harris 2003, Maurer 2005, Medeiros 1997, Medeiros 2002, Medeiros 2004, Michaelsson 1995). The current recommendation for iron intake in women ages 50-70 years is 8 mg/day with a UL of 45 mg/day (Trumbo 2001, Yates 1998).

Animal studies conducted to research the effect of iron on bone have shown that rats with iron deficiency have weaker bones than those with adequate iron intake (Medeiros 2002, Medeiros 2002, Medeiros 2004). In a study by Medeiros *et al*. (2002), rats were either fed a diet deficient in iron, restricted in calcium, deficient in iron and restricted in calcium, or with adequate levels of both nutrients. Bone morphology, mechanical strength and composition were then tested. The cortical widths of both the femurs and tibias of the rats with the iron deficient and calcium restricted diets were significantly lower when compared with the rats in the other groups. These results, in combination with the results from dual energy X-ray absorptiometry (DXA), showed a decrease in BMD in the iron deficient and calcium restricted rats. It can be concluded that calcium restriction alone or in combination with iron deficiency have the greatest effect on bone. However, the iron deficient group had significantly lower bone density when compared with the control group. The same research group also examined the effect of the iron deficient and calcium restricted diets on bone in rats when controlling for caloric intake and body weight (Medeiros 2004). The effect of calcium restriction and iron deficiency was similar to that seen in the previous study but it was also shown that these effects were independent of energy intake and body weight.

Iron's beneficial effect on bone has been confirmed in cross-sectional, as well as longitudinal studies, in women (Harris 2003, Maurer 2005, Michaelson 1995). The effect of iron on BMD was assessed cross-sectionally in a population of 242 postmenopausal women enrolled in the Bone, Estrogen and Strength Training Study (Harris 2003). Iron was shown to have a significant and positive association with bone at five different sites, even when considering calcium and protein intake. A longitudinal study, using the same population of postmenopausal women looked at the relationship between iron intake and BMD after 1 year and the effect of hormone replacement therapy on this association (Maurer 2005). There was a positive association between iron intake and BMD at the femur trochanter ($P < 0.03$) and Ward's triangle ($P < 0.05$) at one year; however, when stratified for women using HT and those who weren't, the relationship between iron intake and BMD was only significant for women using HT. A study by Michaelsson *et al.* (1995), examined the relationship between BMD and dietary iron intake, as assessed by FFQs and four 7-day records, in a population of 175 women ages 28-74. A significant association was seen between iron intake and BMD at the spine, femoral neck and total body.

Magnesium

Magnesium is the fourth most abundant cation in the human body (behind calcium, potassium and sodium) and the second most abundant intracellular cation (Stipanuk 2000). One of its primary roles is in enzymatic reactions where it frequently binds with adenosine triphosphate (ATP). Due to the abundance of magnesium in a

variety of foods, severe deficiency is rare, however, many people do not reach the dietary reference intake (DRI) for magnesium, which is currently set at 320 mg/day for women ages 50 to 70 (Trumbo 2001, Yates 1998, Schaafsma 2001). There are a variety of mechanisms by which magnesium effects bone metabolism: limiting hydroxyapatite crystal formation, which increases bone strength, maintaining the pH of extracellular fluid to help prevent calcium resorption, stimulating calcitonin which will inhibit osteoclasts, as well as stimulating PTH secretion and the hydroxylation of vitamin D in the liver (Schaafsma 2001). While studies have been conducted looking at the effect of magnesium intake and supplementation on bone, the results are inconclusive (New 2000, Tucker 1999, Ryder 2005, Abraham 1990).

A number of animal studies researched the effect of magnesium on bone in rats. In a study by Stendig-Lindberg *et al*. (2004), rats were given either a diet deficient in magnesium or one with an adequate level for one year (Stendig-Lindberg 2004). The rats given an adequate level of magnesium had a significantly higher bone density at both the vertebral and femoral bone than the magnesium deficient group of rats. In addition, the bones in the magnesium deficient rats needed a significantly lower force to break than those of the rats with sufficient magnesium. Due to these results, the authors concluded that prolonged magnesium deficiency in rats leads to osteoporosis.

While animal studies suggest that magnesium deficiency will lead to osteoporosis, human studies have mixed results. Similar to potassium, magnesium may benefit bone by lowering the acid load caused by a high acid diet. In a study by Tucker *et al*., magnesium intake through both diet and supplementation, averaging 300 mg/day and 288

mg/day in men and women respectively, was associated with a higher BMD at one site on the hip in both elderly men and women, and at the wrist in men (Tucker 1999). Additionally, subjects in the highest quartile of magnesium intake exhibited less of a decline in BMD over the 4-year study period. This has been supported by research by New *et al.* (2000), which showed that a higher intake of magnesium was associated with higher total bone in a cohort of 62 healthy women ages 45-55 years (New 2000).

In a cross-sectional study of 2,038 black and white men and women ages 70-79, researchers looked for a correlation between magnesium intake, through either the subject's diet or personal supplements, and BMD (Ryder 2005). This study showed that in the white men and women, magnesium intake, as determined by a semiquantitative FFQ (Block Dietary Data Systems, Berkeley, CA), was positively associated with an increase in BMD. An increase in magnesium of 100 mg per day was associated with a 2% increase in whole body BMD, similar to the effect of calcium on BMD. While most studies suggest that magnesium supplementation will help prevent bone loss, results from the 89,717 postmenopausal women enrolled in the Women's Health Initiative disagreed with previous results (Abraham 1990). The women in the highest quintile of magnesium intake also had the highest rate of fractures in the wrist and lower arm.

Phosphorous

Phosphorus is the second most abundant inorganic element, next to calcium, in the human body (Stipanuk 2000). Eighty-five percent of phosphorus is bound to the skeleton, making it necessary for bone mineralization. Dietary phosphorus' primary role

is to support growth and replaces losses, but due to the effect of phosphorus on PTH, phosphorus may play a role in bone metabolism (Ilich 2000). High dietary intake of phosphorus leads to high levels in the serum, which causes an elevation in PTH, leading to bone resorption in order to maintain adequate calcium levels (Stipanuk 2000). Other researchers report that high phosphorus intake may be beneficial for bone because the high phosphorus load leads to a decrease in serum calcium, caused by an inhibition of PTH-mediated calcium release from the bone (Raisz 1969).

Several human studies examined the effect of phosphorus intake on PTH, calcium and bone metabolism. In one study including young women (20-28 years of age), a dose-dependent increase in serum phosphorus and serum PTH concentrations (due to increased phosphorus intake) was observed (Kemi 2006). In addition, the high intake of phosphorus caused a significant negative effect on bone-specific alkaline phosphatase, a marker of bone formation and a significant increase in N-terminal telopeptide of collage type I, a marker of bone resorption. Serum PTH levels were still elevated the day after the phosphorus load, suggesting that high phosphorus intake can cause secondary hyperparathyroidism when calcium intake is too low. Similarly, a study by Calvo *et al.* (1988), examined the effect of phosphorus on PTH in a group of young adults (Kemi 2006). Subjects in this study were given a diet containing 1660 mg phosphorus and 420 mg of calcium; the current DRI for phosphorus is 700 mg/day and the UL is 4,000 mg/day (Trumbo 2001, Yates 1998). Within 24 hours, the subjects showed an elevation in PTH and this effect was seen throughout the four-week trial. Since the diet only provided 420 mg of calcium it is difficult to tell if the rise in PTH was caused by the high

intake of phosphorus or the low calcium intake. Some research suggests that the effect of phosphorus on bone may be dependent on stage of menopause (Tranquilli 1994, Hernandez-Avila 1993). While a study in postmenopausal women showed that phosphorus was significantly related to BMC (Tranquilli 1994), similar studies in pre- and perimenopausal women found no association (Hernandez-Avila 1993).

Due to the high intake of soda consumption in the United States, researchers are concerned that the ingestion of large amounts of phosphorus-rich carbonated beverages may be another cause for concern (Palacios 2006). Several studies have shown an association between soda consumption and increased fracture as well as low bone mass in adult populations (Petridou 1997, Wyshak 1989), although other studies have not seen this relationship (Kim 1997). Studies including one performed by Heaney and Rafferty show that soda consumption has no effect on calcium excretion, and therefore the high phosphorus in the soda is not harmful (Heaney 2001). Instead, they suggest that soda consumption may be harmful because its consumption is usually replacing that of milk consumption, which is high in calcium.

Potassium

Potassium, along with sodium and chloride, are electrolytes, which are responsible for maintaining the body's fluids (Stipanuk 2000). Their primary roles include electrolyte balance, osmotic control, transportation of organic metabolites by cells and stabilizing polyelectroltyes in cells. Wachman and Bernstein suggested another function of potassium: as a buffer (Wachman 1968). These researchers hypothesized that

acid ingested through the diet will lead to a gradual loss of bone and bone mineral acts as a buffer base. Current research in male and female subjects confirms that low dietary intake of potassium will increase the rate of calcium excretion in the urine (Lemann 1993).

A number of studies have supported the hypothesis advanced by Wachman and Bernstein in 1968 by examining the effect of potassium intake on markers of bone loss (Frassetto 2005, Macdonald 2005, Tucker 1999). In a cross-sectional and longitudinal study that investigated the relationship between potassium intake and BMD in elderly men and women, Tucker *et al.* (1999), reported that increased potassium intake was beneficial to bone, however results were different between the sexes (Tucker 1999). The current recommendation for potassium intake in women is 4700 mg/day in women (Trumbo 2001, Yates 1998). In both men and women, cross-sectional associations were seen between potassium and bone, which suggests that long-term diets high in potassium may help prevent bone loss. However, when observing longitudinally over a 4-year period at the change in BMD, results were only significant in men. The authors suggest that the effect of potassium may not be seen in women because of hormonal factors affecting these postmenopausal women.

While earlier studies have shown that supplementation with potassium, in the form of potassium bicarbonate, will decrease urine calcium excretion (Maurer 2003, Lemann 1999, Sebastian 1994), few studies have looked at this effect over a significant time frame. A recent study by Frassetto *et al.* (2005), studied the effect of a variety of doses of potassium bicarbonate on urine calcium excretion over a 3-year period (Frassetto

2005). The effect of the various doses of potassium was dose dependent, with a greater decrease in urine calcium excretion in those women receiving the highest dosage, 90-mmol/day potassium bicarbonate. In addition, the women with the highest urine calcium excretion at baseline saw the greatest decrease in excretion over the 36 months.

A study by Marangella *et al*. (2004), examined the effect of potassium citrate supplementation (0.08-0.1 grams/kg of body weight per day) on bone turnover in 30 women ages 43-72 years over a three-month period (Marangella 2004). This analysis showed a significant decrease in deoxypyridinoline and hydroxyproline, both markers of bone resorption, supporting the beneficial effect of potassium on bone. Unlike many previous studies, urine calcium excretion decreased but not significantly.

Both potassium and calcium have known benefits on bone but few studies have investigated the effect of supplementation with one or both of these minerals. Sakhaee *et al*. (2005), performed a crossover study in which they compared potassium citrate (4.3 g/day), calcium citrate (800 mg/day), both potassium citrate and calcium citrate, and a placebo's effect on bone turnover in a group of 18 postmenopausal women over a month long period (Sakhaee 2005). While the potassium supplementation caused a decrease in urinary calcium, unlike many other studies, there was no significant change in bone turnover markers. The authors suggested that the lack of change in bone turnover may have been caused by a more moderate intake of protein in this study, leading to less of an acid load, or due to the slightly lower dose of potassium at 4.3 g/day, or 40 mEq/day, when compared with levels of 60-120 mEq/day as seen in other studies. When the subjects were given the combined supplementation of calcium and potassium bone

resorption was decreased by two mechanisms: the potassium's effect on the acid from the diet, and the calcium supplementation increase absorption of calcium.

Sodium

Available studies indicate that dietary sodium, in the form of salt (NaCl) increases calcium excretion in the urine (Zarkadas 1989). For each 2300 mg increased in sodium intake, there is a 23 mg increase in the amount of calcium lost in the urine (Nordin 1993). Strong correlations between urinary sodium and calcium were seen in elderly men and women (Dawson-Hughes 1996) and in preadolescent females (Matkovic 1995). However, these studies found no direct effect of urinary sodium on BMD at the spine, hip, fore arm or total body (Matkovic 1995, Dawson-Hughes 1996). The optimal amount of NaCl for conservation of calcium and that meets the American Heart's Association Guidelines is less than 2300 mg per day.

One possible mechanism is that following a sodium load there would be a fall in extracellular fluid ion concentration. This would reduce a rise in PTH, which would increase the synthesis of 1, 25-dihydroxy vitamin D and calcium absorption efficiency. This change was seen in pre-menopausal not postmenopausal women (Breslau 1982). These findings suggest that sodium intakes may be contributing to postmenopausal osteoporosis.

Other studies have shown that replacing chloride in sodium chloride with acetate or bicarbonate reduces the urine calcium losses dramatically (Berkelhammer 1988, Lutz

1984). However, sodium chloride is overwhelmingly in the diet of Americans and it clearly has a hypercalciuric effect.

Zinc

Zinc, along with copper and manganese, function as metalloenzymes in a wide variety of systems throughout the body (Stipanuk 2000). Zinc is a cofactor for a variety of enzymes including alkaline phosphatase, involved in bone formation, as well as collagenase, which is involved in collagen breakdown. In addition, zinc is necessary for growth and people with zinc deficiency frequently have bone growth retardation. Zinc is also believed to play a role in bone due to its structural function in the bone matrix since bone mineral is composed of hydroxyapatite crystals, which contain zinc (Lowe 2002). Additionally, zinc is necessary for osteoblast function and inhibits bone resorption by osteoclasts. Studies have been conducted in animals, *in vitro* and in human populations, which suggest that zinc deficiencies will lead to problems with bone formation as well as growth (Hosea 2004, Merialdi 2004, Nielsen 2004, Peretz 2001, Rossi 2001, Hall 1999).

Several studies in rats, suggest that zinc deficiency will cause problems in bone (Hosea 2004, Rossi 2001). A study by Rossi *et al.* (2001), compared rats given a zinc deficient diet, a normal diet, and a group with a normal diet with the same quantity of food that was given to the zinc deficient group. The group of rats fed the zinc deficiency diet showed a decrease in body weight as well as long bone growth; these results were caused by the zinc deficiency and not the food reduction. In addition, the growth plate activity was significantly reduced in the zinc deficient rats, compared with the other two

groups of rats, suggesting that zinc deficiency acts by affecting the growth plates. In a different study, researchers examined the effect of zinc deficiency and energy restriction on bone development to determine if repletion of zinc could repair any adverse effects (Hosea 2004). The results of this study showed that both zinc deficiency and diet restriction have a negative impact on bone development in young growing rats that could not be repaired with repletion using a nutritionally complete diet. However, during the repletion phase of the study, the zinc deficient group had better bone recovery than the diet restricted group.

Due to zinc's role in bone formation via alkaline phosphatase, several studies have examined the direct effect of zinc on alkaline phosphatase (Peretz 2001, Hall 1999). An *in vitro* study investigated the effect of zinc and inorganic phosphate on alkaline phosphatase activity in human osteoblast-like cells (Hall 1999). Zinc was shown to cause a time and dose dependent increases in alkaline phosphatase activity in the osteoblast-like cells regardless of how much inorganic phosphate was present. As the active center of alkaline phosphatase, zinc is believed to participate in inorganic phosphate binding which regulates the levels of alkaline phosphatase. A study by Peretz *et al.* (2001) examined the effect of zinc supplementation on bone formation, as measured by alkaline phosphatase activity, in a double-blind placebo-controlled study of 20 men. Men giving a dose of 50 mg of elemental zinc showed a significant increase in total alkaline phosphatase activity when compared with the placebo group. Furthermore, urine calcium and C-terminal collagen peptide, measures of bone resorption, did not change suggesting that zinc increases bone formation while maintaining bone resorption. In this population of men,

the DRI for zinc is 11 mg/day with a UL of 40 mg/day, in our population of interest, the DRI for zinc in women age 50 to 70 years is 8 mg/day and the UL is 40 mg/day (Trumbo 2001, Yates 1998).

Several recent studies have examined the effect of zinc intake on bone metabolism and growth in humans (Merialdi 2004, Nielsen 2004). A study by Nielsen and Milne compared high zinc (53 mg/day) and low zinc (3 mg/day) intake on bone turnover in a population of postmenopausal women (Nielsen 2004). One of the results of the study was that the women consuming the diet high in zinc had an increased excretion of magnesium in the feces and urine, resulting in decreased magnesium balance, when compared with the women consuming the low zinc diet. The researchers suggested this effect was due to competition between zinc and magnesium when zinc is consumed in large quantities, since high intakes of zinc were shown to decrease magnesium balance. However, the women consuming the diet low in zinc, had unfavorable changes in bone as measured by circulating calcitonin and osteocalcin. Due to the commonality of zinc deficiency in developing countries, researchers looked at the effect of zinc supplementation in pregnant women on fetal growth in a population of 242 pregnant women in Peru. The fetuses of women receiving zinc supplementation showed a significantly greater femur diaphysis length than those women not receiving supplementation. However, no significant differences in birth length were seen between the non-supplemented and supplemented fetuses.

Table 3. Summary of mineral relationships with bone.

MINERALS DRI[1]/UL[2]/ NHANES[3]	Function Related to Bone
Calcium (mg/d) DRI: 1200 UL: 2500 NHANES: 799	Calcium comprises 37%-40% of the mineral content of bone and 99% of the body's calcium is found in the bones and teeth.
Iron (mg/d) DRI: 8 UL: 45 NHANES: 13	Iron acts as a cofactor for enzymes in the synthesis of bone matrix such as prolyl and lysyl hydroxylases, involved in collagen bone matrix formation. A cofactor for 25-hydroxycholcalciferl hydroxylase, which transforms vitamin D into its active form. Iron can be toxic to bone cells with iron overload or impaired iron metabolism.
Magnesium (mg/d) DRI: 320 UL: 350 NHANES: 267	Magnesium limits hydroxyapatite crystal formation, which increases bone strength, maintaining the pH of extracellular fluid to prevent calcium resorption, stimulates calcitonin, which inhibits osteoclasts, stimulated PTH secretion and the hydroxylation of vitamin D in the liver.
Phosphorous (mg/d) DRI: 700 UL: 3000 NHANES: 1134	A high intake of phosphorous leads to high levels in the serum, which cause an elevation in PTH, leading to bone resorption in order to maintain adequate calcium levels. However, a high phosphorous intake may decrease serum calcium, caused by an inhibition of PTH-mediated calcium release form the bone.
Potassium (mg/d) DRI: 4700 UL: ND[4] NHANES: 2458	It is theorized that an acid producing meal may cause a disruption in the aid-base balance. Potassium, therefore, functions to decrease plasma acidity thereby decreasing calcium efflux from the bones, decreasing calcium urine excretion, decreasing bone resorption, and increasing the rate of bone formation.
Sodium (mg/d) DRI:1200 UL: 2300 NHANES: 3001	Salt increased urinary calcium excretion.
Zinc (mg/d) DRI: 8 UL: 40 NHANES: 4	A cofactor for a variety of enzymes, such as alkaline phosphatase, involved in bone formation, collagenase, involved in collagen breakdown. It has a structural function in the bone matrix since zinc is found in the hydroxyapatite crystal. It is necessary for osteoblast function and inhibits bone resorption by osteoclasts.

