

**REGULATION OF BONE QUALITY AND CALCIUM ABSORPTION IN
OBESITY AND DURING CALORIC RESTRICTION IN WOMEN**

By

DEEPTHA SUKUMAR

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Sue A Shapses

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ABSTRACT OF THE DISSERTATION

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Dissertation Director:

Sue A Shapses

Weight loss (WL) is associated with loss of bone loss due to several factors such as decrease in intake of calcium, true fractional calcium absorption (TFCA), serum insulin like growth factor -1 (IGF-1) , reduced weight bearing . The aim in this dissertation was to understand whether a higher intake of dietary protein and vitamin D can attenuate bone loss during caloric restriction (CR), and addresses bone quality and altered hormonal milieu in obesity. In the first trial, our goal was to examine how bone responds to CR during a 1 year randomized trial using 2 levels of protein intake with controlled calcium intake of 1.2 g/d in both groups. Forty seven women were randomized to either a normal (18%) or higher (24%) protein intake for 1 year. Our results showed greater loss of BMD at certain sites and lower IGF-1 in the NP compared to HP diet ($p < 0.05$).

The second trial hypothesized that a higher compared to normal vitamin D supplementation will attenuate the decrease in TFCA associated with CR. Eighty four women were supplemented with either 2500 IU/d or 400 IU/d of vitamin D₃ for 6 weeks during WL or weight maintenance (WM). TFCA increased with 2500 IU/d D supplementation in the WM-D group by 3.7% ($p < 0.05$) however did not attenuate the

decrease in TFCA associated with CR, with the greatest increase in 25 hydroxy vitamin D (25OHD) in the WL-D group. These findings suggest that vitamin D supplementation does not attenuate the decrease in TFCA associated with CR. The cross sectional study in this dissertation aimed at evaluating bone quality in obesity using peripheral quantitative computed tomography (pQCT). Our results in 211 women showed that obesity is associated with higher trabecular bone and lower cortical BMD. A separate case control clinical study in 111 women shows that high serum PTH in obesity is also associated with higher monocyte chemoattractant protein (MCP-1). This unique effect of parathyroid hormone (PTH) on MCP-1 is independent of the level of adiposity. Together these studies evaluate nutrient supplementation in attenuating bone loss during CR and help better understand bone quality and higher serum PTH in obesity.

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LIST OF ABBREVIATIONS

CR: Caloric restriction

PTH: Parathyroid hormone

25OHD: 25 hydroxyvitamin D

pQCT : Peripheral quantitative computed tomography

DXA: Dual energy x ray absorptiometry

TFCA: True fractional calcium absorption

BMD: Bone mineral density

BMC: Bone mineral content

vBMD- Volumetric bone mineral density

BMI: Body mass index

Trab: Trabecular

Cort: Cortical

SSI: Stress strain index

Ip: Polar moment of inertia

HP: High protein

NP: Normal protein

MCP-1: Monocyte chemoattractant protein-1

CRP: C reactive protein

PYD: pyridinoline crosslinks

DPD: Deoxy pyridinoline crosslinks

E2: Estradiol

IGF-1: Insulin like growth factor-1

IGFBP-3: Insulin like growth factor binding protein-3

WL: Weight loss

WM: Weight maintenance

TB: Total body

UD: Ultra distal

ANOVA: Analysis of variance

CV: Coefficient of variation

1. INTRODUCTION

Osteoporosis is a disease characterized by excessive skeletal fragility and leads to an increased fracture risk especially in the elderly women. It is an important health concern in the developed society due to related morbidities and costs. Over half of those above the age of 50 are at an increased risk of fracture (1;2), with a 20-24% increased risk of mortality in a year following a hip fracture (3). Worldwide, about 9 million osteoporotic fractures have been reported in the year 2000, and about half of those reported were in the Europe and the Americas, while the other half in the Western Pacific regions and Southeast Asia (4). In the United states, osteoporosis related fractures have increased to about 2 million in the year 2005 and it is expected to rise above 3 million by 2025 (3). The cost of osteoporosis related health care was about 19 billion in 2005 and costs are estimated to increase by 50% in 2025 in the USA (3).

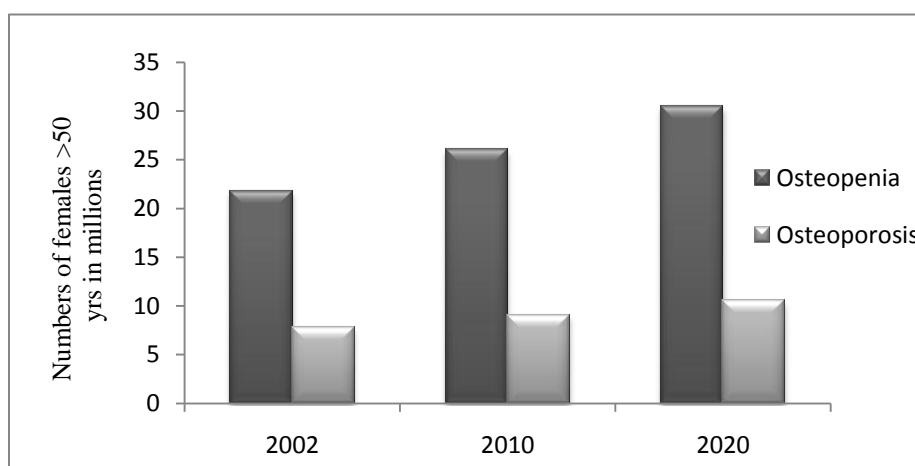


Figure 1: Prevalence of osteopenia and osteoporosis in women over 50 years of age
(CDC 2010, National Center for Health Statistics -NHANES Survey)

Obesity is another epidemic that is also a growing concern worldwide. A combination of excess caloric intake coupled with lower physical activity is an important

contributor to this condition and affected an estimated 1.5 billion in the year 2008 worldwide (5). Obesity is associated with many comorbidities such as cardiovascular disease (CVD), type 2 diabetes and certain cancers (6). Obese subjects on average spend about 40% more in health care costs than normal-weight individuals (7). The incidence of several comorbidities associated with obesity can be reduced with weight loss that is primarily achieved by increasing energy expenditure with exercise and by reducing excess caloric intake.

Both osteoporosis and obesity are important public health concerns. Although weight reduction is recommended to reduce comorbidities related to obesity, it also induces bone loss in postmenopausal women. Calcium supplementation, exercise and use of medications, have shown to attenuate bone loss, however whether or not other nutrient modifications during dieting influence bone health is not completely understood. The role of dietary protein on bone is well established; however its influence during caloric restriction (CR) on bone is unknown. Furthermore, various factors have shown to influence calcium absorption, including a higher vitamin D intake; still whether or not this can attenuate CR mediated decrease in calcium absorption is unknown. This dissertation will address how dietary composition during weight loss impacts bone health, and address bone quality and the altered hormonal milieu in obesity. Overall, it is important that there are nutrient recommendations for the prevention of bone loss since the dieting population is on the rise. The elderly population is on the rise and weight loss being a major determinant of bone loss, fracture risk, and ultimately mortality, the importance of these studies to identify how diet composition can prevent bone loss and understand bone quality in the obese older population is both vital and timely.

2. BACKGROUND

2.1 BONE AND CALCIUM METABOLISM

2.1.1 Composition of the bone matrix

The bone matrix is made up of both mineralized and nonmineralized components. The extracellular matrix consists of mineral, collagen, water, non-collagenous proteins and lipids. The mineral part is primarily composed of hydroxyapatite $[\text{Ca}_{10}(\text{PO}_4)_6(\text{OH})_2]$ that strengthens the collagen and provides a mechanical resistance to the tissue. It is also a reservoir of calcium, phosphate, and magnesium for mineral homeostasis. The collagen in the matrix consists of type 1 collagen; the measurement of bone derived collagen, and is used as a marker of bone turnover. The non-collagenous proteins consist of proteoglycans, glycosylated proteins and gamma-carboxylated (gla) proteins. These proteins regulate deposition of mineral, osteoblast and osteoclastic activity. Water and lipids comprise <10% of the bone (8). The three cell types of the bone are bone forming osteoblasts, bone destroying osteoclasts and osteocytes. The cortical bone is a dense layer of calcified tissue found in the outer part and inner part is composed of less dense network of thin calcified structure termed as the cancellous or trabecular bone.

2.1.2 Hormonal regulation of calcium metabolism

Calcium is a tightly regulated ion in the body and calcium balance (Figure 2) (9) is under the regulation of hormones such as parathyroid hormone (PTH), 25 hydroxy vitamin D (25OHD), 1,25 dihydroxy vitamin D ($1,25(\text{OH})_2\text{D}_3$) and calcitonin. PTH is produced by the chief cells of the parathyroid gland and acts on target cells through PTH

receptors on the cell surface. The primary function of this hormone is to increase calcium concentrations when low concentrations are sensed by the calcium sensing receptor on the parathyroid gland. Higher PTH levels are detected by renal PTH receptors that increase the rate of renal calcium reabsorption. In addition, PTH also increases the activity of the renal 1α -hydroxylase that converts the biologically inactive vitamin D metabolite 25OHD into the active hormonal form of $1, 25(\text{OH})_2\text{D}_3$. This active form of vitamin D increases the intestinal absorption of calcium. In the bone, PTH increases the expression of RANKL that increases osteoclast differentiation ultimately causing calcium resorption from the bone. Through these three distinct but coordinated actions, PTH maintains calcium homeostasis. Calcitonin secreted by C cells of the thyroid gland decreases blood calcium levels by acting on bone by inhibiting the activity of osteoclasts and in the kidney, inhibit tubular reabsorption of calcium and phosphorous. Together these hormones act in concert to maintain calcium homeostasis.

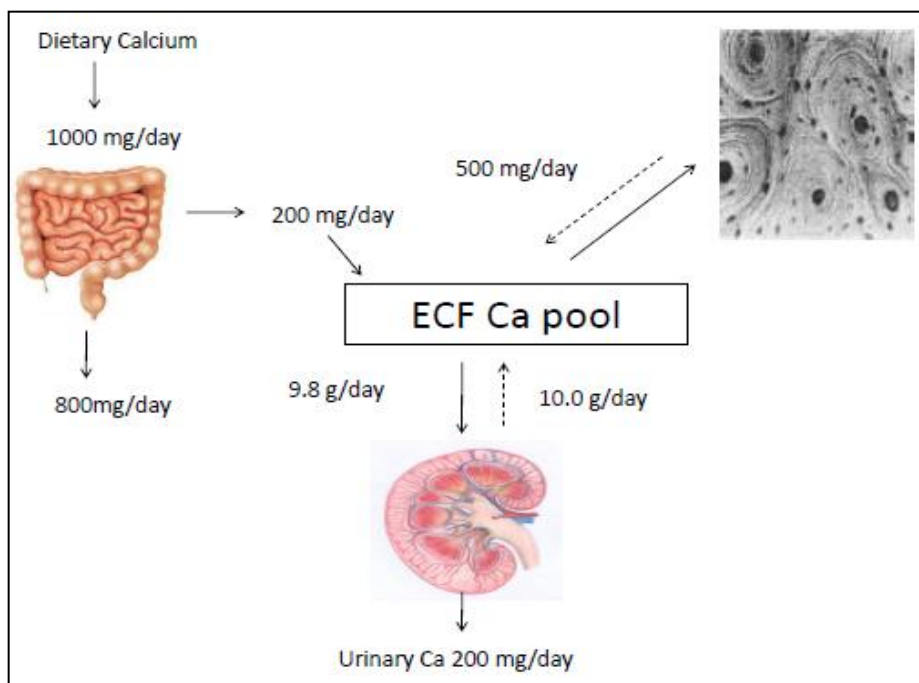


Figure 2: Calcium balance in the body

2.2 OBESITY AND BONE

2.2.1 Hormonal milieu in obesity and influence on bone

A higher body weight is associated with a higher bone mineral density (BMD) and bone mineral content (BMC) (10-14). Several mechanisms have been proposed by which a higher body weight may be associated with a higher bone mass. It is believed that a higher body weight places a greater mechanical load on the bone, and that bone mass increases to accommodate the greater load (15-18). Furthermore, obesity is also associated with an altered hormonal milieu and higher serum levels of adipokines (17;19). Higher levels of serum estrogen, PTH, lower 25OHD, lower 1,25(OH)₂D₃ and sex hormone binding globulin (SHBG), all of which have specific actions on bone are found in obesity (17;19-24). In addition, obesity is also associated with higher levels of pancreatic hormones such as insulin, amylin, preptin (25-27) that are also anabolic to the bone (28-31). Several adipose derived peptides and enzymes such as aromatase, hydroxyl steroid dehydrogenase, leptin and resistin levels are higher in obesity (32-41) and have specific anabolic or catabolic actions on the osteoblast (42-49). In vitro observations suggest an anabolic impact of adiponectin on the osteoblast (50-52) but clinical studies do not always support this (53;54). Adiponectin level increases with moderate weight loss but their role in regulation of bone mass is not completely understood. Obesity is also associated with higher circulating concentrations of inflammatory cytokines, such as Interleukin -6 (IL-6), Monocyte chemoattractant protein-1 (MCP-1), C- reactive protein (CRP) (17;36;55-60). In leaner populations higher inflammatory cytokines have been associated with higher bone turnover (61-63) nevertheless; its role in regulating bone in obesity is not clearly understood.

2.2.2 Bone quality in obesity

A higher body weight is associated with a higher BMD (12;13). Most reports that show a higher BMD in the obese, measure the two dimensional areal BMD (g/cm^2) using dual x ray energy absorptiometry (DXA). Fat mass accounts for approximately 16 - 25% of total body weight in normal-weight men and women, with much greater amounts in the obese. Most reports suggest a positive association between lean mass and BMD (64-66), while some show a negative association between fat mass and BMD in both children and adults (67-70). Interestingly, there are recent reports that show that fracture risk is higher in obese individuals (71;72) in the presence of normal areal BMD. Although obesity is associated with a higher BMD, whether or not obesity is also associated with better bone quality is not known.