[1]Dietary reference Intake (DRI) = the average daily dietary nutrient intake level for healthy women >51 years.

[2]Tolerable Upper Limits (UL)= The highest level of daily nutrient intake that is likely to pose no risk of adverse health effects to almost all individuals in the general population.

[3] National health and nutrition examination survey (NHANES) = Nutrient Intakes from food: mean amounts consumed per female 50-59 years, one day, 2005-2006 (USDA 2008).
[4]Not determinable = ND

OTHER NUTRIENTS

Alcohol

Alcoholism leads to lower BMD and higher fractures risk due to poor nutrition, malabsorption, of nutrients, liver disease, toxicity to osteoblasts, and increases the risk of falling (Ilich 2003). A prospective study with 85,000 women showed that those who consumed more than 25 g alcohol per day had increased risk for hip and forearm fractures compared to those who did not drink (Hernandez-Avila 1991). One 12 fl oz beer has about 13 g of alcohol, 3.5 fl oz of wine about 10 g, and 1.5 fl oz of liquor about 15 g. (Ilich 2003). A study in postmenopausal women found moderate alcohol consumption to have a positive association with bone mass (Holbrook 1993). A possible mechanism may be that alcohol stimulates the conversion of androstenedione to estrone, an estrogenic compound with bone-preserving properties. The aromatization of androgens to estrogens in postmenopausal women is the only source of their estrogen. Results from a study in postmenopausal women revealed higher estradiol levels with moderate alcohol consumption (Gavaler 1991). Health professionals recommend no more than one drink a day for women.

Caffeine

Intake of caffeine causes short-term (1-3 hours) increase in calcium excretion in the urine, but sustained effects in controlled studies have not be shown (Harris 1994). A study in postmenopausal women found that two or more cups of coffee a day was associated with a reduced BMD in those who did not drink milk daily (Barrett-Conner 1994). In a subgroup of postmenopausal women, calcium intakes lower than 800 mg a day (more than two cups of brewed coffee per day) saw more rapid bone loss than those who consumed adequate calcium (Harris 1994). The evidence suggests avoiding intakes of greater than four cups of caffeinated beverages a day and include adequate calcium intake (Nieves 2003).

Fiber

Although dietary fiber is not an essential nutrient, it is beneficial for a variety of reasons, most likely because it's indigestible (Stipanuk 2000). It has been well established that increased fiber intake increases feelings of satiety, slows gastric emptying, improves glucose tolerance and lowers serum cholesterol levels. Recent research suggests that fiber intake may also be related to bone health (Nzeusseu 2006, Abrams 2005, Raschka 2005, Weaver 2005). Most studies have shown increased calcium absorption and retention with fiber intake through inulin-type fructans. A number of theories have been suggested regarding the mechanism by which inulin-type fructans enhance calcium absorption (Weaver 2005). The most common theory is that the fermentation of the inulin-type fructans by the microflora in the large intestine lower

the pH by forming short chain fatty acid, the lowered pH leads to calcium absorption or it is possible that the fatty acids may directly promote absorption (Weaver 2005).

While a number of studies, in both humans and animals, have shown that consumption of inulin-type fructans increase the absorption of calcium, these studies show inconsistent results and are primarily short term (Nzeusseu 2006, Abrams 2005, Raschka 2005). Therefore, Abrams *et al.* (2005) designed a study to look at the effect of inulin-type fructan supplementation on calcium absorption and bone over a one-year period in pubertal adolescents (Abrams 2005). Subjects given the inulin-type fructan had greater calcium absorption and bone mineralization than the subjects given a placebo. The magnitude of increase in calcium absorption seen with the inulin-type fructan supplementation could be achieved with calcium supplementation, but the authors still suggest that it would be beneficial include inulin-type fructan in the diets of pubertal adolescents. A study by Nzeusseu *et al*. (2006), examined the effect of inulin and oligofructose intake on bone density in a group of growing rats over a three-month period (Nzeusseu 2006). At the end of the three-month period, both inulin and oligofructose were shown to increase BMC and BMD, although the effect was greater with inulin supplementation. However, BMD at the cortical bone site was increased only by inulin intake, not oligofructose. One finding that may suggest a mechanism for fructan's effect on bone was a significant increase in cecal wall weight with fructan intake; the authors suggest that this increase in surface area may allow greater for calcium absorption. In order to determine the effect of inulin-type fructans on bone and calcium absorption, Raschka and Daniel, performed three experiments on rats, providing them with different

intakes of inulin-type fructans (Raschka 2005). Rats, who consumed a diet with 10% inulin-type fructans, had higher levels of calcium, magnesium and zinc absorption as well as higher levels in the bone. However, when inulin-type fructans were added to a diet that already contained inulin-type fructans, even in very small quantities, the beneficial effect of inulin-type fructans was no longer observed.

Table 4. Summary of alcohol, caffeine and fiber and their relationships with bone.

OTHER REC[1] NHANES[2]	Function Related to Bone
Alcohol (g/d) REC: moderate NHANES: 5	Chronic alcoholism leads to lower BMD and higher fracture risk due to poor nutrition and malabsorption of nutrients, liver disease, abnormal vitamin D metabolites and parathyroid function, direct toxicity to osteoblasts and increased propensity to fall. Moderate alcohol consumption my increase BMD. The alcohol may stimulate androgstenedione conversion into estrone.
Caffeine (mg/d) REC: < 4 cups/day NHANES: 225	It is negatively correlated with intestinal calcium absorption with the net result being a more negative calcium balance.
Fiber (g/d) REC: 21 NHANES: 15	The most common theory is that fermentation of inulin-type fructans by the microflora in the large intestine lower the Ph by promoting formation of short chain fatty acids, the lowered pH leads to calcium absorption or it is possible that the fatty acids may directly promote absorption.

[1]Health professional's recommendations.
[2] National health and nutrition examination survey (NHANES) = Nutrient Intakes from food: mean amounts consumed per female 50-59 years, one day, 2005-2006 (USDA 2008).

METHODS

Design

The three papers in this dissertation were completed using cross-sectional data that was collected from the first (Fall 1995-1997) through the fourth (Fall 1995-2002)

year of the BEST study, a block- randomized, clinical trial. The BEST study investigated the effect of exercise on BMD in healthy, postmenopausal women (Going 2003). Participants were stratified by use of HT and then randomized to exercise or control conditions. They were provided with and requested to consume 800 mg of calcium citrate supplements each day during the trial to minimize variability in calcium intake. Year one dietary intake was assessed from eight randomly assigned days of DR throughout the year. Dietary intake was assessed at the end of the first year and annually thereafter using the AFFQ to capture the overall pattern of food intake during the previous 12 months. The BMD was measured at the femur neck, femur trochanter, lumbar spine (L2-L4), and total body using DXA (Going 2003, Cussler 2005). The University of Arizona Institutional Review Board (Human Subjects Committee) approved the study and the subjects provided written and informed consent.

Subject Recruitment, Entry Criteria, and Run In

Participants were recruited through television, radio and newspaper advertisements and flyers distributed in the community. Initial screening was done by telephone followed by small group meetings during which the requirements of the study were explained and informed consent, self-reported height and weight, recent weight change, and menopausal status were obtained. Initial screening assessment of medical history including eating disorders, physical activity history, diet and medication including HT were obtained by questionnaire. The BEST study enrolled 6 cohorts of women (Fall 1995- Fall 1997) that met the following inclusion criteria: 40-65 years; surgical or natural menopause (3.0-10.9 years); body mass index (BMI) > 19.0 kg/m^2 and < 32.9

kg/m^2; non-smoker; no history of osteoporotic fracture and an initial BMD greater than Z-score of –3.0 at all bone sites of interest; taking HT (1.0-5.9 years) or not taking HT (>1 year); weight gain or loss ≤ 13.6 kg in the previous year; cancer and cancer treatment-free ≥ 5 years (excluding skin cancer); not taking BMD-altering medications, beta-blockers, or steroids; dietary calcium intake >300 mg/day; performing <120 minutes of low intensity, low impact exercise per week and no weightlifting or similar physical activity. Participants agreed to accept randomization to exercise or no-exercise groups, continue their baseline level of physical activity (if not randomized to exercise), continue their usual dietary practices, maintain their HT status, and to take daily calcium each day of the trial.

Eligible women were enrolled in an eight-week run-in phase designed to test adherence and encourage early drop out. Initially, participants underwent a detailed medical history and physical exam by the study physician (or physician's assistant) a maximal graded exercise stress test with 12 lead electrocardiogram, orthopedic and postural analysis by a physical therapist, and osteoporosis screening by DXA. Women who met entry criteria went on to complete baseline assessments of body composition, muscle strength, physical activity, and dietary intake. Questionnaires to assess quality of life and psycohosocial variables such as depression, body image and self-cathexis were also administered, and fasting (12 hours overnight) samples of serum, plasma, and first void urine were collected for analysis of hormones and markers of bone turnover. Quality of life, psychosocial variables, and blood and urine chemistries are not reported herein. With the exception of physical exam (baseline only) and exercise stress tests

(baseline and 12 months), all assessments were repeated at 6 and 12 months intervals from baseline measurements (Going 2003).

Hormone Therapy

Women using HT were asked to continue to follow the regimen prescribed by their physicians and to report any changes every year. Consequently, a variety of hormone combinations were used, although most women took oral estrogen or estrogen and progesterone. Participants were encouraged to maintain the same regimen throughout the study and to report changes if they occurred.

Anthropometry

Trained anthropometrists took three measurements of each variable at each assessment time point (baseline, 6 months, 12 months and annually thereafter), which were averaged to obtain the criterion measures. Subjects wore lightweight clothing without shoes for the height and weight (WT) measurements. Standing height was measured to the nearest 0.1 cm during a maximal inhalation using a Schorr measuring board (Schorr Products, Olney, MD). Weight was measured on a calibrated digital scale (SECA, model 770, Hamburg, Germany) accurate to 0.1kg. Body mass index (BMI) in kilograms per meter squared was calculated from WT (kg) and height (m^2).

Dual Energy X-ray Absorptiometry

The DXA measured total body, lumbar spine (L2-L4), femur neck, trochanter, and Ward's triangle BMD (Lunar, Model DPX-L; software version 1.3y, extended research analysis, pencil beam densitometer, Lunar Radiation Corp, Madison, Wisc., USA). Standardized data acquisition and analysis techniques were used (Going, 2003,

Cussler 2005). Each subject was scanned twice at each measurement period (baseline, 6 month, 12 month and annually thereafter) and the mean of the two measurements was used in the analysis. Soft tissue composition was also derived from DXA whole body scans. Percent fat was derived as the ratio of fat mass to whole body mass estimated by DXA. Lean soft tissue mass measured by DXA is the equivalent of whole body mass minus the fat and bone masses.

Exercise Program

Participants randomized to the exercise intervention were asked to attend training sessions three days per week, on non-consecutive days, in one of four community facilities under the supervision of study on-site trainers. Sessions lasted 60-75 minutes and included stretching, balance, and weight-bearing activities for warm-up, weightlifting an additional weight-bearing circuit of moderate-impact activities (e.g. walk/jog, skipping, hopping), and stair-climbing/step boxes with weighted vests. Exercise frequency, weightlifting loads, sets and repetitions, steps with weighted vests, and minutes of aerobic activity were recorded in exercise logs that were monitored regularly by on-site trainers.

The participant-to-trainer ratio was five-to-one in the first year. Supervision was reduced during the second year; and in the third and fourth years, trainers were available at each facility one morning of afternoon per week. Crossover exercisers received supervision comparable to randomized exercisers because new cohorts with trainers were present in all facilities during the entire study (Cussler 2005).

Weightlifting was done using free weights and machines. Eight core exercises focused on major muscle groups with attachments on or near BMD measurements sites. These exercises included the seated leg press, lat (latissimus dorsi) pull down, weighted march, seated row, back extension, one-arm military press (right and left), squats (wall squats initially, progressing to Smith or hack squats), and the rotary torso machine.

Women completed two sets of six to eight repetitions (four to six repetitions for the military press to decreased injury to the shoulder) at 70% (2 days per week) or 80% (1 day per week) of the on-repetition maximum, determined by monthly testing (Metcalfe 2001).

Diet Assessment

Dietary intake was assessed from diet records collected from eight randomly assigned days throughout the year. Three days of DR were collected and analyzed at baseline, two days at six months and three days at 12 month. Eight days of DR has been shown to be a sufficient number of days to measure most of the nutrients and dietary components of interest (protein, fat, carbohydrate, calcium, iron, phosphorus, potassium, sodium, vitamin C, and fiber) based on the sample size for the group mean intakes (Basiotis 1987). Alcohol, caffeine, magnesium, zinc, vitamin E, and vitamin D were included in the analyses because of their possible impact on BMD. Participants completed an intensive 90 minute DR training prior to each DR recording period. Training consisted of participatory portion size and dimension estimation, directions on recording food descriptions, and evaluation of portion size estimation accuracy Weber 1997). Examples of individual food and recipe items, including combination dishes,

were prepared and portioned out for use in all training sessions to increase the accuracy of portion size estimation. Participants did not receive dietary advice and were instructed to refrain from changing their diets during the study. Each 2-3 week recording period included one weekend day and 1-2 nonconsecutive, random weekdays. Seasonal eating, consecutive day food leftovers, and weekend eating were taken into account by assessing intake at three time points throughout the year and recording 1 day per week over a 2-3 week period at each collection time point.

Completeness and accuracy of the DR were fostered by personal interviews given by trained technicians. Recipes, labels, and restaurant information were collected to enhance food item entry. The DR were analyzed for dietary intakes using the Minnesota Nutrient Data System (NDS) 93 (versions 2.8-2.92, 1995-1999, Nutrition Coordinating Center, Minneapolis, MN). Foods not in the database were substituted with a similar food item that had ≤10% disagreement for energy, carbohydrate, protein, fat and sodium of the original food. A master control sheet for each cohort, by test period, tracked each DR through the data entry process. This process included: initial entry of the data, checking the NDS analysis with the original DR, correcting any errors to the data, checking the corrections, final corrections, and the filing of completed DR.

Quality assurance of the DR was completed after each diet recording session for each cohort. Individual DR dietary intakes were calculated for energy, cholesterol and the nutrients of interest (protein, fat, carbohydrate, alcohol, caffeine, calcium, iron, magnesium, phosphorus, potassium, sodium, zinc, vitamin E, vitamin D, vitamin C, and fiber). Individual dietary intakes were compared to the group dietary intakes ± 3 standard

deviations (SD). If an individual dietary intake was above or below 3 SD of the group dietary intake, the original record was rechecked with the original NDS analysis. Corrections were made as necessary and all dietary intakes were again compared to the group dietary intakes. If no corrections were needed, documentation of the inflated or deflated dietary intake was made and the DR would be considered completed.

Dietary intake was assessed at the end of the first year and annually thereafter used the previously validated AFFQ (Martínez 1999). Participants were asked to complete the questionnaire in relation to their overall pattern of food intake during the previous 12 months. The AFFQ is based on the Block Model and is a 153 item, semi-quantitative, scannable questionnaire on the frequency of food consumption, using age and gender specific estimates of portions. The AFFQ was modified to include southwestern foods (Taren 1999). They were distributed with verbal and written instructions at the one-year DR training, completed at home, and collected during the 12-month anthropometry and BMD testing. Completeness and accuracy of the AFFQ were fostered by personal or telephone interviews. The AFFQ were checked a second time for completeness before they were sent to the Arizona Diet and Behavioral Assessment Center for analysis. Questionnaires missing more than 10 items were excluded from the analysis. Nutrient analysis for the AFFQ was completed using a proprietary software program called Metabolize (version 2.7, 2003, Arizona Diet and Behavioral Assessment Center, Tucson, AZ), which was updated to include version 17 of the USDA food composition database in 2005.

Calcium Supplementation

In the first and second year, all participants received blister packs of elemental calcium tablets in the form of calcium citrate (Citracal®, Mission Pharmacal, San Antonio, TX). Instructions on calcium supplement intake were given at each of the DR training sessions. The subjects were instructed to take 2 tablets (200 mg elemental calcium/tablet), twice a day (800 mg/day), without food, with a minimum of 4 hours between doses. Calcium supplement compliance was monitored through tablet counts. Participants brought in their unused calcium tablets before they received their new supply. The unused calcium tablets were counted. Participants were considered compliant if they consumed 80% or more of their expected calcium tablet intake. At the start of the third year and annually thereafter, participants were asked to continue calcium supplementation on their own by purchasing and taking a comparable calcium supplement. Calcium supplement compliance in year 3 and annually thereafter was monitored through quarterly self- reports. The quarterly self-reports collect information on the subjects' average supplement use by type, dosage per pill, and pills taken per week.

Participants in the third year of the study received the calcium quarterly self-report via mail or email. Every quarterly self-report was sent out with an accompanying cover letter which included the purpose and directions. Any tracking sheets not returned by the subjects after three weeks following the mailing date were sent a second mailing.

If the quarterly report is not returned after the second mailing, a trained technician makes a follow-up phone call. Any unreturned tracking sheets after this point are considered missing.

Statistical Methods

All data analyses were performed using the Statistical Package for the Social Sciences (version 11.5, 2002 and version 16, 2007, SPSS Inc., Chicago, IL). Year-one and year four average nutrient intake values were calculated from estimates of dietary intake alone, assessed by DR and/or the AFFQ, except for total calcium and total iron which included supplemental intakes. Year one average total calcium intake was calculated as the sum of the mean calcium intakes obtained from the DR or AFFQ plus mean intakes from the calcium supplements calculated through tablet count compliance. Year one total iron intake was calculated as the sum of the mean iron intake obtained from the diet only, assessed by DR, and the mean of any supplemental iron recorded in the DR. Total calcium and iron are the only variables in the analysis that include supplemental intake in the estimate of total intake. Four year dietary intakes were calculated from the yearly AFFQ, except for total calcium. Dietary intakes were averaged over the four years to provide a more stable and representative estimate of dietary intake. Four year total calcium was calculated using the yearly mean dietary calcium intake from AFFQ plus calcium supplement intake from tablet counts (years 1-2) and quarterly self- reports (years 3-4).

Nutrient intake distributions were examined and log-transformed, when appropriate to meet the assumptions of the statistical tests. The different statistical tests

for the three dissertation studies include descriptive statistics, student's independent t-test, paired t-test, Pearson's correlations, repeated measures analysis of variance, and multiple linear regressions. Significance was evaluated at the $p \leq 0.05$ level. Only significant associations from multiple linear regressions are reported using the standardized β coefficient.

FIRST STUDY RATIONALE, HYPOTHESES, AND STATISTICAL BACKGROUND

Rational

Determining the relationship between dietary intake and BMD is important for identifying nutritional strategies for minimizing age-related bone loss (Ilich 2003). The relationship between dietary intake and BMD has been studied by several investigators in pre and postmenopausal women (Okubo 2006, Ožeraitienė 2006, Salminen 2006, Maurer 2005, Macdonald 2004, Harris 2003, Ilich 2003, Whiting 2002, Sasaki 2001, New 2000, Tucker 1999, Teegarden 1998, New 1997) with conflicting results due, in part, to differences in the age of participants and the dietary assessment methods used. Identifying significant associations between dietary intake and BMD requires accurate dietary assessment methods, and these associations may vary by menopausal status.