Bone quality is a composite of BMD, bone geometry and bone strength, and is considered a major determinant of fracture risk [Figure 3, (73-80)]. In adolescents, obesity has been shown to be associated with lower cortical bone and bone strength (81;82). Obesity is associated with a higher serum PTH (83;84) that has known catabolic actions on the trabecular (trab) and cortical (cort) bone (85;86). It is thus important to examine how adult adiposity influences bone quality. In obese rodent models with growth hormone deficiency it has been shown that adiposity does not confer a significant influence over the strength of cortical bone (87). Furthermore, there is a moderate increase in cortical vBMD with fat loss during weight reduction in obese premenopausal women (88). Dual X ray energy absorptiometry is the most popular method to estimate BMD. However, measurement of bone mass in obesity using DXA is a concern given the excess padding around the bone (89-91) due to an artificial decrease in BMD and an

increase in BMC associated with a greater fat thickness surrounding an axial site (90). Peripheral quantitative computed tomography (pQCT) may have an advantage over DXA in that it measures a peripheral site such as the tibia with less surrounding fat thickness compared to axial sites. The pQCT assesses the trabecular and cortical compartments of the bone, with measures of BMD, (Total, cortical and trabecular), bone geometry (area, circumference), bone mineral content (BMC) and bone strength indices. Hence pQCT may be considered as a useful method to assess the bone quality and ultimately bone strength (92). In children, although a higher body weight is associated with a higher trabecular bone, it does not provide additional benefit to the cortical vBMD (81;82;93). The influence of excess body weight on trabecular and cortical compartments of bone in adult obesity is not clear and has been elucidated in this dissertation.

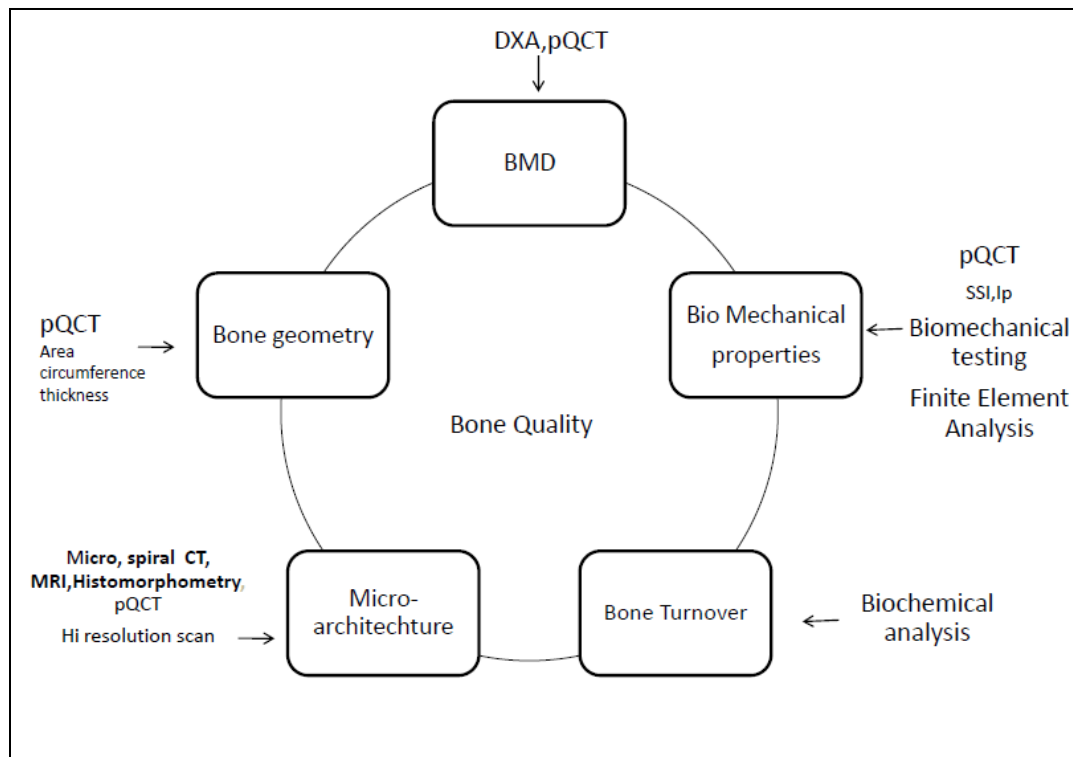


Figure 3: Variables contributing to bone quality and assessment methods

DXA: Dual X ray energy absorptiometry, SSI: Stress strain Index, Ip: Polar moment of inertia, pQCT : peripheral quantitative computed tomography, MRI : Magnetic Resonance Imaging, CT : Computed tomography (74-81)

2.3 PARATHYROID HORMONE IN OBESITY AND ACTIONS ON BONE

Parathyroid hormone plays a central role in the regulation of calcium metabolism. Secreted by the chief cells of the parathyroid gland, this hormone increases in response to lower calcium concentrations in the serum that are sensed by the calcium sensing receptors in the parathyroid gland. Mammalian PTH is a single-chain 84 amino acid polypeptide hormone that is expressed almost exclusively in the parathyroid gland, with lesser expression in the rodent hypothalamus and thymus (94). PTH maintains calcium homeostasis by promoting bone resorption, thus releasing calcium from the skeleton; by inducing renal conservation of calcium and excretion of phosphate; and by increasing the renal production of calcitriol by upregulating 1α hydroxylase, thereby enhancing intestinal calcium absorption. Serum levels of ionized calcium and $1,25(\text{OH})_2\text{D}_3$ produce feedback inhibition for the secretion of PTH, whereas serum phosphate increases PTH secretion. The concerted interplay between serum calcium, PTH, $1,25(\text{OH})_2\text{D}_3$, 25OHD , and phosphate permit serum ionized calcium levels to be maintained within very narrow limits (8.5-11.0 mg/dL), even over a wide range of dietary calcium intake. A normal serum level of PTH is <65 pg/mL and levels >65 pg/mL is defined as hyperparathyroidism. Patients with swelling of one or more of the parathyroid glands leading to an increase in secretion of PTH have primary hyperparathyroidism, and those whose elevation in PTH is secondary to renal disease (such as renal insufficiency of 1α hydroxylase) that leads to increase in PTH have secondary hyperparathyroidism.

PTH has a marked effect on the cortical and trabecular compartments in women with $\text{BMI} < 27\text{kg/m}^2$ (85;86;95;96). Patients with hyperparathyroidism have a

compromised cortical BMD, but a well preserved trabecular bone (85;86;97-99). There is some evidence suggestive of an anabolic effect of PTH on trabecular bone by increasing osteoblast recruitment (100;101), but not all reports support this (85). In addition, PTH has shown to have some anabolic effect on the bone when administered intermittently (102-104). Nevertheless, the effects of excess PTH on bone in the obese is not clear and given these specific actions of PTH on bone compartments, the dissertation also aims to examine the influence of a higher PTH at different levels of adiposity on trabecular and cortical bone.

Serum levels of PTH are positively correlated with fat mass (83;84). Interestingly, reports have shown that patients with primary and secondary hyperparathyroidism usually weigh higher throughout their adult lives in comparison to age matched controls (105-107). It has been thus suggested that a higher body weight may promote PTH excess which may be mediated via altered vitamin D metabolism (83). Serum levels of vitamin D are lower in obesity possibly due to increased sequestration in adipose tissue, lower exposure to sunlight because of choice of clothing in the obese, and/or decreased mobility in heavier individuals (108-112). A lower level of serum 25OHD may thus lead to a rise in PTH to maintain calcium homeostasis. Also a lower serum ionized calcium level in the overweight (84), or possibly damage to the parathyroid glands and/or a mild renal dysfunction may contribute to higher PTH levels in obesity. However this chronic elevation of PTH on bone or other metabolic effects in obesity is poorly understood. A higher PTH has been shown to promote weight gain by inhibiting catecholamine induced lipolysis and causes an increase in intracellular calcium levels in adipocytes, thereby promoting insulin resistance (113). But, recently it has been shown that body weight is

not reduced after correction of excess PTH by parathyroidectomy(114). Thus whether or not an increased PTH in obesity is due to excessive fat mass and /or due to a lower vitamin D is not entirely clear.

2.3.1 Monocyte chemoattractant protein-1 (MCP-1) and regulation by parathyroid hormone

MCP-1 is a chemokine and a member of the small inducible cytokine family. It plays a role in the recruitment of monocytes and T lymphocytes to sites of inflammation (115) and is produced predominantly by macrophages (116). Various cell types including skeletal muscle, smooth muscle and endothelial cells express this protein. Serum MCP-1 levels are higher in patients with atherosclerosis (117) and both protein and mRNA levels of MCP-1 are higher in atherosclerotic lesions (118). MCP-1 levels are also increased in obesity (119-121) showing higher mRNA expression and protein levels in adipose tissue and MCP-1 levels decrease with weight loss (122;123).

More recently it has been shown that MCP-1 is expressed by mature osteoblasts (124). PTH (1-34) administration rapidly induces MCP-1 mRNA expression in osteoblast. Microarray expression experiments by Li et al show that expression of MCP-1 in the osteoblast by PTH is higher than any other gene, following intermittent injections of PTH. The induction occurs very quickly, and there is a 32-fold increase in MCP-1 mRNA expression 1 h after a PTH injection (124;125). In addition to the increase in the mRNA expression in the osteoblast, serum levels of MCP-1 also respond to higher PTH. Rats treated with PTH (1-34) for 14 days show a significant increase in serum MCP-1 levels 2 h after PTH injection from 65pg/mL to 159 pg/ml compared to vehicle treatment.

PTH (1-34) treatment in rats for 1, 7 and 14 days showed a 24%, 53% and 79% increase in serum MCP-1 levels 2 h after injection on these days, respectively (126).

Obesity is a state of low grade inflammation with upregulation of proinflammatory cytokines such as IL-6, Tumor Necrosis Factor-alpha (TNF- α), MCP-1 and CRP. It is interesting to note that circulating levels of these adipokines decreases following weight loss (127-131). It is thought that some of these cytokines may mediate the influence of obesity on development of CVD and insulin resistance (55;132). Interestingly, serum levels of some of these inflammatory markers i.e IL-6 and TNF- α are also upregulated in patients with hyperparathyroidism. The levels of these cytokines respond to parathyroidectomy and are reduced after surgery (133). Also, these cytokines have also shown to be correlated with markers of bone resorption in patients with primary hyperparathyroidism (133) and those with osteoporosis (134). Preliminary evidence from rodent studies show that MCP-1, similar to IL-6 and TNF- α is also upregulated by PTH, but whether this relationship exists in patients with high serum PTH was not known.

Since both serum levels of MCP-1 and PTH are higher in obesity, it was interesting to examine whether higher PTH differentially influences serum MCP-1 levels in obese vs leaner women. Whether or not a higher PTH in obesity plays a more important role compared to adiposity in regulating MCP-1 has not been addressed. In addition, it was interesting to examine whether a higher MCP-1 is associated with trabecular and cortical bone and greater bone turnover. These questions are also addressed in this dissertation.

2.4 WEIGHT LOSS AND BONE

A weight loss goal of ~10% often has a positive effect on many of the co-morbidities associated with obesity, but is also associated with bone loss of about 1-2% at most sites, an effect more pronounced in older women and men (19;135-142).

Importantly, weight loss of about 5% is associated with bone loss and increased fracture risk (140;143-147). Many factors contribute to loss of bone mass during CR including a decrease in calcium intake and/or other nutrients (148-150), a decrease in calcium absorption (151), reduced weight bearing and/or hormonal changes (19). For example, a reduction in adiposity has been shown to also reduce estrogen levels that influences the osteoblast (152-155) and may contribute to bone loss in postmenopausal women (21;156-159). Also, CR is associated with reduced serum insulin like growth factor (IGF-1) (160-162). Serum IGF-1 has shown to be osteotrophic (163) and a decrease in IGF-1 associated with CR would negatively influence bone. Other mechanisms such as reduced weight bearing due to a reduction in body weight may also play a role in mobilizing bone during CR. Clinical interventions such as exercise program and/or nutrient supplementation have shown to minimize bone loss during CR. Weight reduction induced by increasing physical activity and exercise, especially strength training has shown to minimize bone loss (164-166) (138). Furthermore, use of osteoporosis medications during weight reduction also is beneficial (167). Nutrient supplementation such as calcium supplementation have been shown to attenuate the bone loss associated with CR (137;148;168), but the role of other macro and micro nutrients is poorly understood.

2.5 DIETARY PROTEIN AND BONE METABOLISM

(Adapted from Shapses S.A and Sukumar D “Protein Intake during Weight Loss: Effects on Bone”. In Nutritional Aspects of Bone health. Springer London 2010: 27-33) (169)

Dietary protein plays an important role in maintenance of skeletal health. A daily supply of amino acids is needed to offset loss due to proteolysis in the collagen cross links. An increase in dietary protein intake has been shown to increase serum levels of IGF-1 (170;171) , which promotes osteoblast proliferation and matrix formation (163) and may in turn increase bone mass and reduce fracture risk (170;172). Protein intake of 2.1g/kg has also been shown to increase intestinal calcium absorption that leads to a parallel increase in urinary calcium and decrease in bone turnover markers compared to a moderate protein intake (1.0g/kg) (173). With a higher protein intake, there is greater calcium retention and this has been shown in both a 10 day study (174) and another 8 week isotopic tracer study examining protein intake (175). In addition, the positive effect of a higher protein intake on muscle mass and in promoting collagen synthesis, may have a positive influence on acquisition of bone mass (176;177). However, the negative influence of higher protein intakes on bone is primarily due to a higher acid load caused by sulfur containing amino acids, which leads to a buffering response by the skeleton and causing an increased calcium resorption.

2.5.1 High protein intake and Bone

The influence of protein on bone has been addressed in several population and clinical trials. Epidemiological studies have shown a positive influence of dietary protein on bone (Framingham osteoporosis study, NHANES III) (170;178). A higher protein intake (84-152g/d) has been positively associated with changes in femoral neck and spine

BMD over a 4 year period in the Framingham osteoporosis study (178). Similarly, the NHANES III showed a positive association between femoral neck BMD and total protein intake (>75 g) (179), as did another study that found a decreased risk of hip fracture and wrist fracture (170) with higher protein intake. In clinical intervention studies, protein supplementation increases serum levels of IGF-1 and femur BMD; and a shorter hospital stay in patients with hip fracture (172;180), although in one of these trials, the protein supplement group was also given more calcium and vitamin D (180). However, it has been estimated that for every 1 g rise in dietary protein, approximately 1 mg calcium is lost in urine (181). Some epidemiological studies show that high protein diets reduce bone mass, and this has been attributed to a higher acid load, and can be measured using dietary calculations such as potential renal acid load (PRAL) and Net endogenous acid production (NEAP), leading to a buffering response by the skeleton and greater urinary calcium excretion. There has also been considerable debate on plant vs animal protein sources, with some large epidemiological findings (182) attributing the negative effect to a higher content of sulfur containing amino acids in animal sources. In the Nurses Health Study, no association was observed between protein intake and the risk of hip fracture, yet the risk of forearm fractures was higher in women with a higher (>95 g) protein intake (183). This is consistent with an epidemiological study showing that countries with higher protein intakes also have a greater fracture risk (182). However, in all cross sectional studies, protein intake was measured using self assessed food frequency questionnaires and/or largely using diet recalls. Intervention studies to date have not reported a negative influence of protein on bone, and there are potential mechanisms that would support either a positive or negative influence of protein on fracture risk. Furthermore, controlled

intervention studies (174;175) have shown no adverse effects of animal protein on calcium retention. Also, a normal to high calcium intake along with a HP diet will offset loss of urinary calcium, attenuate bone turnover (181;184;185) and is associated with greater femoral neck and total body BMD in a 3 year study (178). Consistent with this, a recent study (186) showed that high protein diets (including meat) were associated with increased fracture risk only when the calcium intake was low (< 400 mg/1000 kcal) and has been consistently reported in other large population trials (187;188) .