Diet records and FFQ are common instruments used to assess dietary intakes. Multiple day DR can be burdensome to the participant, are costly to administer and analyze, and may cause changes in eating behavior. They are, however, the most accurate and feasible method to measure food intake in adults (McKeown 2001). The FFQ, on the other hand, have a limited cost, low burden on the participant, and are easier

to administer and analyze (Thomson 2003). The validity of the FFQ has been questioned in recent research, particularly related to disease endpoints such as osteoporosis and cancer (Bingham 2003). Accurate, reliable, time efficient, low-cost dietary intake assessment methods are needed to determine associations between dietary intake and disease (Hartman 1992).

Previous research using the BEST dietary data showed mean dietary intakes of calcium and iron, assessed by DR, had positive associations with BMD, at baseline and one year, in postmenopausal women. Baseline BEST dietary data demonstrated iron intake (>20 mg), assessed by three days DR, was positively associated with greater BMD at the femur neck, femur trochanter, ward's triangle, spine, and total body when mean calcium intakes were 800-1200 mg/day (Harris, 2003). There was a positive association with iron intake, assessed by eight days of DR, at one year and BMD at the femur trochanter and Ward's triangle (Maurer 2005). However, when analyzed by HT status associations, which were positive, remained significant only for women using HT. Calcium was associated with a positive change in femur neck and femur trochanter BMD only in women not using HT (Maurer 2003). At four years in the BEST study, total calcium was positively associated with change in BMD at the femur trochanter and the femur neck for women not using HT (Cussler 2005).

Hypotheses

The first study in this dissertation research investigated two relationships. The fist examined if the DR and AFFQ gave equivalent estimates of the same year of dietary

nutrient intake in postmenopausal women. The second examined if the DR and AFFQ resulted in similar dietary nutrient intake associations with BMD.

Aim #1:

Compare DR and AFFQ and their estimates of the same year of dietary nutrient intake.

Hypothesis #1:

DR and AFFQ will have equivalent estimates of the same year of dietary nutrient intake.

Aim #1a:

Compare dietary nutrient intake associations with BMD using DR and AFFQ.

Hypothesis #1a:

When determining dietary nutrient intake associations with BMD, DR and AFFQ will provide equivalent estimates.

In other words, DR and the AFFQ, assessing the same year of dietary nutrient intake, provided equivalent estimates of nutrient intakes when determining the associations of dietary nutrient intakes with BMD in healthy, post-menopausal women.

Statistical background

All data analyses were performed using the Statistical Package for the Social Sciences (version 11.5, 2002, SPSS Inc., Chicago, IL). As stated previously, participants were not excluded from the total samples based on implausible energy intake as

described by Harris *et al.* (2003) in order to maintain sample size and to have a untreated comparison of the dietary nutrient intake captured by DR and AFFQ. However, outliers were evaluated.

Year-one average nutrient intake values for DR and AFFQ were calculated from estimates of dietary intake alone and from supplements recorded in the DR or AFFQ for the nutrients of interest energy, protein, carbohydrate, fat, alcohol, vitamin C, vitamin E, vitamin D, calcium, phosphorous, iron, magnesium, sodium, potassium, zinc, caffeine, fiber, and zinc.

The nutrient intakes from DR and AFFQ were further analyzed using the non log-transformed data and then by log-transforming the average nutrient intakes to meet the assumptions of the statistical tests (normal curve). Pearson's correlations, student's t-test, frequencies, were all performed on the un-supplemented log transformed, un-supplemented non log transformed, supplemented log transformed, supplemented non log transformed data sets. The final analysis used log transformations on the un-supplemented nutrient intakes obtained from DR and AFFQ because the difference between the supplemented and un-supplemented dietary intake was negligible. Paired t-tests were used to detect significant differences between DR and AFFQ dietary nutrient intakes.

Pearson's correlations were completed on log-transformed mean nutrient intake data and on log-transformed, energy adjusted mean nutrient intake data. The average energy intake for each dietary assessment method was used to make the energy adjustments. For the final analysis, using the residual method, Pearson's correlations

between energy-adjusted mean nutrient intake estimates from DR and AFFQ were computed (Willet 1998). Standardized residuals were used in calculations to allow comparability across the two diet assessment methods.

A comparison of quintile ranking was completed on average nutrient intakes between DR and AFFQ, with the DR being the standard. The number of AFFQ estimated mean nutrient intakes within the same quintile as DR estimated mean nutrient intakes were summarized. "Percent in same quintile" was the percent and number of estimated mean nutrient intakes assessed by both dietary assessment methods that are in the same quintile. This approach was previously used in the dietary assessment research to calibrate across instruments (Jain 1996) and to test the reliability of quintile placement of nutrient intake estimates for epidemiological studies testing diet-disease associations. About a third of the AFFQ mean nutrient intakes are within the same quintile as the DR mean nutrient intakes. This section was not reported on in the final study results because of the ambiguous results when compared to the other statistical test such as Pearson's correlation and the multiple linear regressions.

Separate multiple linear regression was used to test the associations between mean nutrients intakes and BMD, adjusting for the effects of exercise, HT use, body weight at 1 year, years post menopause and total energy intake. The covariates had been selected previously from prior analyses. Significant associations ($p \leq 0.05$) were reported using the standardized ß coefficient to allow direction of the association to be seen. Significance was evaluated at the $p \leq 0.05$ level. With a sample size of 244, correlations

as low as r=0.15 could be detected at a power of 99% and regression models were able to detect an adjusted R-squared of 0.10 at a power of 98%.

SECOND STUDY RATIONALE, HYPOTHESES, AND STATISTICAL BACKGROUND

Rational

Experts predict that osteoporosis related fractures would increase health care cost by approximately $25.3 billion in 2025 (NOF 2008). Diet is a key lifestyle factor that can modify risk and facilitate the prevention of osteoporosis (Albertazzi 2002). The amount and type of fat consumed has been linked to bone loss (Corwin 2006, Watkins and Li 2001). Of the different types of fats, PUFAs are receiving recognition for having beneficial roles in the prevention and treatment of osteoporosis (Salari 2008, Albertazzi 2002, Das 2002, Watkins and Lippman 2001).

The PUFAs are found in all cell membranes as structural phospholipids and contain more than one double bond on their unbranched hydrocarbon chain. There are two families of PUFA, n-6 FA and n-3 FA. PUFAs, particularly n-6 FA and n-3 FA, are the primary precursors of eicosanoids, the signaling molecules, which modulate intracellular signal transduction and cell-to-cell interactions (Albertazzi 2002). The eicosanoids include prostaglandins, thromboxane, and leukotrienes (Albertazzi 2002). Eicosanoids, derived from n-6 FA, promote cell proliferation and inflammation and those from n-3 FA have an anti-inflammatory action and inhibit the effects on cell growth (Genuis 2007).

A high intake of n-6 FA, particularly arachidonic acid (20:4 n-6, AA), increases the formation of PGE_2. Cyclooxyngenase 2 (Cox 2), an enzyme that oxidizes AA to form PGE2, is unregulated by n-6 FA and down regulated by n-3 FA (Watkins and Lippman 2001). A high intake of n-3 FA produces anti-inflammatory cytokines that decrease bone resorption, increase calcium absorption in the gut, decrease the amount of calcium lost in the urine, increase calcium deposition in the bone, and increase collagen synthesis (Genuis 2006, Watkins and Lippman 2001, Watkins and Li 2001).

For many years HT has been used for the prevention of osteoporosis. Estrogen and PUFA have similar effects on BMD. The loss of estrogen during menopause increases the production of certain eicosanoids, particularly proinflammatory cytokines Il-1, Il-6, and TNF alpha (Das 2000) and also increases osteoclastogenesis (Das 2002). The beneficial effect of estrogen therapy on bone includes inhibition of osteoclast growth and activity, blocking inflammatory cytokines IL-6 and TNF alpha, and production of osteoblasts. Inflammatory cytokine, IL-1, can stimulate cartilage degradation, which can be prevented or reversed by estrogen (Das 2002). Like estrogen the PUFAs, LA and ALA, the essential fatty acids, can inhibit the production of the proinflammatory cytokines (IL-1, IL-2, TNF alpha) and thus, aid in the prevention of postmenopausal osteoporosis (Das 2002, Das 2000).

Previous findings from the Rancho Bernardo Study showed postmenopausal women with a higher ratio of n-6 FA:n-3 FA, assessed by FFQ, was associated with a lower BMD at the hip and at the spine, assessed by DXA, in women not using HT (Weiss, 2005). Another study in women (50-59 y), not categorized by HT use, reported

that total PUFA intake, assessed by FFQ, was associated with lower BMD loss at the femur neck, assessed by DXA, particularly among women with lower calcium intakes (Macdonald 2004).

Hypotheses

The second study in this dissertation investigated PUFA intakes and their association with BMD in postmenopausal women who completed year one in the BEST study. The first hypothesis was that n-3 FA will have a positive association and n-6 FA will have a negative association with regional and total body BMD in healthy, postmenopausal women. The second hypothesis was that PUFA dietary intake associations with BMD at year one will be seen in the HT and no HT groups.

Aim # 2:

To evaluate if dietary PUFA intake has a positive n-3 FA association with BMD or negative n-6 FA associations with BMD in postmenopausal women at one year

Hypothesis #2:

Dietary PUFA intakes will have positive n-3 FA or negative n-6 FA associations with regional and total body BMD sites in postmenopausal women at one year.

Aim #2a:

To evaluate if HT will influence the PUFA association with regional and total body BMD in postmenopausal women at one year.

Hypothesis #2a:

Dietary PUFA intake associations with regional and total body BMD will occur in both the HT and the no HT groups at one year.

Statistical background

All data analyses were performed using the Statistical Package for the Social Sciences (version 16, 2007, SPSS Inc., Chicago, IL). Mean nutrient intake values were calculated from estimates of dietary intake for total PUFA, n-6 FA, LA, AA, n-3 FA, ALA and ratios LA/ALA, and n-6 FA/n-3 FA. Total calcium and total iron included dietary nutrient intakes plus supplemental intakes from calcium supplements assessed by tablet counts and iron from supplement intake assessed by the DR. Participants were not excluded from the total samples based on implausible energy intake as described by Harris *et al.* (2003) in order to maintain sample size.

Nutrient intake distributions were examined and log-transformed, when appropriate, to meet the assumption of the statistical tests (normal curve). Descriptive characteristics for body composition and mean nutrient intakes from DR were calculated. Student's t-tests at one year were used to detect statistically significant differences in nutrient intakes between the subjects who used HT and those who did not use HT in both the baseline and year one data. Pearson's correlations were computed between all nutrients and covariates at baseline and one-year. Separate multiple linear regression was used to examine the associations between dietary PUFA and BMD. Separate multiple linear regressions were performed in baseline and one year data as summarized in Table 5. The shaded areas are the separate linear regressions that are reported in study number two.

Previous analysis in the BEST sample showed, total calcium and total iron were significant predictors of BMD (Maurer 2005, Harris 2003) and thus these nutrients were

added to Models 2, 3, and 4 and these models are summarized in Table 5. Models 1, 2, and 3, summarized in Table 5, had similar significant dietary PUFA intake associations with BMD when categorized by HT. So Model 3, which takes into account total calcium and total iron was reported in study two. Total fat was added to Model 4 to test if the dietary PUFA intake associations would remain significant in the HT group, suggesting the dietary PUFA intake association with BMD is independent of fat.

In the final sample, the multiple linear regression used with the total sample included three *a priori* coded contrasts (exercise contrast 1=exercise versus no exercise within HT; exercise contrast 2=exercise versus no exercise within no HT groups; HT contrast=HT versus no HT) that were used to test for significant group differences in BMD due to exercise and HT, while controlling for other potentially important covariates such as year 1 weight, years post menopausal, and energy intake. When the sample was stratified by HT use, energy intake, exercise, year 1 weight, years post menopausal, total calcium and total iron were the covariates that were controlled. Significance was evaluated at the $p \leq 0.05$ level. The power for the whole model, including all independent variables for 266 participants is ≥ 0.99 for all adjusted R^2.

Table 5. Summary of separate multiple linear regression analysis in study number two.

Models	Baseline Bone (N=295)	Year 1 Bone (N=266)	Baseline HT(n=148)/no HT (n=147)	Year 1 HT(n=136)/no HT (n=130)
Model 1 Basic model	Total energy HT contrast Baseline weight Years post-menopausal	Total Energy HT contrast Year 1 weight Years post-menopause Contrast 2 Contrast 3	Total energy Baseline weight Years post-menopausal	Total Energy Year 1 weight Years post-menopause Contrast 2 Contrast 3
Model 2 Basic model plus calcium and iron	Total Energy HT contrast Baseline weight Years post-menopausal Calcium Iron	Total Energy HT contrast Year 1 weight Years post-menopause Contrast 2 Contrast 3 Calcium Iron	Total Energy HT contrast Baseline weight Years post-menopausal Calcium Iron	Total Energy HT contrast Year 1 weight Years post-menopause Contrast 2 Contrast 3 Calcium Iron
Model 3 Basic model plus total calcium, total iron	Total Energy HT contrast Baseline weight Years post-menopausal Total calcium Total iron	Total Energy HT contrast Year 1 weight Years post-menopause Contrast 2 Contrast 3 Total Calcium Total iron	Total Energy HT contrast Baseline weight Years post-menopausal Total calcium Total iron Fat	Total Energy HT contrast Year 1 weight Years post-menopause Contrast 2 Contrast 3 Total Calcium Total iron
Model 4 Basic model plus total calcium, total iron and total fat	Total Energy HT contrast Baseline weight Years post-menopausal Total calcium Total iron Total fat	Total Energy HT contrast Year 1 weight Years post-menopause Contrast 2 Contrast 3 Total Calcium Total iron Total fat	Total Energy HT contrast Baseline weight Years post-menopausal Total calcium Total iron Total fat	Total Energy HT contrast Year 1 weight Years post-menopause Contrast 2 Contrast 3 Total Calcium Total iron Total fat

Baseline or year 1 HT/no HT = analysis were completed in the HT and no HT groups at both time points. Contrast 2 = exercise vs. no exercise within HT= yes;
Contrast 3 = exercise vs. no exercise within HT = no

THIRD STUDY RATIONALE, HYPOTHESES, AND STATISTICAL BACKGROUND

Rational

Experts predict that by 2020, 50% of the population older than 50 will be at risk for a bone fracture (USDHHS 2004); and the cost of osteoporosis related fractures is approaching $25 billion (NOF 2008). Although there are several modifiable risk factors for osteoporosis, adequate dietary nutrient intakes are critical, for the prevention and treatment of this disease. Numerous studies have demonstrated that calcium and vitamin D are related to bone health (Neives 2003, Baeksgaard 1998, Dawson-Hughes 1997). The benefits of the adequate intake of calcium and vitamin D for the prevention of bone loss, decreasing bone turnover, and decreasing non-vertebral fractures has been shown in postmenopausal women (Neives 2003, Cummings 1997, Reid 1995). However, in addition to calcium and vitamin D, a variety of nutrients are needed for normal bone formation, growth, and maintenance (Palacios 2006, Nieves 2005, Nieves 2003, Ilich 2000). Several investigators have examined the associations between BMD measured by DXA with dietary nutrient intakes assessed by FFQ (Okubo 2006, Macdonald 2004, Sellmeyer 2001, New 2000, Teagarden 1998, New 1997) a mini nutritional assessment (Ožeraitienė 2006, Salminen 2006), a diet history questionnaire (Sasaki 2001, Whiting 2002) and DR (Ilich 2003, Harris 2003, Maurer 2005).

There have been limited longitudinal studies that have examined the nutrient-bone relationship. A four year, longitudinal analysis of bone-change rates of postmenopausal women (n=32) found significant correlations with change of radius BMC, assessed by

single-photon absorptiometry, with energy, protein calcium, phosphorus, zinc, and folate, assessed by a diet record form. Higher levels of nutrient intake correlated with a slower BMC loss (Freudenheim 1986). A five-year longitudinal study in postmenopausal women (n=477), showed positive correlations with calcium and modest amounts of alcohol, assessed by a FFQ, with a change in femur neck, measured by DXA, and negative associations with change in femur neck BMD and polyunsaturated fatty acids, retinol, and vitamin E (Macdonald 2004). Studies have shown interactions of nutrients and bones differ by bone sites, menopausal status, and nutrient supplementation (Macdonald 2004, Freudenheim 1986).

Prior research investigating the cross-sectional dietary nutrient intake association with BMD at one year in postmenopausal women (n=244) in the Bone Estrogen Strength Training study, using both diet records and AFFQ, found iron and magnesium were consistently and significantly positively associated with BMD at all bone sites regardless of the dietary assessment method. Zinc, dietary calcium, phosphorous, potassium, total calcium, and fiber intakes were positively associated with BMD at three or more of the same bone sites using each of the dietary assessment method. Protein, alcohol, caffeine, sodium, and vitamin E did not have any similar BMD associations (Farrell 2008 accepted). This previous research at one year led to the investigation of these particular nutrient associations with BMD in this same population of women using the same AFFQ at year four. Previous research in postmenopausal women has also observed HT positively and negatively influencing nutrient associations with BMD (Weiss 2006, Maurer 2003).

Previous analyses from study number one of this dissertation showed dietary nutrient intake, assess by DR and the AFFQ, associations that were mostly positive with the total body and regional BMD sites (Farrell 2008, accepted). Study number one concluded that DR and AFFQ were acceptable dietary tools that could determine associations of iron, magnesium, phosphorous, potassium, calcium, and total calcium with BMD sites in a sample healthy, post-menopausal women in long-term studies (Farrell 2008, accepted). Study number two, also examined the one-year dietary PUFA intakes, assessed by eight days of DR, and identified significant associations with PUFA and BMD. When categorized by HT use all significant PUFA associations with BMD remained in the HT group, but were lost in the no HT group (Farrell PhD dissertation 2008). This was seen in previous papers with positive associations with iron and calcium, assessed by eight days of DR, at one year and BMD (Maurer 2003). Previous research also showed that at four years in the BEST study, total calcium was positively associated with change in BMD for women not using HT (Cussler 2005).

Hypotheses

The third study in this dissertation investigated dietary intakes and BMD associations in postmenopausal women completing four years of the BEST study. The third hypothesis was that some dietary nutrient intakes, assessed by AFFQ, at four years, would have positive associations in the regional and total body BMD in healthy, postmenopausal women. The next hypothesis was that dietary intake associations with BMD at year four would be seen in both the HT no HT group. The estimated dietary nutrients of interest included: energy, protein, carbohydrate, fat, PUFA, n-6 FA, LA, AA,

n-3 FA, ALA, LA:ALA, n-6 FA:n-3 FA, DHA, DPA, EPA, alcohol, vitamin B_{12} vitamin C, vitamin E, vitamin D, calcium, phosphorous, iron, magnesium, sodium, potassium, caffeine, zinc, fiber.

Aim #3:

To examine nutrient and BMD associations in postmenopausal women at four years.

Hypothesis #3:

Dietary nutrient intake will have positive and negative associations with regional and total body BMD sites in postmenopausal women at four years.

Aim #3a:

To evaluate if HT will influence the dietary nutrient intake associations with regional and total body BMD in postmenopausal women at year four.

Hypothesis #3a:

Dietary nutrient intake associations with regional and total body BMD will occur in the HT group and not in the no HT group at year four.

Statistical background

All data analyses were performed using the Statistical Package for the Social Sciences (version 16, 2007, SPSS Inc., Chicago, IL). Four year dietary intakes were calculated from the yearly AFFQ.

Nutrient intake distributions were examined and log-transformed, when appropriate, to meet the assumption of the statistical tests (normal curve). Student's t-tests at year four were used to detect statistically significant differences in participant

characteristics and dietary intakes between the subjects who took HT and those who did not take HT. Repeated measures were computed for nutrient intakes from year one through year four. Dietary nutrient intakes were averaged over the four years to obtain a representative estimate of dietary intake used in the analyses.

Separate multiple linear regression was used to examine the associations between the average nutrient intakes and four year BMD, since the 4 years of dietary nutrient intake represented the four years prior to the BMD measurements. Table 6 summarizes the models examined at each bone site. The covariates that were changed are in italics.

Table 6. Summary of separate multiple linear regression models in study number three.

Models	Covariates
Model A	HT during the diet assessment period, years post menopausal, exercise history over four years, average energy intake, *year four weight*
Model B	HT during the diet assessment period, years post menopausal, exercise history over four years, average energy intake, *year 4 total body fat, year 4 total lean soft tissue*
Model C	HT during the diet assessment period, years post menopausal, exercise history over four years, average energy intake, year 4 total body fat, *year 4 total lean soft tissue, year 4 change in total fat, year 4 change in lean soft tissue*

The covariates used in the separate multiple linear regression included years on HT during the dietary assessment period, years post menopausal, exercise history over four years, average energy intake, year four total lean soft tissue, and year four total body fat (Model B). When the sample was categorized by HT, years post menopausal, exercise history over four years, average energy intake, year four total lean soft tissue, and year four total body fat were controlled. Significance was evaluated at the $p \leq 0.05$ level and significant associations are reported using the standardized β coefficient. The

power from the whole model, including all independent variables is ≥ 0.80 for R^2 ≥0.1045.

FIRST STUDY:

COMPARISON BETWEEN DIETARY ASSESSMENT METHODS FOR DETERMINING ASSOCIATIONS BETWEEN DIETARY INTAKE AND BONE MINERAL DENSTIY IN POSTMENOPAUSAL WOMEN

Abstract

It is important to identify the role of nutrition in the treatment and prevention of osteoporosis. The goal of this study is to compare the equivalency of nutrient intakes assessed by diet records (DR) and the Arizona Food Frequency Questionnaire (AFFQ) and the associations of these nutrients with bone mineral density (BMD). This is a secondary analysis of cross-sectional data that was analyzed from 6 cohorts (Fall 1995-Fall 1997) of postmenopausal women (n=244; 55.7±4.6 years) participating in a 12 month, block-randomized, clinical trial. One-year dietary intakes were assessed using eight days of DR and the AFFQ. Participant's BMD was measured at the lumbar spine (L2-L4), femur trochanter, femur neck, Ward's triangle and total body using dual energy X-ray absorptiometry. Linear regression analyses ($p \leq 0.05$) were adjusted for the effects of exercise, hormone therapy use, body weight at one year, years post menopause and total energy intake. Significant correlations ($r=0.30$-0.70, $p \leq 0.05$) between dietary assessment methods were found with all dietary intake variables. Iron and magnesium were consistently and significantly positively associated with BMD at all bone sites regardless of the dietary assessment method. Zinc, dietary calcium, phosphorous, potassium, total calcium, and fiber intakes were positively associated with BMD at three

or more of the same bone sites regardless of the dietary assessment method. Protein, alcohol, caffeine, sodium, and vitamin E did not have any similar BMD associations. The DR and AFFQ are acceptable dietary tools used to determine the associations of particular nutrients and BMD sites in healthy postmenopausal women.

Introduction

Determining the relationship between dietary intake and bone mineral density (BMD) is important for identifying nutritional strategies for minimizing age-related bone loss (Ilich 2003). The relationship between dietary intake and BMD has been studied by several investigators in pre and postmenopausal women (Ilich 2003, New 1997, Teegarden 1998, Tucker 1999, Whiting 2002, New 2000, Sasaki 2001, Harris 2003, Macdonald 2004, Maurer 2005, Okubo 2006, Ožeraitienė 2006, Salminen 2006) with conflicting results due, in part, to differences in the age of participants and the dietary assessment methods used. Identifying significant associations between dietary intake and BMD requires accurate dietary assessment methods, and these associations may vary by menopausal status.

Diet records (DR) and Food Frequency Questionnaires (FFQ) are common instruments used to assess dietary intakes. Multiple day DR can be burdensome to the participant, are costly to administer and analyze, and may cause changes in eating behavior. They are, however, the most accurate and feasible method to measure food intake in adults (McKeown 2001). The FFQ, on the other hand, have a limited cost, low burden on the participant, and are easier to administer and analyze (Thomson 2003). The validity of the FFQ has been questioned in recent research, particularly related to disease

endpoints such as osteoporosis and cancer (Bingham 2003). Accurate, reliable, time efficient, low-cost dietary intake assessment methods are needed to determine associations between dietary intake and disease (Hartman 1992).

This study examined both DR and the Arizona Food Frequency Questionnaire (AFFQ) in postmenopausal women, an age group that is at high risk for osteoporosis. No other study has simultaneously compared these two dietary assessment methods and their estimated dietary nutrient intake associations with BMD in postmenopausal women. It is hypothesized that DR and AFFQ, assessing the same year of dietary intake, provide equivalent estimates of nutrient intakes when determining the associations of dietary nutrient intakes with BMD in healthy, post-menopausal women.

Methods

Study Design and Anthropometry

This is a secondary analysis of cross-sectional data that was collected from the first year of the Bone Estrogen Strength Training (BEST) a blocked- randomized, clinical trial. The BEST study investigated the effect of exercise on BMD in healthy postmenopausal women (Going 2003, Metcalfe 2001). Participants were stratified by use of hormone therapy (HT) and then randomized to exercise or control conditions. They were provided with and requested to consume 800 mg of calcium in supplemental form each day during the trial to minimize variability in calcium intake. Subject inclusion criteria, 12-month measurements of BMD at the 5 sites of interest (lumbar spine L2-L4 (mean=1.130.16 g/cm^3), femur trochanter (mean=0.75±0.11 g/cm^3), femur neck (mean=0.88±1.12 g/cm^3), Ward's triangle (mean=0.76±1.14 g/cm^3), and total body

(mean=1.11±0.08 g/cm^3) along with anthropometric and body composition measurements were described previously (Going 2003). The University of Arizona Internal Review Board approved the study and the subjects provided written and informed consent.

Dietary Assessment

Dietary intake was assessed from diet records collected from eight randomly assigned days throughout the year. Three days of DR were collected and analyzed at baseline, two days at six months and three days at 12 month. Eight days of DR has been shown to be a sufficient number of days to measure most of the nutrients and dietary components of interest (protein, fat, carbohydrate, calcium, iron, phosphorus, potassium, sodium, vitamin C, and fiber) based on the sample size for the group mean intakes (Basiotis 1987). Alcohol, caffeine, magnesium, zinc, vitamin E, and vitamin D were included in the analyses because of their possible impact on BMD. Participants completed an intensive 90 minute DR training prior to each DR recording period. Training consisted of participatory portion size and dimension estimation, directions on recording food descriptions, and evaluation of portion size estimation accuracy (Weber 1997). Examples of individual food and recipe items, including combination dishes, were prepared and portioned out for use in all training sessions to increase the accuracy of portion size estimation. Participants did not receive dietary advice and were instructed to refrain from changing their diets during the study. Each 2-3 week recording period included one weekend day and 1-2 nonconsecutive, random weekdays. Seasonal eating, consecutive day food leftovers, and weekend eating were taken into account by assessing

intake at three time points throughout the year and recording 1 day per week over a 2-3 week period at each collection time point.

Completeness and accuracy of the DR were fostered by personal interviews given by trained technicians. Recipes, labels, and restaurant information were collected to enhance food item entry. The DR were analyzed for dietary intakes using the Minnesota Nutrient Data System (NDS) 93 (versions 2.8-2.92, 1995-1999, Nutrition Coordinating Center, Minneapolis, MN). Foods not in the database were substituted with a similar food item that had ≤10% disagreement for energy, carbohydrate, protein, fat and sodium of the original food. A master control sheet for each cohort, by test period, tracked each DR through the data entry process. This process included: initial entry of the data, checking the NDS analysis with the original DR, correcting any errors to the data, checking the corrections, final corrections, and the filing of completed DR.

Quality assurance of the DR was completed after each diet recording session for each cohort. Individual DR dietary intakes were calculated for energy, cholesterol and the nutrients of interest (protein, fat, carbohydrate, alcohol, caffeine, calcium, iron, magnesium, phosphorus, potassium, sodium, zinc, vitamin E, vitamin D, vitamin C, and fiber). Individual dietary intakes were compared to the group dietary intakes ± 3 standard deviations (SD). If an individual dietary intake was above or below 3 SD of the group dietary intake, the original record was rechecked with the original NDS analysis. Corrections were made as necessary and all dietary intakes were again compared to the group dietary intakes. If no corrections were needed, documentation of the inflated or deflated dietary intake was made and the DR would be considered completed.

Dietary intake was assessed at the end of the first year using the previously validated AFFQ (Martínez 1999). Participants were asked to complete the questionnaire in relation to their overall pattern of food intake during the previous 12 months. The AFFQ is based on the Block Model and is a 153 item, semi quantitative, scannable questionnaire on the frequency of food consumption, using age and gender specific estimates of portions. The AFFQ was modified to include southwestern foods (Taren 1999). They were distributed with verbal and written instructions at the one-year DR training, completed at home, and collected during the 12-month anthropometry and BMD testing. Completeness and accuracy of the AFFQ were fostered by personal or phone interviews. The AFFQ were checked a second time for completeness before they were sent to the Arizona Diet and Behavioral Assessment Center for analysis. Questionnaires missing more than 10 items were excluded from the analysis. Nutrient analysis for the AFFQ was completed using a proprietary software program called Metabolize (version 2.7, 2003, Arizona Diet and Behavioral Assessment Center, Tucson, AZ), which was updated to include version 17 of the USDA food composition database in 2005.

The subjects were instructed to take two tablets of calcium citrate (200 mg elemental calcium/tablet) (Citracal®, Mission Pharmacal, San Antonio, TX), twice a day (800 mg/day), without food, with a minimum of four hours between doses. Calcium supplement compliance was monitored through tablet counts. Participants were considered compliant if they consumed 80% or more of their expected calcium tablet intake.

Statistical Methods

All data analyses were performed using the Statistical Package for the Social Sciences (version 11.5, 2002, SPSS Inc., Chicago, IL). Year-one average nutrient intake values were calculated from estimates of dietary intake alone except for total calcium. Average total calcium intake was calculated as the sum of the mean calcium intakes obtained from the DR or AFFQ plus mean intakes from the calcium supplements calculated through tablet count compliance.

Dietary intake distributions were examined and log-transformed, when appropriate, to meet the assumption of the statistical tests. Paired t-tests were used to detect statistically significant differences in dietary intakes between the two dietary intake assessment methods. Pearson's correlations between energy-adjusted mean nutrient intake estimates from DR and AFFQ were computed using the residual method (Willet 1998). The average energy intake for each dietary assessment method was used to make the energy adjustments. Standardized residuals were used in calculations to allow comparability across the two diet assessment methods. Linear regression was used to test the associations between nutrients of interest and BMD, adjusting for the effects of exercise, HT use, body weight at 1 year, years post menopause and total energy intake. Significance was evaluated at the $p \leq 0.05$ level. With a sample size of 244, correlations as low as $r=0.15$ could be detected at a power of 99% and regression models were able to detect an adjusted R-squared of 0.10 at a power of 98%.

Results and Discussion

Three hundred twenty-one women were enrolled in the primary study. The current investigation excluded participants who had less than five days of DR (n=28), and missing or incomplete AFFQ (n=20). Five participants were excluded for having AFFQ mean energy intake twice that of the DR. Twenty-four were excluded because they did not have valid dual-energy X-ray absorptiometry measurements. Two hundred and forty-four women were included in these analyses.

Mean age of the subjects was 55.7 ± 4.6 years. Participants were on average nearly six years past menopause (5.7±3.0 years) and had a 1-year mean body mass index that classified them as being slightly overweight (68±11.5 kg, 163±6.6 cm, 25.6±3.9 kg/m^2). No significant change in weight or body mass index over the year indicated the women maintained their weight as instructed.

Diet records and AFFQ dietary intakes, % difference, and Pearson's correlations at one year are reported in **Table 7**. Nutrients are arranged by highest Pearson's correlation to lowest**.** Paired sample t-test between the dietary intakes from eight random days of DR and the AFFQ at 12 months showed no significant differences between mean values for any nutrients. This suggests both methods capture similar dietary intakes in this sample.

Pearson's correlations from the two dietary assessment methods (r=0.33-0.71, $p<0.05$) are also reported in **Table 7**. Estimates of fat, carbohydrate, alcohol, caffeine, potassium, vitamin C, and fiber from the two dietary assessment methods had the highest

correlations ($r \geq 0.6$). The lowest correlations ($r \leq 0.41$) were for energy, protein, sodium, and total calcium.

Compared with results from other studies that compared nutrient intakes with DR and FFQ, this study showed stronger positive correlations for carbohydrate, fat, magnesium (Ambrosini 2003), vitamin D (Tokudome 2001) vitamin E (Tokudome 2001, Roddam 2005), and zinc (Ambrosini 2003). In contrast, results from this study showed weaker positive correlations with protein (Tokudome 2001, Rimm 1992), calcium, magnesium (Tokudome 2001), phosphorous (Tokudome 2001), and vitamin D (Jain 1996). There were comparable positive associations with alcohol (Ambrosini 2003, Roddam 2005), fiber, iron (Tokudome 2001, Rimm 1992), phosphorus (Ambrosini 2003), and vitamin C (Roddam 2005, Jain 1996). Differences in findings can be attributed to dietary assessment methods, sample sizes, gender, age, ethnicity, nutrients assessed, nutrient databases, methodology, and quality assurance of the collection of dietary information.

Multiple linear regression analyses were conducted to test the robustness of the correlations between dietary intakes, using the two dietary assessment methods, and BMD collected at 12 months. Linear regressions were adjusted for the effects of exercise, HT, body weight at one year, years post menopause, and total energy intake. **Table 8** summarizes the significant associations of dietary intakes from DR and AFFQ at five BMD sites. Significant associations ($p \leq 0.05$) are reported using the standardized ß coefficient.

Iron and magnesium were significantly associated with all BMD sites regardless of the dietary assessment method used. Iron, magnesium, zinc, fiber, calcium, total calcium, phosphorous, and potassium, were each associated with three or more of the same BMD sites. Protein and caffeine only had significant association with BMD using the DR. Alcohol, vitamin E, and sodium were not associated with any BMD site using either dietary assessment method. Except for dietary fat and caffeine, all dietary intakes had positive associations with the BMD sites regardless of the dietary assessment method used. Better agreement with similar significant BMD associations was found with the micronutrients than with the macronutrients. The magnitude of associations among the BMD sites and the dietary intakes from both diet assessment methods tended to be similar.

A study by Ilich (2003), showed dietary intake associations with different BMD sites using a 3 day DR in 136 healthy Caucasian, postmenopausal women. Using stepwise regression, Ilich *et. al.* found dietary intakes associated with three or more skeletal BMD sites for calcium (total body, Wards, hand), magnesium (neck, Ward's, trochanter), protein (total body, Ward's, hand), vitamin C (Ward's, trochanter, femur, shaft) and zinc (trochanter, femur, shaft). In comparison, the current study showed, using DR and AFFQ, magnesium, calcium, and zinc having three or more of the same significant associations with the same BMD sites.

Using a FFQ, New (1997), found that higher intakes of potassium (spine, neck, trochanter, Ward's), magnesium (spine), vitamin C (spine), alcohol (spine), and fiber (spine) were associated with higher bone mass in 994 healthy premeonopausal women.

Compared to New (1997), this study found potassium (neck, Ward's, total body), magnesium (neck, Ward's, trochanter, spine, total body), and fiber (neck, Ward's, trochanter, total body) had nutrient associations with three or more BMD sites, using both DR and AFFQ. Even though the results were similar between the two studies, hormonal status may have an effect on nutrient associations with BMD and; therefore, pre and postmenopausal women may have different dietary intake associations with BMD (Maurer 2003).

The participants were asked to keep their DR with them throughout the day and to measure their food and record it in their DR as they ate. It is unknown whether many participants actually recorded their food choices as they ate or recorded what they ate at the end of the day or before the diet record interview, thus turning the DR into a dietary recall. The AFFQ is limited in food choices and vague in serving size options, which make the portion size reference different for each individual thus, decreasing the accuracy of the diet assessment method. Actual nutrient estimates used within and between dietary assessment methods used different versions of the USDA databases. This can lead to incomplete estimated nutrient intake information and missing nutrient values. Although these dietary assessment methods have their limitations, databases are updated and, FFQ continue to be revised to gather more accurate information.

Conclusion

This study suggests that both DR and AFFQ, assessing the same year of dietary intake, provided equivalent estimates of particular nutrient intakes when determining the associations of dietary nutrient intakes with BMD in healthy, postmenopausal women.

This analysis showed iron and magnesium were consistently and significantly associated with BMD at all bone sites and zinc, fiber, phosphorous, potassium, calcium, and total calcium were significantly associated with BMD at three or more of the same bone sites regardless of the dietary assessment methods. Protein, alcohol, caffeine, sodium, and vitamin E, assessed by DR and AFFQ, did not have any similar BMD associations in these analyses; therefore, caution should be used if either tool is used to investigate these nutrient associations with BMD. This type of research can be used to examine the associations of nutrients in bone health, which can lead to valuable information in the prevention and treatment of osteoporosis.

Table 7. Diet record (DR) and Arizona Food Frequency Questionnaire (AFFQ) nutrient intake estimates, percent differences, and Pearson's correlations in 244 postmenopausal women.

Nutrient	**Diet Records Mean±SD[a]**	**AFFQ Mean±SD**	**% Difference[b]**	**Pearson's Correlation[c]**
Alcohol (g)	5 ± 8	3± 5	40	0.71
Caffeine (mg)	189 ± 163	224 ± 210	-19	0.69
Fat (g)	58 ± 20	50 ± 23	14	0.63
Carbohydrate (g)	228 ± 53	238 ± 102	-4	0.62
Fiber (g)	20 ± 6	21 ± 11	-5	0.62
Potassium (mg)	2823 ± 695	3228 ± 1290	-14	0.60
Vitamin C (mg)	133 ± 65	156 ± 102	-17	0.60
Vitamin E (mg)	9 ± 4	8 ± 5	11	0.59
Magnesium (mg)	302 ± 74	324 ± 126	-7	0.59
Calcium (mg)	776 ± 261	942 ± 468	-21	0.56
Iron (mg)	15 ± 5	14 ± 6	7	0.55
Zinc (mg)	10 ± 3	10 ± 5	0	0.54
Phosphorus (mg)	1136 ± 277	1247 ± 533	-10	0.48
Vitamin D (mcg)	5 ± 3	3 ± 3	40	0.47
Protein (g)	70 ± 18	64 ± 26	9	0.41
Sodium (mg)	2698 ± 810	2693 ± 1076	0	0.33
Energy (kcal)	1707 ± 365	1631 ± 637	5	0.31[d]
Total Calcium[e] (mg)	1483 ± 316	1598 ± 539	-8	0.31

[a]SD = standard deviation

[b]% difference estimated mean nutrient intakes from DR-AFFQ/DR. Percent differences are not statistically different between methods for any nutrient, $p \leq 0.05$.