2.5.2 High protein diets and weight loss

High protein (HP) diets are popular and often result in greater short term weight loss compared to high carbohydrate low fat diets. HP diets are also associated with greater fat loss and preservation of lean mass (189-193). The positive influence of dietary protein on weight loss may be mediated by its higher thermic effect and sleeping metabolic rate (194) compared to carbohydrate and fat, that increases total energy expenditure to ultimately promote a greater weight loss (195;196). In addition, higher protein (or dairy) diets also promote satiety (197) possibly mediated by higher postprandial cholecystokinin (CCK) levels (198), higher circulating concentrations of certain amino acids (199) or greater diet induced thermogenesis (195). Certain appetite regulatory hormones are also triggered by higher protein intake, including an increase in postprandial ghrelin, glucagon like peptide-1 and insulin secretion (200). These mechanisms collectively may mediate the effect of higher protein intake on short-term weight loss (201-205) and fat loss (203;206;207) as compared to normal protein diets. Hence these diets are popular and are increasingly accepted by the dieting population. The greater weight loss on a HP compared to a normal protein (NP) diet may contribute

to the improved lipid profile, greater loss of fat, and improved insulin sensitivity (204;208;209). However, longer term studies show that the greater weight loss on a higher protein diet is not sustained and is similar to that seen with a standard high carbohydrate diet after 1-2 years (210). It is also possible that HP weight loss diets have adverse effects on renal function. A 6 month weight loss study showed greater glomerular filtration rate (GFR) and kidney volume on a HP (108 g/d) compared to NP diet (70 g/d) (211). However, the specific GFR, which is an expression of the filtration rate per unit kidney volume, and albumin levels were not altered by either treatment. It was thus suggested that the changes in GFR were adaptations of the kidney to changes in protein load. In another large 11 year study examining 1624 weight stable women with normal renal function, a high protein intake of 93g/d did not significantly alter GFR (212). Most researchers agree that unlike renal patients, in healthy populations there is little evidence of adverse effects of HP diets on renal function (211;212).

Overall, reports show that while HP diets result in greater loss of body weight over a 6 month period, these diets do not result in any additional benefit to promote weight reduction or improve metabolic parameters compared to a high carbohydrate diet after 1-2 years. Thus in light of the current available literature and in the absence of longer controlled intervention trials, most would agree that there is a beneficial effect of HP intake on bone in older individuals with a normal habitual low intake. Importantly, a positive effect of dietary protein on bone is dependent on the presence of adequate calcium and vitamin D in the diet.

2.5.3 Weight loss, protein intake and bone

HP weight loss diets may preserve bone mass during CR by several mechanisms (Figure 4). Caloric restriction leads to a decrease in IGF-1 levels and IGF-1/IGFBP-3 ratio (160;162), whereas a high protein intake raises these levels (171;213;214). Therefore it is possible that a HP diet may attenuate the decrease in IGF-1 associated with CR. The rise in IGF-1 may also increase skeletal muscle mass and indirectly preserve bone mass (191;215;216). A higher protein intake (2.1g/kg) has also been shown to increase intestinal calcium absorption that leads to a parallel increase in urinary calcium and decrease in bone turnover markers compared to a moderate intake (1.0g/kg) (173;217). This too may attenuate the decrease of TFCA associated with CR. Finally, due to reduced food intake during CR, protein intake may be compromised (< 0.8 g/kg), and it is well established that low protein diets reduces IGF-1 (218;219), which in turn has a negative effect on calcium and phosphate metabolism, and bone (179;220).

Interestingly, several hormones that influence bone are also influenced by dietary protein intake. Following a high protein meal, leptin sensitivity has been shown to improve (221). In addition, postprandial ghrelin secretion as well as incretins- specifically GLP-1 that eventually triggers insulin release, increase following a high protein meal. Similarly PYY concentrations also increase in response to a protein meal (222). However, there is no evidence to show that such short-term influences of HP diets on these hormones will mediate its effects on bone.