[c]Pearson's correlations between DR and AFFQ estimated mean nutrient intakes are log transformed and energy-adjusted (standardized residual method) $p \leq 0.05$.

[d]Pearson's correlation between DR and AFFQ for Energy. Energy is Log transformed $p \leq 0.05$.

[e]Dietary calcium plus calcium supplement (mg).

Table 8. Significant associations between bone mineral density and nutrients intakes estimated by 8 days of diet records and the Arizona Food Frequency Questionnaire (AFFQ) at one year (N=244).

	BSA[b]	Standardized Coefficient ß[a]									
		DIET RECORDS					AFFQ				
Nutrient		**Neck[c]**	**Wards[c]**	**Troc[c]**	**Spine[c]**	**Total[c]**	**Neck[c]**	**Wards[c]**	**Troc[c]**	**Spine[c]**	**Total[c]**
Iron (mg)	5	0.289	0.380	0.252	0.214	0.240	0.334	0.426	0.232	0.265	0.358
Magnesium (mg)	5	0.310	0.315	0.140	0.175	0.257	0.564	0.514	0.276	0.291	0.384
Zinc (mg)	4	0.267	0.307	0.227	0.146	0.204	0.335	0.374	0.196	--	0.219
Fiber (g)	4	0.257	0.267	0.122	--	0.171	0.294	0.272	0.192	0.225	0.230
Phosphorus (mg)	3	0.393	0.413	0.258	0.196	0.327	0.413	0.433	--	--	0.291
Potassium (mg)	3	0.238	0.231	--	--	0.184	0.442	0.388	--	--	0.246
Calcium (mg)	3	0.204	0.222	--	--	0.165	0.330	0.324	0.172	--	0.249
Total Calcium[d](mg)	3	0.209	0.154	--	--	0.130	0.290	0.279	0.177	--	0.147
Fat (g)	2	-0.188	-0.259	--	-0.299	-0.293	--	--	--	-0.241	-0.293
Vitamin D (IU)	2	0.151	0.175	--	--	--	0.155	0.158	--	--	--
Vitamin C (mg)	1	0.151	0.155	--	0.155	0.177	--	--	--	0.170	--
Carbohydrate (g)	1	--	--	--	0.227	--	--	--	--	0.388	0.376
Protein (g)	0	0.305	0.312	0.236	--	0.245	--	--	--	--	--
Caffeine (mg)	0	--	--	--	--	-0.118	--	--	--	--	--
Alcohol (g)	0	--	--	--	--	--	--	--	--	--	--
Sodium (mg)	0	--	--	--	--	--	--	--	--	--	--
Vitamin E (mg)	0	--	--	--	--	--	--	--	--	--	--

[a] $p \leq 0.05$

[b] The number of the same nutrient and bone site agreements (BSA) between 8 days of diet records and the AFFQ, both representing one year.

[c] Participant's bone mineral density was measured at the femur neck (Neck), Ward's triangle (Wards), femur trochanter (Troc), lumbar spine L2-L4 (Spine), and total body (Total).

[d] Dietary calcium plus supplemental calcium (mg)

SECOND STUDY:

POLYUNSATURATED FATTY ACID INTAKE IS ASSOCIATED WITH BONE MINERAL DENSITY IN POSTMENOPAUSAL WOMEN

Abstract

A secondary analysis of cross-sectional, 12 month, data that was analyzed from 6 cohorts (Fall 1995- Fall 1997) of postmenopausal women (n=266; 56.6±4.7 years) participating the Bone Estrogen Strength Training (BEST) Study a 12 month, block-randomized, clinical trial. Bone Mineral Density (BMD) was measured at the femur neck and trochanter, lumbar spine (L2-L4), and total body BMD using dual energy X-ray absorptiometry. Mean dietary PUFA intakes were assessed 8 days of diet record. Multiple linear regression was used to examine the associations between dietary PUFAs (total PUFA, total n-6 fatty acids (n-6 FA), linoleic acid (LA), arachidonic acid, total n-3 fatty acids (n-3 FA), alpha linolenic acid (ALA), and the ratios: LA:ALA, and n-6 FA:n-3 FA) and BMD. Covariates included in the models were total energy intake, body weight, and years post menopause, exercise, the use of hormone therapy (HT), total calcium, total iron, and total fat intakes. In the total sample, femur trochanter, lumbar spine, and total body BMD had significant associations with dietary PUFA intake. In the HT group no significant associations between dietary PUFA intake and BMD were seen. In the HT group, inverse associations with dietary PUFA intake were seen in the spine (n-3 FA, ALA) and total body (n-3 FA, ALA, LA:ALA) BMD. Dietary PUFA intakes had the strongest associations with lumbar spine and total body BMD. When stratified by HT

use the associations remained in the HT group ($p \leq 0.05$), but were lost in the no HT group, suggesting that HT may influence PUFA associations with BMD.

Introduction

Experts predict that osteoporosis related fractures would increase health care cost by approximately $25.3 billion in 2025 (NOF 2008). Diet is a key lifestyle factor that can modify risk and facilitate the prevention of osteoporosis (Albertazzi 2002). The amount and type of fat consumed has been linked to bone loss (Corwin 2006, Watkins and Li 2001). Of the different types of fats, polyunsaturated fatty acids (PUFA) are receiving recognition for having beneficial roles in the prevention and treatment of osteoporosis (Salari 2008, Albertazzi 2002, Das 2002, Watkins and Lippman 2001).

PUFAs are found in all cell membranes as structural phospholipids and contain more than one double bond on their unbranched hydrocarbon chain. There are two families of PUFA, omega 6 fatty acid (n-6 FA) and omega 3 fatty acid (n-3 FA). PUFAs, particularly n-6 FA and n-3 FA, are the primary precursors of eicosanoids, the signaling molecules, which modulate intracellular signal transduction and cell-to-cell interactions (Albertazzi 2002). The eicosanoids include prostaglandins, thromboxane, and leukotrienes (Albertazzi 2002). Eicosanoids, derived from n-6 FA, promote cell proliferation and inflammation and those from n-3 FA have an anti-inflammatory action and inhibit the effects on cell growth (Genuis 2007).

In many animal and in vitro cell culture experiments a decrease in the n-6 FA:n-3 FA ratio can protect against loss of bone mineral density (BMD) and the production of prostaglandin E_2 (PGE2), a lipid compound derived from fatty acids (Holick 2004).

Osteocytes and osteoblasts release PGE2 when there is strain on bone and this stimulates the production of proinflammatory cytokines, which activate osteoclasts and bone resorption. A high amount of PGE2 promotes bone resorption and a low amount PGE2 plus mechanical loading promotes bone formation (Poulson 2007, Watkins and Li 2001, Albertazzi 2002, Das 2002).

A high intake of n-6 FA, particularly arachidonic acid (20:4 n-6, AA), increases the formation of PGE_2. Cyclooxyngenase 2, an enzyme that oxidizes AA to form PGE2, is unregulated by n-6 FA and down regulated by n-3 FA (Watkins and Lippman 2001). A high intake of n-3 FA produces anti-inflammatory cytokines that decrease bone resorption, increase calcium absorption in the gut, decrease the amount of calcium lost in the urine, increase calcium deposition in the bone, and increase collagen synthesis (Genuis 2006, Watkins and Lippman 2001, Watkins and Li 2001).

For many years hormone therapy (HT) has been used for the prevention of osteoporosis. Estrogen and PUFA have similar effects on BMD. The loss of estrogen during menopause increases the production of certain eicosanoids, particularly proinflammatory cytokines Il-1, Il-6, and TNF alpha (Das 2000) and also increases osteoclastogenesis (Das 2002). The beneficial effect of estrogen therapy on bone includes inhibition of osteoclast growth and activity, blocking inflammatory cytokines IL-6 and TNF alpha, and production by osteoblasts. Inflammatory cytokine, IL-1, can stimulate cartilage degradation, which can be prevented or reversed by estrogen (Das 2002). Like estrogen the PUFAs, linoleic acid (18:2 n-6, LA) and alpha linolenic acid (18:3 n-3, ALA), the essential fatty acids, can inhibit the production of the

proinflammatory cytokines (IL-1, IL-2, TNF alpha) and thus, aid in the prevention of postmenopausal osteoporosis (Das 2002, Das 2000).

In humans, dietary intervention and epidemiological studies assessing the role of PUFA in bone health are limited in number (Macdonald 2004, Weiss 2004, Bassey 2000, Kruger 1998, van Papendorp 1995) and show equivocal results. The purpose of this analysis was to determine whether the dietary PUFA intakes and the ratios of specific PUFAs have associations with femur neck and trochanter, lumbar spine, and total body BMD in a sample of 266 postmenopausal women. Secondarily, the relationships between BMD and dietary PUFA intakes and their ratios categorized by HT use were investigated.

Methods

Design

This is a secondary analysis of cross-sectional data that was collected during the first year of the Bone Estrogen Strength Training (BEST) study, a blocked- randomized, clinical trial. The BEST study investigated the effect of exercise on BMD in postmenopausal women (Going 2003). Participants were categorized by use of HT and then randomized to exercise or control conditions. Participants were instructed to consume 800 mg of calcium supplements daily (provided by the study) during the trial to minimize variability in calcium intake. The University of Arizona Internal Review Board approved the study and the subjects provided written and informed consent.

Subject Entry Criteria

The BEST study enrolled 6 cohorts of women (Fall 1995- Fall 1997) who met the following inclusion criteria: 40-65 years of age; surgical or natural menopause (3.0-10.9

years); body mass index (BMI) > 19.0 kg/m^2 and < 32.9 kg/m^2; non-smoker; no history of osteoporotic fracture and an initial BMD greater than Z-score of –3.0 at all bone sites of interest; taking HT (1.0-5.9 years) or not taking HT (>1 year); weight gain or loss ≤ 13.6 kg in the previous year; cancer and cancer treatment-free ≥ 5 years (excluding skin cancer); not taking BMD-altering medications, beta-blockers, or steroids; dietary calcium intake >300 mg/day; performing <120 minutes of low intensity, low impact exercise per week and no weightlifting or similar physical activity. Participants agreed to accept randomization to exercise or no-exercise groups, continue their baseline level of physical activity (if not randomized to exercise), continue their usual dietary practices, maintain their HT status, and take 800 mg of calcium supplements each day of the trial (Going 2003, Metcalfe 2001).

Three hundred twenty-one women were enrolled in the primary study. The current analyses with one-year data (n=266), excluded participants who had < 5 days of diet records (DR) (n=26) or did not have valid year-one dual-energy X-ray absorptiometry (DXA) measurements (n=29).

Anthropometry

Trained anthropometrists took three measurements of each variable at each assessment (baseline, 6 months, and 12 months), which were averaged to obtain the criterion measures. Twelve-month means were used in these analyses. Subjects wore lightweight clothing without shoes for measurements of height and weight. Standing height was measured to the nearest 0.1 cm during a maximal inhalation using a Schorr measuring board (Schorr Products, Olney, MD). Weight was measured on a calibrated

digital scale (SECA, model 770, Hamburg, Germany) accurate to 0.1kg. Body mass index (BMI) in kilograms per meter squared was calculated from weight (kg) and height (m).

Dual Energy X-ray Absorptiometry

The DXA was used to measure femur neck and trochanter, lumbar spine (L2-L4), and total body BMD (Lunar, Model DPX-L; software version 1.3y, extended research analysis, pencil beam densitometer, Lunar Radiation Corp, Madison, Wisc., USA). Standardized data acquisition and analysis techniques were used (Going 2003). Each subject was scanned twice at each measurement period (baseline, 6 month, 12 month) and the mean of the two measurements taken at 12 months was used in this analysis. Soft tissue composition was also derived from DXA whole body scans.

Diet Assessment

Dietary intake was assessed from eight randomly assigned days of DR collected at baseline (3 days), 6 months (2 days), and 12 months (3 days). Diet data was averaged over a year to provide a more stable more representative average of dietary intake, hence it makes sense to use the 12 month BMD estimates rather than baseline because the years worth of dietary behavior led up to the end of the year BMD. Participants completed an intensive 1½ h of DR training prior to each recording period. Training consisted of participatory portion size and dimension estimation, directions on recording food descriptions, and evaluation of portion size estimation accuracy (Weber 1997). Participants did not receive dietary advice and were instructed to refrain from changing their diets during the study. Each 2 to 3 week recording period included one weekend

day and 1-2 nonconsecutive, random weekdays. Seasonal eating, consecutive day food leftovers, and weekend eating were taken into account by assessing intake at three time points, at which one day per week was recorded over a 2 to 3 week period throughout the year.

Completeness and accuracy of the DR were fostered by personal interviews given by trained technicians. Recipes, labels, and restaurant information were collected to enhance food item entry. The DR were analyzed for estimated mean nutrient intakes using the Minnesota Nutrient Data System (NDS) 93 (versions 2.8-2.92, 1995-1999, Nutrition Coordinating Center, Minneapolis, MN). Foods not in the database were substituted with a similar food item that was within 90% agreement for energy, carbohydrate, protein, fat and sodium of the original food. A master control sheet for each cohort, by test period, tracked each DR through the data entry process. This process included: initial entry of the data, checking the NDS analysis with the original DR, correcting any errors to the data, checking the corrections, final corrections, and filing of completed records. (Farrell 2008 accepted)

Every two months, participants received blister packs of calcium citrate tablets (Citracal®, Mission Pharmacal, San Antonio, TX). Instructions on calcium supplement intake were given at each of the DR training sessions. The subjects were instructed to take 2 tablets (200 mg elemental calcium/tablet), twice a day (800 mg/day), without food, with a minimum of 4 hours between doses. Calcium supplement compliance was monitored through tablet counts. Participants were considered compliant if they consumed at least 80% of their calcium tablets. Efficacy of adequate calcium intake from

diet and supplementation to improve bone health has been previously reported for this population (Cussler 2005, Maurer 2005, Harris 2003). Calcium supplementation is evaluated in this investigation as a potential confounder of the primary analysis regarding dietary PUFA intake associations with BMD.

Iron and calcium intakes have been shown to have a relationship with BMD in this sample (Maurer 2005, Harris 2003). Because of these previous associations, calcium from diet only, total calcium, iron from diet only and total iron were included in the analysis. Total calcium intake was calculated as the sum of the mean calcium intake obtained from the diet only and the mean intake from the calcium supplements calculated through tablet count compliance. Total iron intake was calculated as the sum of the mean iron intake obtained from the diet only and the mean of any supplemental iron recorded in the DR. Total calcium and total iron are the only variables that include supplemental intake in this analysis.

Statistical Methods

All data analyses were performed using the Statistical Package for the Social Sciences (version 16, 2007, SPSS Inc., Chicago, IL). Mean nutrient intake values were calculated from estimates of dietary intake only except for total calcium and total iron, which included dietary and supplemental intakes. The independent variables included total PUFA, n-6 FA, LA, AA, n-3 FA, ALA and ratios LA:ALA, and n-6 FA:n-3 FA. The dependent variables included BMD from femur neck and trochanter, lumbar spine (L2-L4), and total body BMD.

Descriptive characteristics for body composition and mean nutrient intakes from DR were calculated. Student's t-tests at one year were used to detect statistically significant differences in mean nutrient intakes between the subjects who used HT and those who did not use HT. Nutrient intake distributions were examined and log-transformed, when appropriate, to meet the assumption of the statistical tests. Pearson's correlations were computed between all nutrients and covariates at one-year. Separate multiple linear regressions were used to examine the dietary PUFA associations with BMD. The multiple linear regression used with the total sample included three *a priori* coded contrasts (exercise contrast 1=exercise versus no exercise within HT; exercise contrast 2=exercise versus no exercise within no HT groups; HT contrast=HT versus no HT) that were used to test for significant group differences in BMD due to exercise and HT, while controlling for other potentially important covariates such as year 1 weight, years post menopausal, and total energy intake. When the sample was stratified by HT use, energy intake, exercise, year 1 weight, years post menopausal, total calcium and total iron were the covariates that were controlled. Significance was evaluated at the $p \leq 0.05$ level. With n=266, power is ≥ 0.99 for all adjusted R^2.

Results

Table 9 summarizes twelve-month subject characteristics for 266 postmenopausal women as a whole group then by HT status. Women not using HT were older, had been menopausal longer, and had significantly lower BMD at all bone sites compared to women on HT. Based on BMI, participants on average were slightly overweight

(25.7±3.9). There was no significant change in weight or BMI over the year indicating the women, as instructed, did not lose or gain weight.

The one-year nutrient intake associations with BMD using multiple linear regression analyses are summarized in **Table 10**. Significant associations between nutrient intake and BMD were found for the femur trochanter, lumbar spine and total body. No significant nutrient associations were found with BMD at the femoral neck. The significant associations between dietary PUFA intakes and BMD at the spine and total body at one-year were negative and included total PUFA, n-6 FA, LA, n-3 FA, ALA. Arachidonic acid was positively significantly associated with BMD only at the trochanter.

We further determined that the relationship between mean PUFA intakes and BMD would vary with HT use. In the no HT group, a significant, positive association between total fat intake and trochanter BMD was found (data not shown). **Table 11** summarized findings that indicate that in the HT group, significant inverse associations were seen with n-3 FA, ALA and total fat at the lumbar spine BMD. Significant inverse associations were found between total body BMD and n-3 FA, ALA, n-6 FA:n-3 FA, and total fat. The ratio LA:ALA was positively associated with total body BMD. Adjusting for total fat in the model resulted in only one significant positive association in the HT group, which was between LA:ALA and total body BMD (data not shown). This suggests that there is an association with the dietary intake between the ratio of LA:ALA and total body BMD regardless of total fat intake.

Discussion

This cross-sectional analysis showed that PUFA intakes and their ratios assessed by repeat DR over a 12-month period were significantly associated with BMD at the femur trochanter, lumbar spine, and total body in postmenopausal women. No association with dietary PUFA and femur neck BMD was observed in any of these analyses. It was also determined that the relationship between dietary PUFA intakes and their ratios were seen in the HT group but not in the no HT group. This study sample was unique in the analysis because of the stratifications of the results by HT use and only one other study to our knowledge examined this relationship between dietary PUFA and BMD in users and non-users of HT (Weiss 2005).

The BEST study was conducted before the Women's Health Initiative trial reported increased risk of coronary events and breast cancer in women on HT (The Writing Group 2002) providing a unique opportunity to study bone-nutrient interactions. Thus, this group of women maintained their HT status throughout the study. In the total sample, the results indicated an inverse BMD association with PUFA intakes (PUFA, n-6 FA, LA, n-3 FA, and ALA) assessed by 8 days of DR with BMD in the lumbar spine and total body. There was a positive association with AA and the BMD at the trochanter. When stratified by HT, only the HT group had significant BMD associations with PUFA intake. In the HT group, n-3 FA and ALA had significant negative associations with spine and total body BMD. The ratio LA:ALA had a significant positive association with total body BMD in the HT group. Other studies found conflicting results, not using a DR, but using food frequency questionnaires (FFQ) (Macdonald 2004, Weiss 2005).