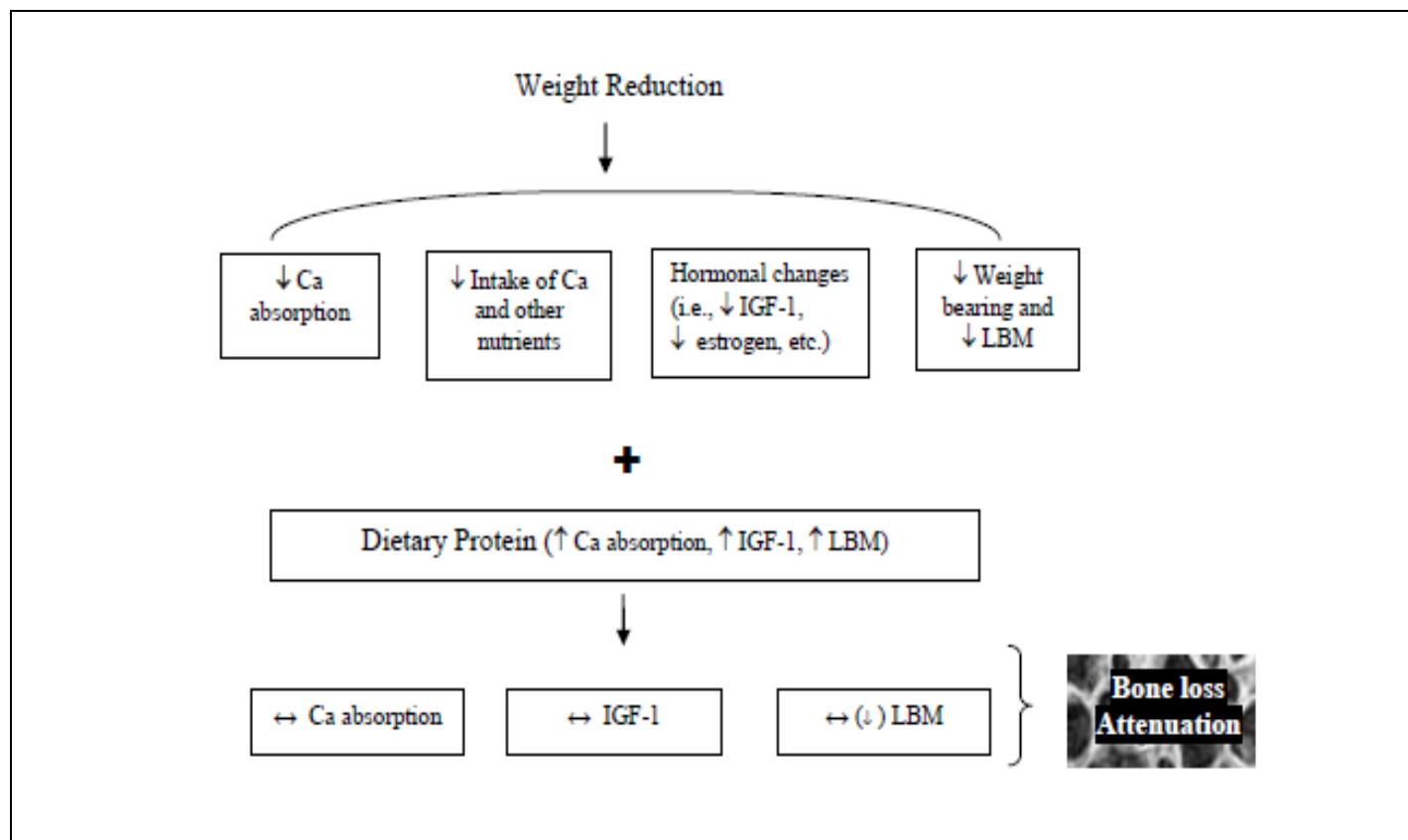


Figure 4: Mechanisms mediating effect of higher protein intake on bone during caloric restriction

Source : Shapses S.A and Sukumar D "Protein Intake during Weight Loss: Effects on Bone". In *Nutritional Aspects of Bone health*. Springer London 2010: 27-33

Several previous studies have evaluated the role of dietary protein on bone and markers of bone turnover during CR (Table 1). In these studies (204;207-209;223-226) both a higher and normal protein diet produce similar weight loss except for one study (207) showing greater weight loss in the HP compared to NP group. Some (207;224) but not all (204;208;209;223) of these studies demonstrate a positive impact of higher dietary protein on bone mass and turnover during CR, but none of these studies control for dietary calcium intake. The importance of dietary calcium on bone are well established (227), as well as during weight loss (148;150;168). Because studies that increase protein intake during dieting have accomplished this by increasing dairy intake, calcium levels

are also higher in these diets. Hence, the HP diets (with adequate or high calcium) were compared to calcium insufficient (~600 mg/d) high carbohydrate diets (207;224) . Not surprisingly, high dairy and protein studies (207;224;225) have shown a positive impact of the diet on maintenance of bone mass during CR, similar to calcium supplementation studies without higher protein intake (148;150;168). One recent study shows a negative effect of HP diet on BMD during 12 weeks of CR, but this study is limited due to absence of measuring other specific sites (226). Thus the role of protein in the maintenance of bone mass during long term CR is unclear, and use of a high carbohydrate control group that is not deficient in calcium intake has not been previously examined. We hypothesized that a protein intake difference of ~20-25g between groups will influence bone during CR.

Table 1: Studies examining higher protein intake during caloric restriction on bone mass and turnover

Author & year	Population N, Age, BMI	Groups (pro intake)	WL duration	Ca intake (mg/day)	Bone site and markers	Weight loss ^a	Effect of HP diet
Skov 2002(207)	n=65 M & F 39 yrs 30 kg/m ²	HP 102g/d NP 71g/d	6 mo	HP 936 NP 659	TB	8.1%	Decreased loss of BMC
Farnsworth 2003 (204)	n=57 M & F 50 yrs 34 kg/m ²	HP (27%) NP (16%)	12wk ^b	HP 1600 NP 600	PYD, DPD	8.0%	No effect
Bowen 2003 (225)	n=50 M & F 50 yrs 33 kg/m ²	2 HP groups Diary (DP, 108g/d) or Mixed (MP 104g/d)	12wk ^b	DP 2371 MP 509	Osteocalcin PYD, DPD	9.9%	DP minimized bone turnover.
Brinkworth 2004 (208)	n=58 M & F 50.2 yrs 34 kg/m ²	HP (30%) NP (15%)	12wk ^b	NA	TB-BMC	8.9%	No effect
Noakes 2005 (223)	n=100 F 49 yrs 32 kg/m ²	HP (31%) NP (18%)	12 wk ^d	HP 777 NP 594	Osteocalcin, PYD,DPD	8.4%	No effect
Thorpe 2008 (224)	n=130 M & F 46 yrs 31 kg/m ²	HP 97g/d NP 61 g/d	4mo ^c	HP 1120 NP 765	TB, LS, TH	8.2%	Decreased loss of BMD at 12 mo
Campbell 2010 (226)	N= 28 F 55 yrs 31 kg/m ²	HP 30% NP 18%	12wk	HP >2000	TB	9.8%	Increased loss of TB BMD at 12 weeks

^a No difference in weight loss between HP and NP groups, except in Skov showing greater loss in HP (-10%) vs. NP (-6%) diet. ^{b-d} The weight loss was followed by a weight maintenance period of ^b4 weeks, ^c8 months, or ^d1 year (this 1 year study showed no BMD difference between groups).
(Adapted from: Shapses S.A and Sukumar D "Protein Intake during Weight Loss: Effects on Bone". In Nutritional Aspects of Bone health. Springer London 2010: 27-3)

2.6 CALCIUM ABSORPTION

The amount of calcium absorbed in the intestine determines its supply for various physiological functions of the body, including maintenance of bone mass (228-230) . Calcium absorption is an important determinant of calcium balance. Calcium absorption is a result of both active transport across the cell in the duodenum and the upper part of the jejunum and passive diffusion throughout the small intestine. In active transport, calcium enters the cell via epithelial calcium selective channels such as Transient receptor potential cation channel, subfamily V, member -TRPV5 (231-233) or TRPV6 (234;235). Entry and diffusion of calcium is calcitriol dependent and is required for expression of calcium channels and the binding protein calbindin D9k that ferries calcium across the cytoplasm. Extrusion of calcium from the cell occurs against an electrochemical gradient via Na/Ca exchanger (NCX1) and Ca-ATPase. The passive diffusion of calcium is calcitriol independent (236), although some studies show an increase in paracellular diffusion of calcium through tight junctions with calcitriol treatment (237-239). It is important that calcium is in its soluble form or bound to a soluble organic molecule for absorption. At low calcium intakes, active transport accounts for 50% of total calcium absorbed, while passive diffusion is increased with higher intakes of calcium (240;241)

Previous studies have shown that elderly women with lower fractional calcium absorption are at an increased risk for hip fracture (242). Indeed, calcium absorption is lower in osteoporotic females compared to age matched controls (228;243). Several factors such as age, endocrine factors and dietary factors influence calcium absorption (244;245). True fractional calcium absorption (TFCA) that estimates intestinal calcium

absorption is an accurate and precise technique to measure absorption (246;247) (See detailed description of TFCA in Methods Section)

2.6.1 Hormonal and dietary factors that influence calcium absorption

The primary hormonal regulators of calcium absorption are estradiol, PTH, $1,25(\text{OH})_2\text{D}_3$ (228;243;248-257) and the role of 25OHD is controversial (258;259). Serum levels of estradiol are a positive regulator of calcium absorption especially in postmenopausal women (151;260;261). The age related decline in TFCA found in women is primarily attributed to a decrease in serum estradiol (248;259;262;263), and this is supported by the rise in absorption observed with estrogen replacement (245;264;265). Animal studies suggest that the action of estradiol is independent of vitamin D (266) . Estradiol treatment has been shown to influence calcium absorption either via its action on calbindin or possibly by its direct interaction with its estrogen receptor in the intestine (267). It has been suggested that there is an intestinal resistance to the positive effect of calcitriol on calcium absorption in estrogen deficiency (259). Furthermore estrogen is also involved in renal calcium reabsorption by increasing mRNA levels and protein levels of ECaC (also known as transient receptor potential cation channel subfamily V member 5; TRPV5). IGF-1 has also been identified as a regulator of intestinal calcium absorption (268;269). The role of serum 25OHD on calcium absorption has been contradictory with some reports showing a positive effect while other showing no effect (258;259;270-275), and it has been suggested that the association between 25OHD and calcium absorption may be an indirect one (276). But, both serum $1,25(\text{OH})_2\text{D}_3$ and PTH have been consistently shown to be positive regulators of TFCA (248;259;277). Glucocorticoids, have been shown to decrease calcium absorption,

especially in animal studies (278) possibly due to downregulation of calcitriol synthesis (279).