Several studies have examined habitual dietary intake of total PUFA and the relationship with BMD. In a study in 891 women (50-59 y), not grouped by HT use, Macdonald *et al.* (2004) reported that total PUFA intake, assessed by FFQ, was associated with lower BMD loss at the femur neck, assessed by DXA, particularly among women with lower calcium intakes. No significant associations were observed in relation to spine BMD (Macdonald 2004). In comparison, the current analyses did not find any significant associations with PUFA intake and femur neck BMD. The current analysis did demonstrate significant inverse associations between dietary PUFA intake and spine and total body BMD. In another study of 890 postmenopausal women, a higher ratio of n-6 FA:n-3 FA (7.9±2.2 g or ~10:1), assessed by FFQ, was associated with a lower BMD at the hip and at the spine in women (N=326) not using HT (Weiss 2005). In contrast, the current analysis did not show the ratio of n-6 FA:n-3 FA (8.16±2.2 g or ~10:1) to be significantly associated with BMD at the hip or spine in the HT group. However, the ratio LA:ALA was positively associated with total body BMD in the HT group.

There have been only a few dietary intervention studies in postmenopausal women investigating the role of supplemental intake of PUFA in osteoporosis and these studies have yielded equivocal results (Paulsen 2007). A 16 week study among 40 osteoporotic women, mean age 80 years, using supplemental 4g/day evening primrose oil (rich in omega-6 GLA, dihomo-γ-linolenic acid, 20:3 n-6) and fish oil (rich in omega-3) reported a rise in osteoblastic activity with increases in serum concentrations of procollagen and osteocalcin. Serum alkaline phosphatase activity decreased and urinary and serum calcium increased (van Papendorp 1995). These measures indicate an increase

in BMD, although BMD was not measured in the study. In another study, 65 women, mean age 79.5, were split into two groups: the treatment group (N=29) was supplemented with 600 mg of calcium and 6 g PUFA (evening primrose oil+ fish oil = 60% LA, 8% ALA, 8% EPA, 3% DPA), and the placebo group (n=36) was supplemented with 600 mg of calcium and 6 g of coconut oil (97% saturated fat, 0.2%LA). After 18 months, the lumbar spine BMD in the treatment group remained the same, while it decreased 3.2 % in the placebo group. At the end of 36 months, the lumbar spine in the treatment group increased 3.1% in postmenopausal women with low calcium intake (Kruger 1998). No significant differences were observed in markers of bone turnover in a randomized control trial in 43 premenopausal (25-40 y) and 42 postmenopausal (50-65 y) women supplemented with Efacal® (4g evening primrose oil, 1 g calcium and 440 marine fish oil per day) (Bassey 2000). In another study, 199 postmenopausal women aged 45-65, received 40 grams of flaxseed (57% ALA) per day. After 12 months no effect was seen in femoral neck or lumbar spine BMD (Dodin 2005). The results from these studies are difficult to compare due to variations in sample size, age of participants, dose of PUFA, type of PUFA, length of interventions, methods of assessing the independent nutrient intake variables, as well as, the diet and baseline bone health of the sample population. These factors likely influence the variability of the results of these studies. This current study was not a dietary intervention and; therefore, could not establish cause and effect. It does, however, add to the limited body of research investigating the roles of dietary PUFAs and bone health.

The optimal ratio of n-6 FA:n-3 FA for bone health is unknown. Simopoulos (2001) has suggested that within the last 100 years Western's society has changed the dietary n-6 FA:n-3 FA intake ratio from 1:1 to 15:1 postulating that the current Western diet is "proinflammatory" and may lack the optimal quantity of n-3 FA to promote bone health (Simopoulos 2001). These analyses showed a 10:1 ratio of n-6 FA:n-3 FA thus, indicating this sample of postmenopausal women's dietary intake of PUFA may not be optimal to promote bone health. A low intake ratio of n-6 to n-3 fatty acids appears to decrease the risk of osteoporosis and slow the rapid rate of postmenopausal bone loss (Genuis 2006, Watkins and Lippman 2001, Watkins and Li 2001). Omega-3 fatty acids are found in fish oil and the chloroplasts of plants. Omega-6 fatty acids are found in plant oils such as palm, soybean, rapeseed, corn, and sunflower. The Dietary Reference Intake, Adequate Intake of n-3 FA in women 14 and older is 1.6 grams/day for men and 1.1 grams/day for women and the Acceptable Macronutrient Distribution Range (AMDR) is 0.6% to 1.2% of total energy (Trumbo 2002). In this study of postmenopausal women, n-3 FA were slightly higher than the recommendation for women (1.30±4.02 grams).

This study is intriguing and supports the associations of dietary PUFA intake and BMD; we were limited by the lack of biological markers of bone turnover for comparison with other studies. Further, the nutrient database used in the analysis while the most comprehensive database for dietary fatty acids did contain incomplete or missing nutrient values for some foods. Dietary assessment methods have their limitations even with frequent updates of food nutrient compositions; however, both report and systemic biases introduced are expected to be equivalent across groups. Biologically it is unclear why

these analysis found n-3 FA and n-6 FA would have an inverse relationship with spine and total body BMD and why AA acid would have a positive association with femur trochanter BMD. One possible explanation may be that sixteen to twenty percent of the values of these nutrients, LA, ALA, n-6 FA, or n-3 FA, are estimated values in NDS nutrient database. Other limitation may include a small sample size or a type 1 error is occurring. Replicate analysis in larger samples and more supplemental studies should be completed to further our knowledge in this area.

This study's strength was the extensive 8 days of DR collected and analyzed with a nutrient database that included extensive fatty acid analyses of foods. Although there have been larger studies associating PUFA's with BMD, they have used the FFQ to assess nutrient intake, which is not as sensitive as the DR (McKeown 2001). Research is emerging showing that different nutrients have associations at different BMD sites (Maurer 2005, Harris 2003, Ilich 2003). Future research needs more controlled, randomized, blinded dietary and supplemental trials to augment these findings. The focus should be on which and the amount of PUFAs and to establish the effect of PUFAs on specific bone sites.

Conclusion

This cross-sectional study among 266 postmenopausal women found dietary intakes of PUFA, n-6 FA, LA, n-3 FA, ALA, had significant inverse associations with lumbar spine and total body BMD. Arachidonic acid had the only positive association, which was at the trochanter BMD. When stratified by HT use all PUFA associations with BMD remained significant in the HT group, but were lost in the no HT group. In

the HT group, n-3 FA, ALA had an inverse relationship with spine and total body BMD and LA:ALA had a positive relationship with total body BMD. This suggests that HT and dietary PUFA intake are modulators of BMD. Dietary intake, a modifiable risk factor for osteoporosis, is a safe and inexpensive way to potentially prevent or treat osteoporosis. With more conclusive dietary studies, especially those that investigate cause and effect, dietary recommendations can aid in the prevention and treatment of osteoporosis.

Table 9. One year characteristics of 266 post-menopausal women using or not using hormone therapy (HT)[a]

		HRT Status	
Characteristics	All Women (N=266)	HRT (N=136)	No HRT (N=130)
Age (years)	56.6±4.7	55.8±4.5	57.4±4.9[b]
Height (cm)	163±6.5	163.2±6.9	162.9±6.1
Weight (kg)	68.4±11.8	68.2±11.9	68.6±11.7
BMI (kg/m^2)	25.7±3.9	25.6±4.2	25.8±3.7
Years HRT	1.4±1.6	2.8±1.1	0
Years postmenopausal	5.7±3.0	5±2.7	6.5±3.1[b]
Nutrient variables			
Energy (kcals)	1703±361	1734±372	1672±349
Total fat (g)	58±21	60±21	56±20
Calcium (mg)	769±258	785±256	753±258
Total calcium (mg)	1524±296	1552±288	1497±302
Iron (mg)	14±5	14±4	15±5
Total iron (mg)	17±9	18±11	16±7
PUFA (g)			
Total PUFA	11.70±4.44	12.11±4.58	11.26±4.25
Total Omega 6 fatty acid (n-6)	10.30±4.02	10.6±4.1	9.89±3.90
Linoleic acid, n-6 (LA)	10.20±4.00	10.60±4.13	9.78±3.84
Arachidonic acid, n-6, (AA)	0.11±0.06	0.10±0.06	0.11±0.06
Total Omega 3 fatty acids (n-3)	1.30±0.56	1.31±0.57	1.30±0.56
Alpha linolenic acid, n-3, (ALA)	1.14±0.50	1.17±0.53	1.11±0.48
n-6:n-3	8.37±2.28	8.58±2.31	8.16±2.2
LA:ALA	9.31±2.17	9.47±2.28	9.15±2.04
Bone Mineral Density (g/cm^3)			
Femur neck	0.88±0.12	0.90±0.12	0.86±0.12[b]
Ward's triangle	0.76±0.14	0.78±0.14	0.74±0.15[b]
Femur trochanter	0.75±0.11	0.77±0.11	0.73±0.11[b]
Lumbar spine L2-L4	1.13±0.16	1.16±0.14	1.10±0.17[b]
Total body	1.11±0.08	1.13±0.08	1.10±0.10[b]

[a]Values are means ± standard deviations; PUFA, polyunsaturated fatty acid; ALA, α-linolenic acid (18:3 n-3) EPA, eicosapentaenoic acid (20:5 n-3); DHA, docsahexaenoic acid (22:6 n-3); total omega 3 (total n-3 = ALA + EPA +DHA); LA, linoleic acid (18:2 n-6); AA, arachidonic acid (20:4 n-6); total omega 6 (total n-6 = LA + AA).
[b]Significantly different from HT group at 1 year by independent t-test $p \leq 0.05$.

Table 10. One year nutrient associations with bone mineral density using multiple regression analysis in 266 postmenopausal women

	Significant Association with Bone Mineral Density					
	Standardized coefficients Beta[a]					
Nutrient (g)	**Troc[b]**	**R^2**	**Spine[b]**	**R^2**	**Total[b]**	**R^2**
Total PUFA	--	--	-0.205	.15	-0.201	.20
Total n-6	--	--	-0.201	.15	0.192	.19
LA	--	--	-0.201	.15	-0.193	.19
AA	0.112	.28	--	--	--	--
Total n-3	--	--	-0.159	.14	-0.173	.19
ALA	--	--	-0.218	.15	-0.246	.21
Total Fat	--	--	-0.303	.18	-0.285	.21

Independent variables: energy, exercise contrast 1, exercise contrast 2, hormone replacement therapy contrast, year 1 weight, years postmenopausal.
Abbreviations: PUFA, polyunsaturated fatty acid; ALA, α-linolenic acid (18:3 n-3); total n-3 =total omega 3; LA, linoleic acid (18:2 n-6); AA, arachidonic acid (20:4 n-6); total n-6 = total omega 6; R^2=adjusted R squared
[a]$p \leq 0.05$
[b]Participant's bone mineral density was measured at the trochanter (Troc), lumbar spine (Spine), and total body (Total).

Table 11. Year 1 polyunsaturated fatty acid associations with bone mineral density adjusted for the basic model plus total calcium and total iron grouped by hormone therapy (HT) status

HRT (N=136)	**Significant Association with Bone Mineral Density**			
	Standardized coefficients Beta[a]			
Nutrient (g)	**Spine[b]**	**R^2**	**Total[b]**	**R^2**
Total n-3	-0.216	.06	-0.258	.15
ALA	-0.318	.09	-0.365	.18
LA:ALA	--	--	0.222	.15
Total Fat	-0.509	.10	-0.509	.17

Independent variables: energy, exercise, year 1 weight, years post menopausal, total calcium, total iron
Abbreviations: ALA, α-linolenic acid (18:3 n-3); total n-3 =total omega 3; LA, linoleic acid (18:2 n-6); total n-6 = total omega 6; R^2=adjusted R squared
[a]$p \leq 0.05$
[b]Participant's bone mineral density was measured at the lumbar spine (Spine), and total body (Total).

THIRD STUDY:

NUTRIENT AND BONE MINERAL DENSITY ASSOCIATIONS IN POSTMENOPAUSAL WOMEN COMPLETING FOUR YEARS OF THE BONE ESTROGEN STRENTH TRAINING STUDY

Abstract

Background: There is a need for research that studies the long-term relationships of nutrient intake with bone mineral density (BMD) in postmenopausal women.

Objective: To determine the associations between the average four-year dietary nutrient intakes and cross-sectional BMD measured at year four in postmenopausal women either using or not using hormone therapy (HT).

Design: This is a longitudinal, secondary analysis of the average of four years of dietary nutrient intake data and cross-sectional BMD data at year four that was analyzed from six cohorts (Fall 1995- Fall 1997) of healthy, postmenopausal women (N=130; 60±4.3 years) participating in the Bone Estrogen Strength Training (BEST) Study a block-randomized, clinical trial.

Methods: Dietary intakes were assessed annually over four years using the Arizona Food Frequency Questionnaire. Dependent variables, femur neck and trochanter, lumbar spine (L2-L4), and total body BMD, were measured by dual energy X-ray absorptiometry.

Statistical analysis: Separate multiple linear regression was used to examine the associations between dietary nutrient intakes and regional and total body BMD. Covariates included years of HT use during the period of dietary assessment, years post

menopausal, exercise history, average total energy intake, year 4 total lean soft tissue and year 4 total body fat.

Results: Average dietary intake of selected bone-related nutrients over four years, is associated with lumbar spine BMD (vitamin B_{12}, polyunsaturated fatty acids (PUFA), omega 3 fatty acids (n-3 FA), alpha-linolenic acid (ALA), omega 6 fatty acids (n-6 FA), linoleic (LA)) and total body BMD (n-3 FA, LA), in a sample of 130 postmenopausal women. When categorized by HT use (N=92), nutrient intake associations with BMD were found in the HT group only for the spine (total fat, vitamin B_{12}, total PUFA, n-3 FA, ALA, docosahexaenoic acid (DHA), docosapentaenoic acid (DPA), eicosapentaenoic acid (EPA), n-6 FA and LA) and total body (n-3 FA, ALA, DHA, DPA, EPA, n-6 FA).

Conclusion: Dietary nutrient intakes had the strongest significant associations with lumbar spine and total body BMD. When stratified by HT use the nutrient and BMD associations remained significant in the HT group ($p \leq 0.05$), but were lost in the no HT group, suggesting that HT modulates the nutrient-BMD association.

Introduction

Experts predict that by 2020, 50% of the population older than 50 will be at risk for a bone fracture (USDHHS 2004); and the cost of osteoporosis related fractures is approaching $25 billion (NOF 2008). Although there are several modifiable risk factors for osteoporosis, adequate dietary nutrient intakes are critical, for the prevention and treatment of this disease. Numerous studies have demonstrated that calcium and vitamin D are related to bone health (Neives 2003, Dawson –Hughes 1997, Baeksgaard 1998). The benefits of the adequate intake of calcium and vitamin D for the prevention of bone

loss, decreasing bone turnover, and decreasing non-vertebral fractures has been shown in postmenopausal women (Neives 2003, Cummings 1997, Reid 1995). However, in addition to calcium and vitamin D, a variety of nutrients are needed for normal bone formation, growth, and maintenance (Palacios 2006, Nieves 2005, Nieves 2003, Ilich 2000). Several investigators have examined the associations between BMD measured by dual energy X-ray absorptiometery (DXA) with dietary nutrient intakes assessed by food frequency questionnaires (FFQ) (Okubo 2006, Macdonald 2004, Sellmeyer 2001, New 2000, Teagarden 1998, New 1997) a mini nutritional assessment (Ožeraitienė 2006, Salminen 2006), a diet history questionnaire (Sasaki 2001, Whiting 2002) and diet records (Ilich 2003, Harris 2003, Maurer 2005).

There have been limited longitudinal studies that have examined the nutrient bone relationship. A four year, longitudinal analysis of bone-change rates of postmenopausal women (N=32) found significant correlations with change of radius bone mineral content (BMC), assessed by single-photon absorptiometry, with energy, protein, calcium, phosphorus, zinc, and folate, assessed by a diet record form. Higher levels of nutrient intake correlated with a slower BMC loss (Freudenheim 1986). A five-year longitudinal study in postmenopausal women (N=477), showed positive correlations with calcium and modest amounts of alcohol, assessed by a FFQ, with a change in femur neck, measured by DXA, and negative associations with change in femur neck BMD and polyunsaturated fatty acids, retinol, and vitamin E (Macdonald 2004). Studies have shown interactions of nutrients and bones differ by bone sites, menopausal status, and nutrient supplementation (Freudenheim 1986, Macdonald 2004).

Prior research investigating the cross-sectional dietary nutrient intake association with BMD at one year in postmenopausal women (N=244) in the Bone Estrogen Strength Training study, using both diet records and AFFQ, found iron and magnesium were consistently and significantly positively associated with BMD at all bone sites regardless of the dietary assessment method. Zinc, dietary calcium, phosphorous, potassium, total calcium, and fiber intakes were positively associated with BMD at three or more of the same bone sites using each of the dietary assessment method. Protein, alcohol, caffeine, sodium, and vitamin E did not have any similar BMD associations (Farrell 2008 accepted). This previous research at one year led to the investigation of these particular nutrient associations with BMD in this same population of women using the same AFFQ at year four. Previous research in postmenopausal women has also observed hormone therapy (HT) somehow influencing nutrient associations with BMD (Maurer 2003, Weiss 2006).

This study was conducted as an extension of a previous cross-sectional and longitudinal analyses that showed dietary intakes of many nutrients were positively and significantly associated with bone (Farrell PhD dissertation 2008, Farrell 2008 accepted, Maurer 2005, Macdonald 2004, Harris 2003, Ilich 2003, Freudenheim 1986). The purpose of this study is to determine whether average nutrient intake over four years and the cross-sectional BMD relationships persisted in year four and whether hormone therapy (HT) use influences these relationships in 130 postmenopausal women who completed the fourth year of the Bone Estrogen and Strength Training Study.

Methods

Design

This is a longitudinal, secondary analysis of the average of four years of dietary nutrient intake data and cross-sectional BMD data at year four that was analyzed from six cohorts (Fall 1995- Fall 1997) of healthy, postmenopausal women enrolled in the fourth year of the Bone Estrogen Strength Training (BEST) study (Cussler 2004). The BEST study in the first year was a blocked, randomized, clinical trial which investigated the effect of exercise on BMD in postmenopausal women (Going 2003). Participants were categorized by use of HT and then randomized to exercise or control conditions. Participants were instructed to consume 800 mg of calcium supplements daily during the trial to minimize variability in calcium intake. The University of Arizona Internal Review Board approved the study and the subjects provided written and informed consent.

Subject Entry Criteria

The BEST study enrolled 6 cohorts of postmenopausal women (Fall 1995- Fall 1997) who met the following inclusion criteria: 40-65 years of age; surgical or natural menopause (3.0-10.9 years); body mass index (BMI) > 19.0 kg/m^2 and < 32.9 kg/m^2; non-smoker; no history of osteoporotic fracture and an initial BMD greater than Z-score of –3.0 at all bone sites of interest; taking HT (1.0-5.9 years) or not taking HT (>1 year); weight gain or loss ≤ 13.6 kg in the previous year; cancer and cancer treatment-free ≥ 5 years (excluding skin cancer); not taking BMD-altering medications, beta-blockers, or steroids; dietary calcium intake >300 mg/day; performing <120 minutes of low intensity,

low impact exercise per week and no weightlifting or similar physical activity. Participants agreed to accept randomization to exercise or usual exercise groups, continue their baseline level of physical activity, continue their usual dietary practices, maintain their HT status, and take 800 mg of calcium supplements each day of the trial (Going 2003).