Several dietary factors have also been shown to influence calcium absorption, such as dietary protein, fat, fiber and vitamin D. Milk Casein (280;281) and amino acids such as lysine and arginine (236) form soluble chelates with calcium and stimulate passive absorption. A higher intake of dietary protein increases transcellular calcium uptake and thus increases absorption (173;282). Furthermore, supplemental vitamin D has been shown to increase calcium absorption in some, but not all studies (274;275;283;284). Dietary fat has also been shown to be a positive predictor, while dietary calcium and fiber are negatively associated with TFCA in pre and perimenopausal women (248). Nevertheless the primary factors that influence calcium absorption are of physiological origin such as growth, pregnancy, lactation and aging (245;285).

2.6.2 Measurement of calcium absorption

The above mentioned dietary and hormonal factors contribute to absorption, but are dependent on dietary calcium availability and absorptive capacity of the intestine. In the past several methods have been used to measure calcium absorption including use of single radioactive isotopes; but safety has been a concern (286). A disadvantage of single oral isotope approach is that it requires an estimate of the endogenous losses during the time from the intake of the isotope until the time that the non-absorbed isotope has been excreted. The dual stable isotope method that measures calcium absorption is widely accepted since it avoids use of radioactivity and with simultaneous oral and intravenous administration of 2 different calcium isotopes; absorption can be estimated from the relative enrichment or appearance of the 2 isotopes in urine (287). The advantage of the

dual tracer method is that the concentration of the intravenous tracer automatically corrects the oral tracer concentration values for variation related to differences in the size of the miscible pool, pool turnover rate, and the effect of time. These isotopes in the urine can be measured using thermal ionization or high-resolution inductively coupled plasma mass spectrometry (ICPMS). The dual isotopes method can thus measure true fractional calcium absorption (TFCA) using specific formulas (288) from relative appearance of oral and IV isotope in a 24 hour urine collection (See Methods Section).

2.6.3 Calcium absorption during caloric restriction

Caloric restriction has shown to decrease TFCA in rodent studies (260), and in postmenopausal women with a normal intake of 1g of calcium and 400 IU/day (d) vitamin D (151). Several factors may contribute to this decrease including a decrease in intake of nutrients such as protein, fat, lactose that positively influence absorption etc. Thus, weight loss reduces the efficiency of calcium absorption. Typically with a low calcium intake, PTH secretion increases that increases calcium absorption, but this is not observed with CR. This suggests that during CR other factors such as stress and increased cortisol (289-291) , reduced estradiol (137;260;292) or reduced intake of nutrients contribute to lower absorption (293;294). In addition, gastric bypass surgery resulting in reduced caloric intake and resulting in massive loss of body weight has also shown to decrease TFCA after 6 months of surgery (295). Overall, both dietary and hormonal factors influence calcium absorption. Whether or not macronutrient and micronutrient supplementation during CR can attenuate the decrease in TFCA decrease is not well understood and in this dissertation, the effect of supplemental vitamin D on TFCA during CR in overweight-obese postmenopausal women has been examined.

2.7 VITAMIN D

Vitamin D, a fat soluble vitamin is unique in that it also functions as a hormone. The classical actions of vitamin D are in the regulation of serum calcium and phosphate homeostasis and, in turn, the development and maintenance of bone health (296-299). Recent reports also suggest important non-classical involvements such as in metabolic syndrome, immunity, certain cancers and neurological conditions (300).

2.7.1 Physiology, metabolism and functions

The active form of vitamin D, $1,25(\text{OH})_2 \text{D}_3$ functions as a hormone and maintains calcium and phosphorous homeostasis. The solar UVB radiation converts 7-dehydrocholesterol (7-DHC) in the skin to previtamin D_3 (preD_3). preD_3 is immediately converted to vitamin D_3 via a heat-dependent process and excess preD_3 is degraded into inactive photoproducts; and hence excessive sun exposure does not lead to vitamin D toxicity. Vitamin D is obtained as either D_2 (ergocalciferol) or D_3 (cholecalciferol) in the diet (See Figure 5) and is incorporated into chylomicrons and transported by the lymphatic system into the venous circulation. In the circulation vitamin D is bound to the vitamin D binding protein (DBP) and is transported to liver where it is acted on by vitamin 25-hydroxylase to yield its major circulating form 25OHD (See Figure 6).

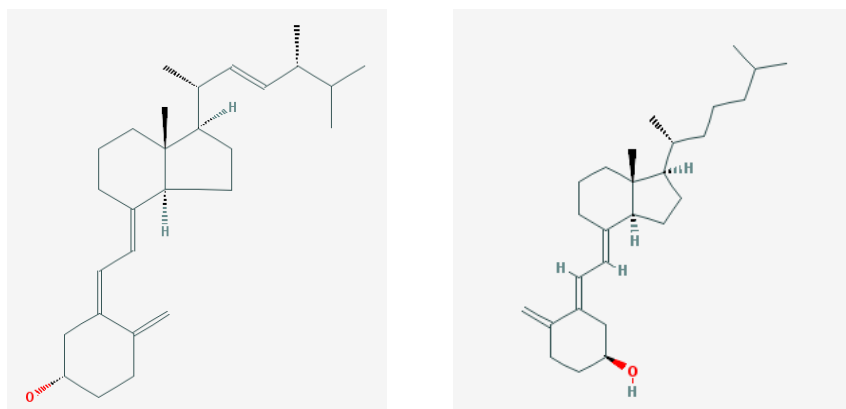


Figure 5: Structure of D_2 and D_3

Serum level of 25OHD is the biological indicator of vitamin D status. The IOM 2011 defines vitamin D sufficiency as serum levels of 25OHD greater than 20ng/mL (50 nmol/L) and insufficiency as serum levels < 16ng/mL (40 nmol/L) (300). 25OHD has a half life of ~ 15 days(301), but is must be converted in the kidneys by the 25-hydroxyvitamin D-1 α -hydroxylase to its biologically active form 1,25(OH)₂D₃ that has a half life of ~ 15 hours (301). The major function of 1,25(OH)₂ D₃ is to enhance intestinal calcium absorption in the small intestine by stimulating the expression of the epithelial calcium channel and the calbindin 9K (calcium binding protein; CaBP). In the skeleton, 1, 25(OH)₂D₃ increases the expression of receptor activator of NF κ B ligand (RANKL) which then binds RANKL and induces the preosteoclast to become a mature osteoclast. Through these coordinated functions of increasing intestinal calcium absorption and promoting bone resorption, vitamin D promotes mineralization of the skeleton. 1,25(OH)₂D₃ can affect bone formation by increasing expression of osteocalcin and osteopontin or resorption. The renal production of 1,25(OH)₂D₃ is tightly controlled by serum phosphorus, calcium, fibroblast growth factor (FGF-23) etc. 1,25(OH)₂ D₃ regulates synthesis of parathyroid hormone PTH in the parathyroid glands and maintains calcium homeostasis. The 24-25 hydroxylase (24 OHase) catabolizes 1,25(OH)₂ D₃ and 25OHD into water-soluble, biologically inactive, calcitroic acid, which is excreted in the bile.