At the end of the first year of intervention, controls (n=78) were allowed to begin or "cross over to" the prescribed exercise regime. Fifty-five women began exercising in the second year (crossovers); 23 women remained controls, and 89 comprised the original randomized exercise group. In year four, 177 women returned for BMD scans (53.3% retention from baseline (n=320), 47 women were excluded in this analysis: six had started taking alendronate; one used steroids; one was hospitalized with a broken leg for 6 months; two women developed cancer (Cussler 2005) and 37 did not return the Arizona Food Frequency Questionnaire (AFFQ) in year 1 (n=1), year 2 (n=5), year 3 (n=8), or year 4 (n=23). Four years of data were obtained across years 1995-2002, for a final sample of 130 postmenopausal women.

Anthropometry

Trained anthropometrists took three measurements, which were averaged to obtain the criterion measures, of each anthropometric variable at the fourth year assessment period. Subjects wore lightweight clothing without shoes for the height and weight (WT) measurements. Standing height was measured to the nearest 0.1 cm during a maximal inhalation using a Schorr measuring board (Schorr Products, Olney, MD). Weight was measured on a calibrated digital scale (SECA, model 770, Hamburg,

Germany) accurate to 0.1kg. Body mass index (BMI) in kilograms per meter squared was calculated from WT (kg) and height (m).

Dual Energy X-ray Absorptiometry

The DXA was used to measure femur neck and trochanter, lumbar spine (L2-L4), and total body BMD (Model DPX-L; software version 1.3y, extended research analysis, pencil beam densitometer, Lunar Radiation Corp, Madison, WI., USA). Standardized data acquisition and analysis techniques were used (Going, 2003, Cussler 2005). Each subject was scanned twice at the four-year measurement period. Lean soft tissue and body fat composition was also derived from DXA whole body scans.

Diet Assessment

Dietary intake was assessed at the end of the first year and annually thereafter using the previously validated Arizona Food Frequency Questionnaire (AFFQ) (Martínez, 1999). Participants were asked to complete the questionnaire in relation to their overall pattern of food intake during the previous 12 months. The AFFQ is based on the Block Model and is a 153 item, semi quantitative, scannable questionnaire on the frequency of food consumption, using age and gender specific estimates of portions. The AFFQ was modified to include southwestern foods (Taren, 1999). The AFFQ were distributed with verbal and written instructions at the end of the first year. During years two through four, participants were mailed the AFFQ annually with written instructions and returned the AFFQ during the assessment period. Each year the trained technicians reviewed the AFFQ once with the participants and a second time without the participant for completeness and accuracy before they were sent to the Arizona Diet and Behavioral

Assessment Center for analysis. Nutrient analysis for the AFFQ was completed using a proprietary software program called Metabolize (version 2.7, 2003, Arizona Diet and Behavioral Assessment Center, Tucson, AZ), which was updated to include version 17 of the USDA food composition database in 2005. All AFFQ were reanalyzed with this newer version of the USDA food composition database.

Calcium Supplements

In their first and second years, all participants received 800mg/day of elemental calcium (calcium citrate) (Citrical, Mission Pharmacal, San Antonio, TX, USA). At the start of the third year, participants were asked to continue calcium supplementation on their own by purchasing and taking a comparable calcium supplement. Total calcium was calculated using the yearly mean dietary calcium intake from AFFQ plus calcium supplement intake from tablet counts (years 1-2) and quarterly self- reports (years 3-4) of average calcium intake.

Statistical Methods

All data analyses were performed using the Statistical Package for the Social Sciences (version 16, 2007, SPSS Inc., Chicago, IL). Four year dietary intakes were calculated from the yearly AFFQ, except for total calcium.

Descriptive characteristics for body composition at year four and mean dietary nutrient intakes, from AFFQ averaged over four years (to obtain a representative estimate of dietary intake used in the analysis) were calculated. Student's t-tests at year four were used to detect statistically significant differences in participant characteristics and dietary intakes between the subjects who took HT and those who did not take HT. Repeated

measures were computed for nutrient intakes from year one to year four. Dietary nutrient intakes were averaged over the four years to obtain a representative estimate of dietary intake used in the analyses. Nutrient intake distributions were examined and log-transformed, when appropriate, to meet the assumption of the statistical tests.

Separate multiple linear regression was used to examine the associations between the average 1-4 years nutrient intakes and year four BMD, since the 4 years of dietary nutrient intake represented the four years prior to the BMD measurements. The covariates used in the multiple linear regression included years on HT during the dietary assessment period, years post menopausal, exercise history over four years, average energy intake, year 4 total lean soft tissue, and year 4 total body fat. When the sample was stratified by HT, years post menopausal, exercise history over four years, average energy intake, year 4 total lean soft tissue, and year 4 total body fat were controlled. Significance was evaluated at the $p \leq 0.05$ level. Only significant associations are reported using the standardized β coefficient. The power from the whole model, including all independent variables is ≥ 0.80 for $R^2 \geq 0.1045$.

Results

The characteristics of participants at year four are presented in **Table 12**. There were no significant differences between the HT and no HT group except that the no HT group was slightly older (62±4 years vs. 59±3.9) and had been menopausal longer (11±3.8 years vs. 9.5±3). The mean nutrient intake of the ratio of linoleic acid (LA) to alpha-linolenic acid (ALA) was significantly lower in the no HT group. The mean nutrient intake values for all women were similar across all four years ($p \leq 0.05$).

Significant differences were found among the four years for energy (p=0.032), carbohydrate (p=0.008), caffeine (p=0.003), fiber (p=0.032) and sodium (p=0.034) among years one through four. The highest intakes of energy, carbohydrate, caffeine, fiber and sodium were in year one. The lowest intakes for these nutrients were in year three, except for caffeine where the lowest intake was in the fourth year. There was less than 20% percent difference between the highest and the lowest values for these specific nutrient intakes, except for caffeine, which was 29% different, but caffeine is notoriously skewed. Clinically these differences between these particular nutrients are minimal. Average dietary intake over four years is similar to NHANES (USDA 2008) population estimates for women aged 50-59 years, except for vitamin C and folate which were about half of this populations average dietary intake over four years.

The nutrient associations with BMD in 130 postmenopausal women are presented in **Table 13**. Only spine and total body BMD have significant associations with nutrients. Spine BMD was inversely related to intakes of vitamin B_{12}, total PUFA, total omega 3 (n-3 FA), ALA, total omega 6 (n-6 FA), and LA. Similarly, total body BMD was inversely related to n-3 FA and LA. When the sample was categorized by HT (N=92) and no HT (N=38), the only significant associations appeared in the HT group (**Table 14**). As in the total sample, nutrients were inversely related to spine and total body BMD. In the HT group, spine BMD was significantly related to total fat, vitamin B_{12}, total PUFA, n-3 FA, ALA, docosahexaenoic acid (DHA), DPA, eicosapentaenoic acid (EPA), n-6 FA and LA. In the HT group, total body BMD was significantly related to n-3 FA, ALA, DHA, DPA, EPA, n-6 FA.

Discussion

This study examined the longitudinal dietary nutrient intake associations, assessed over a four-year period, with cross-sectional four year regional and total BMD of a healthy, nonsmoking population of postmenopausal women who participated in the BEST clinical trial. Previously published results measured at baseline and at the end of the first year of the BEST study demonstrated that mean dietary nutrient intakes were associated with BMD (Farrell PhD dissertation 2008, Farrell 2008 accepted, Cussler 2005, Maurer 2005, Harris 2003). Baseline cross-sectional BEST study data (N=242) demonstrated iron intake (>20 mg/day), assessed by 3 days of DR, was associated with greater BMD at the femur neck and trochanter, Ward's triangle, lumbar spine, and total body when mean calcium intakes were 800-1200 mg/day (Harris 2003). A study by Maurer *et al.* (2003) examined the relationships between mean nutrient intake and BMD after one-year of participation in of the BEST study (N= 228). A positive association with iron intake, assessed by 8 days of DR, and BMD at the femur trochanter and Ward's triangle was identified. However, when analyzed by HT status, associations remained significant only for women using HT. Calcium intake was positively associated with the change of femur neck and trochanter BMD in the first and fourth year in women not using HT (Maurer 2003, Cussler 2005). The current analysis did not find any associations of BMD with dietary iron or total calcium. However, the current analysis did show significant nutrient associations with the lumbar spine and total body BMD in the total sample (N=130) and in the HT group (N=92) when the sample was categorized by HT.

In a prior study with data from the BEST study (N=266), one-year dietary PUFA intakes (PUFA, n-3 FA, ALA, n-6 FA, LA), assessed by 8 days of DR, were significantly inversely associated with lumbar spine and total body BMD. Arachiconic acid had the only positive association, which was with trochanter BMD. When stratified by HT use all PUFA associations with BMD remained in the HT group, but were lost in the no HT group. In the HT group, n-3 FA, ALA had an inverse relationship with spine and total body BMD and LA:ALA had a positive relationship with total body BMD (Farrell PhD dissertation 2008). These same PUFA associations were seen with the lumbar spine BMD and only n-3 FA and LA associations were seen with total body BMD at 4 years. Maurer *et al.* (2005) and Farrell *et al.* (dissertation, 2008), reported associations with nutrients and BMD remained only in the HT group. In one year (N=136, nutrient intake assessed by diet records) and 4 year (N=92; nutrient intake assessed by AFFQ) cross sectional data, the same PUFAs, n-3 FA and ALA, were inversely related and significant in both year one and year four. This adds to the evidence of the interaction of HT use and nutrient and BMD relationships.

Other researchers using a FFQ to estimate diet and DXA to measure BMD found that higher intakes of potassium were positively correlated with BMD at the lumbar spine, femur neck, femur trochanter, and Ward's triangle (Farrell 2008 accepted, Tucker1999, New 1997). Higher intakes of magnesium, vitamin C, alcohol, and fiber, correlated with BMD at the spine in 994 healthy premeonopausal women (New 1997). Tucker *et al.* (1999) also showed in 512 women aged, 69-97 years, that there were significant associations between potassium and magnesium intakes and trochanter BMD.

The current study showed the nutrient intakes assessed by the AFFQ had significant inverse associations of total fat, vitamin B_{12}, and the PUFAs with lumbar spine and total body BMD in postmenopausal women.

Few studies have examined the relationship between habitual dietary intake of PUFA and BMD. It is thought that the n-3 FA has a favorable affect on skeletal health (Vanek 2007). However, the current analysis shows negative associations with BMD. In a study of, 891 women (50-59 years), not categorized by HT use, Macdonald *et al.* (2004) reported that total PUFA intake, assessed by FFQ, was associated with lower BMD loss at the femur neck, particularly among women with lower calcium intakes, assessed over the previous 12 months. No significant associations were observed in relation to spine BMD (Macdonald 2004). In comparison, the current analyses did not identify any significant associations between PUFA intake and femur neck BMD. The current analyses did demonstrate significant inverse associations between dietary PUFA intake and spine BMD. In another study of 890 postmenopausal women, a higher ratio of n-6 FA:n-3 FA (7.9±2.2 g or ~10:1), assessed by FFQ, was associated with a lower BMD at the hip and at the spine in women (45-95 years) (N=326) not using HT (Weiss 2005). In contrast, the current analysis did not show the ratio of n-6 FA:n-3 FA (8.16±2.2 g or ~10:1) to be significantly associated with BMD at the hip or spine in the HT group. However, dietary PUFA had significant inverse associations with spine BMD which included total PUFA, n-3 FA, ALA, DHA, DPA, EPA, n-6 FA and LA, and total body BMD included n-3 FA, ALA, DHA, DPA, EPA, n-6 FA. These inconsistent findings among studies may be due, in part, to differences in age of participants, dietary

assessment methods, and the co-dependency and interactions among nutrients and genetic and environmental factors (Ilich 2003).

When the sample was stratified by HT, total fat and vitamin B_{12} had significant inverse associations with BMD. Since intestinal calcium absorption normally decreases with age, consumption of high-fat diets, particularly those rich in saturated fatty acids may put the elderly at risk for increased calcium excretion and associated bone loss (Corwin 2003). Fat consumption was negatively associated with BMD in the lumbar spine and radius in 218 postmenopausal women (Michaëlsson 1995). Research suggests that the type and amount of fat consumed are important in bone health. Of the different types of fats, PUFA are receiving recognition for having beneficial roles in the prevention and treatment of osteoporosis (Salari 2008, Watkins and Lippman 2001). Osteocytes and osteoblasts release prostaglandin E_2 (PGE2), a lipid compound derived from fatty acids, when there is strain on bone and this stimulates the production of proinflammatory cytokines which activate osteoclasts and bone resorption. Arachidonic acid (AA), increases the formation of PGE2 and n-3 FA decrease this formation (Watkins and Lippman 2001). A high intake of n-3 FA produces anti-inflammatory cytokines that decrease bone resorption, increase calcium absorption in the gut, decrease the amount of calcium lost in the urine, increase calcium deposition in the bone, and increase collagen synthesis (Genuis 2006, Watkins and Lippman 2001). However, in this study all PUFA associations with BMD are negative.

Compared to the NHANES 2005-2006 fat intake for women 50-59 years (68 grams), this sample consumed ~18 grams less fat on average (50 ± 21 g) than the general

population of their age and gender. Low levels of vitamin B_{12} correlate with elevated homocysteine levels. Homocysteine may inhibit the formation of osteoblasts, decrease bone resorption, cause structural changed with the extracellular matrix protein, and interfere with the formation of collagen cross-links and fibrils (Hermann 2005). Low vitamin B_{12} levels, high homocysteine concentrations or a combination of both were shown to increase risk for factures by three times in a population of 1267 men and women, ages 55-85 years (Dhonukshe-Rutten 2005). This sample (average over 4 years, 4 ± 3 mcg) consumed a comparable amount of vitamin B_{12} when compared to the NHANES 2005-2006 vitamin B_{12} intakes (assessed by 24 recall, 4 mcg).

Although DR are a more accurate method than FFQ to measure food intake in adults (McKeown, 2001), DR are usually cost prohibitive to use when collecting 4-year data, so FFQ were utilized. The FFQ is known to have a low burden on the participant and are easier to administer and analyze than DR (Thomson 2003). Cross-sectional BEST data was analyzed for associations between one-year dietary intakes, assessed by 8 days of DR and AFFQ, and BMD (N=244) (Farrell 2008 accepted). The investigation suggested that certain bone sites have more associations with certain nutrients than other sites, and the AFFQ could be used interchangeably with DR for certain nutrients, and that mean dietary intake associations with BMD did occur after one-year of study participation. The current study investigating four-year nutrient intakes with BMD compared to previous work at one year with the same population of women and found no similar nutrient associations with nutrient intakes, assessed by the AFFQ and BMD. While nutrient associations were found in all of the bone sites in the fist year of the study,

the nutrient associations in year four were found only in the lumbar spine and total body BMD.

Other limitations of this study include the number of subjects particularly after categorizing by HT use is relatively small. This study design only allows for association and not causal relations between diet and BMD to be inferred. A couple of reasons why these results report a negative association of dietary intake on BMD is the accuracy of self-reported dietary intake is limited and the database used in the nutrient analysis may have incomplete nutrient intake information and missing nutrient values. A constantly changing food supply, the advent of new compounds of health interest, and limited resources are limitations in all analyses of dietary nutrient intake assessment methods and the USDA database used for analyses of nutritive values of foods (Dwyer 2003). Another possibility, although not proven, may be that the dietary intakes, particularly the PUFAs, may be over-reported or underabsorped, thus when the associations with BMD are made in reality lower intakes of PUFAs are actually being consumed or absorbed. A negative BMD association with PUFAs is possible if more n-6 FA and less n-3 FA are consumed because a high n-6:n-3 FA ratio is desirable (Simopoulos 2006). Biological markers for fatty acid concentrations would enhance the interpretation of these types of results.

The BEST clinical trial was not designed to evaluate the biochemical mechanisms underlying the observed nutrient-BMD associations therefore, the analysis can only be used to speculated that HT, most likely estrogen, which inhibits bone resorption, has some direct association with bone metabolism in relation to dietary intake in

postmenopausal women. The relationship was identified in several previous studies (Farrell PhD dissertation 2008, Weiss 2006, Maurer 2003), but no potential HT-nutrient-BMD mechanism has been identified. Hopefully, future research studies will investigate the nutrient-bone-hormone relationship.

Conclusion

In conclusion, the results of this study demonstrated that the means of dietary nutrient intakes, assessed by AFFQ over a four year period, are inversely associated with BMD at the lumbar spine (vitamin B_{12}, PUFA, n-3 FA, ALA, n-6 FA, LA) and total body (n-3 FA, LA), in a sample of healthy postmenopausal women N=130. When categorized by HT use (N=92), nutrient intake associations with BMD remained significant and negative for spine (total fat, vitamin B_{12}, total PUFA, n-3 FA, ALA, DHA, DPA, EPA, n-6 FA and LA) and total body (n-3 FA, ALA, DHA, DPA, EPA, n-6 FA) BMD in the HT groups. The current results support the one-year findings that dietary PUFAs intakes, assessed by diet records, have mostly inverse association with BMD. The results also add to the growing body of evidence of a nutrient-bone-HT relationship as seen by the retention of nutrient associations with BMD in the HT group. It is important for future studies to explore the biological mechanism to determine the role of dietary intake on bone.

Table 12. Mean characteristics of 130 postmenopausal women using or not using HT at year 4[a]

			HRT Status	
Characteristics	**All Women[a] (N=130)**	**NHANES[c]**	**No HRT[a] (n=38)**	**HRT[a] (n=92)**
Age (years)	60±4.3		62±4[e]	59±3.9
Post menopause (years)	10±3.3		11±3.8[e]	9.5±3
Height (cm)	162±6.9		163.6±5.9	162.2±7.2
Weight (kg)	68±11		67.5±10.6	68.9±10.8
BMI ($kg/m^{2)}$	25.8±3.7		25.2±3.4	26±3.8
BMD from DXA (g/cm^2)				
Femur neck BMD	0.870±0.122		0.869±0.109	0.871±0.127
Femur trochanter BMD	0.749±0.111		0.726±0.104	0.759±0.113
Lumbar spine L2-L4	1.133±0.161		1.11±0.146	1.144±0.167
Total body BMD	1.103±0.085		1.08±0.082	1.111±0.085
Nutrients				
Energy (Kcal)	1609±552[b]	1718	1576±560	1623±551
Protein (g)	65±24	70	65±24	65±24
Fat (g)	50±21	68	47±20	52±21
Carbohydrate (g)	232±86[b]	205	232±86	232±86
Alcohol (g)	3±5	5	2±3	3±6
Caffeine (mg)	188±164[b]	225	214±172	177±160
Fiber (g)	21±8[b]	15	21±8	21±9
Calcium (mg)	955±379	799	1006±421	934±361
Total Calcium (mg)	1695±388	--	1750±429	1672±370
Iron (mg)	14±5	13	14±5	14±4
Magnesium (mg)	324±103	267	333±109	321±101
Phosphorus (mg)	1260±455	1134	1283±475	1250±449
Potassium (mg)	3232±1106	2458	3410±1110	3158±1101
Sodium (mg)	2628±975[b]	3001	2553±950	2659±988
Zinc (mg)	10±4	10	10±4	10±4
Vitamin B_{12} (mcg)	4±3	4	4.2±1.8	4.2±3.1
Vitamin C (mg)	153±85	75	167±86	147±85
Vitamin D (IU)	127±83	--	139±91	122±79
Vitamin E (mg)	8±3	6	8±4	8±3
Total Poly Unsaturated Fatty Acid (g)	10±4	15	9±4	11±4
Omega 3 Fatty Acid (n-3) (g)	1.1±0.44	--	1.0±0.41	1.1±0.48
Alpha-Linolenic Fatty Acid (ALA) (g)	0.93±0.40	1.3	0.88±0.35	0.95±0.42
Docosahexaenoic Acid (g)	0.08±0.06	0.07	0.08±0.06	0.08±0.06

Docosapentaenoic Acid (g)	0.01±0.01	0.02	0.01±0.01	0.01±0.01
Eicosapentaenoic Acid (g)	0.04±0.03	0.04[d]	0.04±0.03	0.04±0.03
Omega 6 Fatty Acid (g)	9±4	--	8.3±3	9.3±4
Arachidonic Fatty Acid (g)	0.09±0.05	--	0.08±0.05	0.09±0.05
Linoleic Fatty Acid (LA) (g)	9±4	13	8±3	9±4
n-6/n-3 (g)	8.5±1.4	--	8.3±1.3	8.7±1.4
LA/ALA (g)	9.6±1.3	--	9.3±1.3[e]	9.8±1.2

[a] Values are means ± SD; [b] $\rho \leq 0.05$ by repeated measures from AFFQ years one through four; [c] National health and nutrition examination survey (NHANES) = Nutrient Intakes from food: mean amounts consumed per female 50-59 years, one day, 2005-2006 (USDA 2008).; [d]indicates an estimate with a relative standard error greater than 30%; [e]significantly different from HRT $\rho \leq 0.05$;

Table 13. Mean nutrient associations with bone mineral density (BMD) in postmenopausal women at year four (N=130).

	Standardized Beta coefficients			
Nutrient	**Spine (L1-L4) BMD**	**Adjusted R^2**	**Total Body BMD**	**Adjusted R^2**
Vitamin B_{12}	-0.260	0.077		
Total Poly Unsaturated Fatty Acid	-0.389	0.096		
Omega 3 Fatty Acid	-0.374	0.095	-0.309	0.150
Alpha Linolenic Fatty Acid	-0.342	0.085		
Omega 6 Fatty Acid	-0.344	0.087		
Linoleic Fatty Acid	-0.343	0.087	-0.343	0.087

Independent variables: years of HT use during the period of dietary assessment, years post menopausal, exercise history, average total energy intake, year 4 total lean soft tissue and year 4 total body fat.
$p \leq 0.05$

Table 14. Mean nutrient associations with bone mineral density (BMD) in postmenopausal women on hormone therapy at year 4 (N=92).

	Standardized Beta Coefficients			
Nutrient	**Spine (L1-L4) BMD**	**Adjusted R^2**	**Total body BMD**	**Adjusted R^2**
Total Fat	-0.390	0.049		
Vitamin B_{12}	-0.347	0.065		
Total Poly Unsaturated Fatty Acid	-0.474	0.084		
Omega 3 Fatty Acid	-0.499	0.098	-0.360	0.078
Alpha-Linolenic Fatty Acid	-0.428	0.071	-0.526	0.137
Docosahexaenoic Acid	-0.263	0.062	-0.434	0.101
Docosapentaenoic Acid	-0.270	0.060	-0.322	0.118
Eicosapentaenoic Acid	-0.230	0.050	-0.305	0.104
Omega 6 Fatty Acid	-0.440	0.075	-0.275	0.098
Linoleic Fatty Acid	-0.438	0.075		

Independent variable: years of HT use during the period of dietary assessment, years post menopausal, exercise history, average total energy intake, year 4 total lean soft tissue and year 4 total body fat.
$p \leq 0.05$

SUMMARY AND CONCLUSION

The first study in this dissertation compared the equivalency of nutrient intakes estimated by two dietary assessment methods and the comparability of the associations of these nutrient intakes with BMD in postmenopausal women who completed year one in the BEST study (n=244). The first hypothesis stated that DR and AFFQ would have equivalent estimates of the same year of dietary nutrient intake. Hypothesis #1a stated that when determining nutrient intake associations with BMD, DR and AFFQ would provide equivalent estimates. In other words, DR and AFFQ will provide equivalent estimates of nutrient intakes that could be used interchangeably to determine the associations of dietary nutrient intakes with BMD in healthy, post-menopausal women.

Pearson's correlations were used to determine any significant differences between dietary nutrient intakes of energy, protein, carbohydrate, fat, alcohol, vitamin C, vitamin E, vitamin D, calcium, phosphorous, iron, magnesium, sodium, potassium, zinc, caffeine, fiber, and zinc assessed by DR and AFFQ. This study observed that there were no significant differences between the dietary nutrient intakes assessed from DR and AFFQ. Thus, DR and AFFQ have relatively equivalent estimates of dietary nutrient intakes.

Further analysis, using multiple linear regression, investigating the dietary nutrient intake associations with BMD, using DR and AFFQ. This study showed that iron and magnesium, estimated either from DR or AFFQ, were consistently and significantly associated with BMD at all bone sites (femur neck, Ward's triangle, femur trochanter, lumbar spine (L2-L4), total body). Other nutrient relationships that showed statistically significant associations with BMD, assessed by DR and AFFQ, in three or

more of the same skeletal sites included zinc and fiber which were significantly associated with BMD at the femur neck, Ward's triangle, femur trochanter, and the total body. Phosphorous, potassium, calcium, and total calcium were significantly associated with BMD at the femur neck, Ward's triangle, and total body when estimated by both dietary assessment methods. Protein, alcohol, caffeine, sodium, and vitamin E did not have any similar associations with any of the BMD sites in these analyses. This study showed that either DR or AFFQ provided relatively equivalent estimates and found similar significant associations of dietary nutrient intakes of iron, magnesium, zinc, fiber, calcium, total calcium, phosphorous, and potassium with BMD in postmenopausal women at one year. **Table 15** gives a summary of the one-year dietary nutrient intakes, assessed by DR and AFFQ, that are associated with three or more of the same BMD sites.

The conclusions of this study were that either DR or AFFQ, assessing the same year of dietary intake, provided relatively equivalent estimates of nutrient intakes for the bone-related nutrients investigated. When determining the dietary nutrient intake associations with BMD, the nutrients that had three or more of the same significant bone site associations included iron, magnesium, zinc, fiber, calcium, total calcium, phosphorous, and potassium. The DR and AFFQ are acceptable dietary assessment tools used to determine the associations of particular nutrients and BMD sites in healthy postmenopausal women.

Table 15. Summary of the one-year dietary nutrient intakes, assessed by DR and AFFQ, that are associated with three or more of the same BMD sites.

Nutrients	Ward's Triangle		Femur Neck		Femur Trochanter		Lumbar Spine (L2-L4)		Total Body	
	All		All		All		All		All	
Iron	+X		+X		+X		+X		+X	
Magnesium	+X		+X		+X		+X		+X	
Zinc	+X		+X		+X				+X	
Fiber	+X		+X		+X				+X	
Phosphorous	+X		+X						+X	
Potassium	+X		+X						+X	
Calcium	+X		+X						+X	
Total Calcium	+X		+X						+X	

All= all women (N=244)
+/- = indicates direction of association
X= study number one significant dietary nutrient intake associations with BMD at 3 or more bone sites.
$p \leq 0.05$

The second study in this dissertation investigated PUFA intakes, assessed by 8 days of DR, and their association with BMD in postmenopausal women who completed year one in the BEST study (N=266). The first hypothesis (#2) of this study was that dietary PUFA intakes would have positive n-3 FA or negative n-6 FA associations with regional and total body BMD sites in postmenopausal women at one year. Hypothesis #2a was that dietary PUFA intake associations with regional and total body BMD would occur in both the HT (n=136) and no HT (n=130) groups. The following nutrients served as the independent variables for the multiple linear regression analyses, total PUFA, n-6 FA, LA, AA, n-3 FA, ALA, and the ratios: LA:ALA, and n-6 FA:n-3 FA.

The findings of this study showed that in the total sample dietary intakes of PUFA, n-6 FA, LA, n-3 FA, ALA, had significant inverse associations with lumbar spine and total body BMD. Arachidonic acid had the only positive association with BMD, which was at the trochanter. Dietary PUFA intake did have mostly significant inverse associations with BMD in the femur trochanter, lumbar spine (L2-L4), and total body in healthy, postmenopausal women. However, dietary PUFA intake did not have any associations with BMD in the Ward's triangle or the femur neck.

In the no HT group, no significant associations between dietary PUFA intake and regional and total body BMD were observed. In the HT group, inverse associations with dietary PUFA intake were seen with BMD in the spine and total body for n-3 FA, ALA. The ratio of LA:ALA had a positive association with total body BMD. Dietary PUFA intake associations with BMD at year one only occurred in the HT group. There were no significant dietary PUFA intake associations with BMD at year one in the no HT group. **Table 16** summarizes the one-year dietary PUFA intakes, assessed by DR, associated with BMD.

This cross-sectional study among 266 postmenopausal women found dietary intakes of PUFA, n-6 FA, LA, n-3 FA, ALA, had significant inverse associations with lumbar spine and total body BMD. Arachidonic acid had the only positive association, which was at the trochanter BMD. When stratified by HT use all PUFA associations with BMD remained significant in the HT group, but were lost in the no HT group. In the HT group, n-3 FA, ALA had an inverse relationship with spine and total body BMD and LA:ALA had a positive relationship with total body BMD. This suggests that HT

and dietary PUFA intake are modulators of BMD. Dietary intake, a modifiable risk factor for osteoporosis, is a safe and inexpensive way to potentially prevent or treat osteoporosis. With more conclusive dietary studies, especially those that investigate cause and effect, dietary recommendations can aid in the prevention and treatment of osteoporosis.

Table 16. Summary of significant dietary polyunsaturated fatty acid intake associations with BMD, using the DR, at year one.

Nutrients	Femur Trochanter		Lumbar Spine (L2-L4)		Total Body	
	All	HT	All	HT	All	HT
PUFA			-Y		-Y	
n-6 FA			-Y		-Y	
LA			-Y		-Y	
AA	+Y					
n-3 FA			-Y	-Y	-Y	-Y
ALA			-Y	-Y	-Y	-Y
LA:ALA						+Y
Total fat			-Y	-Y	-Y	-Y

All= all women (N=266)
HT = women on hormone therapy (n=136)
+/- = Indicates direction of association
Y= Study number two significant dietary nutrient intake associations with BMD
$p \leq 0.05$

The third study in this dissertation investigated dietary nutrient intake, assessed by the AFFQ, associations with regional and total body BMD in postmenopausal women completing four years of the BEST study. The first hypothesis (#3) was that dietary

nutrient intake at four years, will have positive and negative associations with regional and total body BMD (N=130) at year four. The second hypothesis (#3a) was that dietary nutrient intake associations with regional and total body BMD would occur in the HT group (n=92) but not in the no HT group (n=38) at year four. The independent variables used in the multiple linear regression included energy, protein, carbohydrate, fat, alcohol, vitamin B_{12}, vitamin C, vitamin E, vitamin D, calcium, phosphorous, iron, magnesium, sodium, potassium, zinc, caffeine, fiber, and zinc total PUFA, n-6 FA, LA, AA, n-3 FA, ALA, DPA, DHA, EPA and the ratios: LA:ALA, and n-6 FA:n-3 FA.

The findings from this study showed that the mean of four years of dietary intake was negatively associated with BMD at the lumbar spine for vitamin B_{12}, PUFA, n-3 FA, ALA, n-6 FA, LA and total body for n-3 FA, LA, in postmenopausal women. Dietary nutrient intake had some associations with the lumbar spine and total body BMD in postmenopausal women at year four. When categorized by HT use, negative associations with BMD were seen in the lumbar spine for total fat, vitamin B_{12}, total PUFA, n-3 FA, ALA, DHA, DPA, EPA, n-6 FA and LA and in total body for n-3 FA, ALA, DHA, DPA, EPA, n-6 FA. There were no significant nutrient associations with BMD in the no HT group. Dietary nutrient intake associations with BMD were found only in the HT group at four years. **Table 17** summarizes the dietary nutrient intake associations with BMD.

The conclusions of this study were that significant dietary nutrient intake, assessed by the AFFQ, at four years, had significant inverse associations with BMD at the lumbar spine and total body. Dietary nutrient intake associations with regional and total body BMD were found in the HT group but not in the no HT group at year four.

Furthermore, there were no significant dietary nutrient intake associations in the femur neck or femur trochanter BMD. The current results supports the one-year findings from study number two that dietary PUFA intakes, assessed by diet records, have mostly inverse association with BMD. The results also add to the growing body of evidence of a nutrient-BMD-HT relationship as seen by the retention of nutrient associations with BMD in the HT group. It is important for future studies to explore the biological mechanism to determine the role of dietary intake on bone.

Table 17. Summary of the dietary nutrient intake associations with BMD, using the AFFQ, at year four.

Nutrients	Lumbar Spine (L2-L4)		Total Body	
	All	HT	All	HT
PUFA	-Z	-Z		
n-6 FA	-Z	-Z		-Z
LA	-Z	-Z	-Z	
AA				
n-3 FA	-Z	-Z	-Z	-Z
ALA	-Z	-Z		-Z
LA:ALA				
DHA		-Z		-Z
DPA		-Z		-Z
EPA		-Z		-Z
Vitamin B_{12}	-Z	-Z		
Total fat		-Z		

All= all women (N=130)
HT = women on hormone therapy (n=92)
+/- = Indicates direction of association
Z= Study number three significant dietary nutrient intake associations with BMD
$p \leq 0.05$

The three studies included participants that were a well-nourished population of postmenopausal women. Equivalent dietary nutrient intake associations were seen with regional and total body BMD at one year, assessed by DR and AFFQ. Furthermore, the PUFA had primarily negative associations with regional and total body BMD at one year. However, HT seems to be influencing dietary nutrient intake associations with BMD. It

is evident that different dietary nutrient intakes are associated with different BMD sites. **Table 18** summarizes the significant dietary nutrient associations with BMD across all three studies.

Table 18. Summary of dietary nutrient intake associations with BMD across three studies.

Nutrients	Ward's Triangle		Femur Neck		Femur Trochanter		Lumbar Spine (L2-L4)		Total Body	
	All	HT	All	HT	All	HT	All	HT	All	HT
Iron	+X		+X		+X		+X		+X	
Magnesium	+X		+X		+X		+X		+X	
Zinc	+X		+X		+X				+X	
Fiber	+X		+X		+X				+X	
Phosphorous	+X		+X						+X	
Potassium	+X		+X						+X	
Calcium	+X		+X						+X	
Total Calcium	+X		+X						+X	
PUFA							-Y, -Z	-Z	-Y	
n-6 FA							-Y, -Z	-Z	-Y	-Z
LA							-Y, -Z	-Z	-Y, -Z	
AA					+Y					
n-3 FA							-Y, -Z	-Y, -Z	-Y, -Z	-Y, -Z
ALA							-Y, -Z	-Y, -Z	-Y	-Y,- Z
LA:ALA										+Y
DHA								-Z		-Z
DPA								-Z		-Z
EPA								-Z		-Z
Vitamin B_{12}							-Z	-Z		
Total fat							-Y	-Y,-Z	-Y	-Y

X= 12 month (3 or more of the same BMD sites with DR and AFFQ agreement) (N=266)

Y=12 month (N=266; n of HT users=136)
Z= 4 year (N=130; n of HT users=92)
+/- = Indicates direction of association
$p \leq 0.05$

There are several limitations of these studies. After an intensive, thorough training, participants were asked to keep their DR with them throughout the day and to measure their food and record it in their DR as they ate. It is unknown whether many participants actually recorded their food choices as they ate or recorded what they ate at the end of the day or before the diet record interview, thus turning the DR into a dietary recall. Different versions of the USDA and propriety databases were used to estimate nutrient intakes from DR and AFFQ. Discrepancies occur because of the constantly changing food supply, the advent of new compounds of health interest and, limited resources (Dwyer 2003). These discrepancies can lead to incomplete estimated nutrient content information and missing nutrient values. Further, the DR nutrient database used in the analysis, while the most comprehensive database for dietary fatty acids, did contain incomplete or missing nutrient values for some foods. For example sixteen to twenty percent of the values of LA, ALA, n-6 FA, or n-3 FA, were estimated values in version 28 of the NDS Nutrient Database.

Other limitations of this study include the number of subjects, which, particularly after categorizing by HT, was relatively small, thus decreasing the power of the study and increasing the risk of a type 1 error. Also, the PUFA had a high amount of colliniarity among the different fatty acids. Another issue was that the dietary nutrient intakes might not reflect the actual amount of the nutrient being ingested. This may be because of

mislabeling of the product and/or insufficient nutrient information. Thus, in reality lower or higher dietary intakes are actually being consumed than are reported from the dietary analysis which could confound the ability to detect relationships with BMD. Although dietary nutrient assessment methods have their limitations, subjective errors in self-reporting, changes in nutrient intake over time, and limitations of nutritional databases used for calculation (Ilich 2003), reliable trends in nutrient intakes can be collected and useful information obtained from cross-sectional studies with large number of participants to provide for statistical power (Ilich 2003). Other limitations include the co-dependency of nutrients, influence of genetic and hormonal factors and the interactions of lifestyles (e.g. smoking, drinking) (Ilich 2003).

This dissertation research led to further research questions regarding this population of BEST postmenopausal women. Possible questions that have yet to be answered include: 1) What are the dietary nutrient intake associations with BMD assessed by DR at one year categorized by HT?, 2) What are the dietary PUFA intake associations with BMD assessed by the DR at one year?, 3) What are the dietary PUFA intake associations with BMD assessed by the DR at one year categorized by HT?, 4) Does dietary vitamin B_{12} intake have significant associations with BMD, assessed by DR and AFFQ, at one year?, 5) Does dietary vitamin B_{12} intake have significant associations with BMD, assessed by DR and AFFQ, at one year categorized by HT?, 6) Using the 10 year BEST data, what dietary nutrient intake assessed from the AFFQ will have associations with BMD? 7) What is the association of the dietary nutrient intakes and change in BMD at one year, four year and beyond?

The research in this dissertation will add to the growing body of evidence of dietary nutrient intake and associations with BMD at specific bone sites, however without biomarkers cause and effect will be elusive. Another area that needs more research is the interaction among HT, dietary nutrient intake, and BMD. Future research needs large, controlled, randomized, blinded dietary trials that look at dietary nutrient intake and appropriate biomarkers for nutrient intake, BMD and HT to augment the findings from this dissertation. The focus should be on type and the amount of a specific nutrient and to establish the effect of specific nutrients on the specific bone sites while on or not on HT. Many studies show a relationship with nutrients, HT and BMD, yet no potential mechanisms have been investigated or identified (Farrell PhD dissertation 2008, Weiss 2006, Maurer 2005). The analyses from this dissertation, however, can be used to speculate that HT, most likely estrogen, which inhibits bone resorption, has some direct association with dietary nutrient intake and bone metabolism in postmenopausal women. These results provide additional evidence that future studies need to explore the biological mechanism to determine the HT-dietary nutrient intake-BMD relationship. The results from this dissertation and future more robust studies can lead to valuable information regarding the role of nutrient intake in the prevention and treatment of osteoporosis.

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