The synthesized 1,25(OH)₂ D₃ acts on tissues that contain the vitamin D receptor (VDR) through a binding protein. The DBP is produced in the liver and is a member of the albumin family of proteins. It is the specific chaperone for vitamin D and its metabolites in the serum (302;303). It has a high affinity (nM range) to the 25-

hydroxylated metabolites 25OHD, 24,25(OH)₂D₃, and 1,25(OH)₂D₃. DBP has a serum half-life of 2.5-3.0 days. (304;305)

The skeletal effects of vitamin D are very well established. Vitamin D deficiency has shown to cause osteopenia, exacerbate osteoporosis, increases fracture risk and muscle weakness (300;306-309). 1,25(OH)₂D₃ acts both directly on the osteoclast and osteoblast via the RANK ligand and the transcription factor RUNX2 to influence mineralization or acts indirectly through its effects on increasing calcium absorption (See section on calcium absorption). Epidemiological studies show an association between low serum levels of 25OHD and low BMD (310-313). However, the RCT's do not consistently show a positive effect of vitamin D alone on BMD (300;314-316), whereas a combination of calcium and vit D has shown a positive outcome on BMD and fractures (300). Several target tissues are influenced by 1,25(OH)₂D₃, since VDR is present in several tissues. There is some evidence for the positive effect of supplemental vitamin D and/or higher serum levels of 25OHD on certain types of cancer, cardiovascular disease and hypertension, diabetes, metabolic syndrome, falls, immune response, neuropsychological functioning, physical performance, preeclampsia, and reproductive outcomes (300).

The dietary reference intake (DRI) for vitamin D intake in the USA is 600 IU/d for adults and 800 IU/d for elderly (300). The major source of vitamin D is from sunlight (317), although some oily fish such as salmon contain about 500-1000 IU of vitamin D. More recently, dairy bread and other breakfast cereals are also fortified with vitamin D. There has been a considerable debate on the bioavailability of vitamin D₂ vs D₃, however

it has been suggested that both D₂ and D₃ are equally effective in maintaining vitamin D status (318).

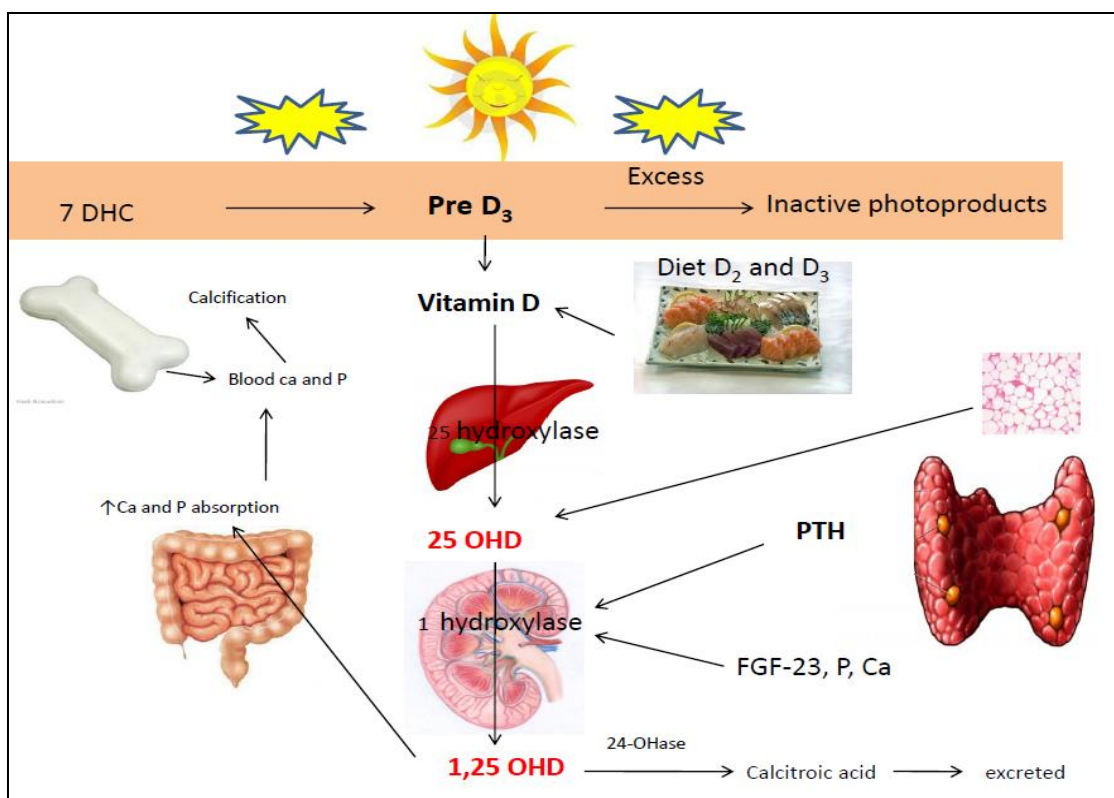


Figure 6: Vitamin D metabolism in the body

2.7.2 Vitamin D and calcium absorption

The mechanism by which vitamin D supplementation has a positive effect of BMD, is due to its effects on calcium absorption. The active form 1, 25(OH)₂D₃ facilitates the intestinal absorption of calcium by mediating active calcium transport across the intestinal mucosal brush border across to the basolateral side of the mucosal cell (249;250;319). The synthesis of 1,25(OH)₂D₃, is increased during certain physiological conditions that require higher calcium absorption such as pregnancy or lactation or also due to variation in intake of calcium. The active and passive transport of

calcium are both influenced by $1,25(\text{OH})_2\text{D}_3$. The transcellular or active transport involves steps that are all regulated by $1,25(\text{OH})_2\text{D}_3$ such as the entry of calcium across the brush border membrane, intracellular diffusion, and the energy requiring extrusion of calcium across the basolateral surface. Calbindin, the calcium binding protein is also induced by $1,25(\text{OH})_2\text{D}_3$ and, acts to facilitate the diffusion of calcium through the cell interior toward the basolateral membrane. $1,25(\text{OH})_2\text{D}_3$, also affects the plasma membrane calcium pump (PMCA) and thus influences calcium extrusion from the enterocyte (320-322). The rate of calcium entry into the enterocyte is also increased by $1,25(\text{OH})_2\text{D}_3$ primarily mediated via TRPV6.

Vit D supplementation raises serum levels of 25OHD and several studies have investigated the influence of supplementation and/ or a higher serum level on calcium absorption. In a study that examined the serum level of 25OHD in reference to calcium absorption showed that absorption was significantly lower when serum levels of 25OHD were 56nmol/L as compared to 86nmol/L (323). In another study TFCA was examined after supplementation of 50000 IU/d of vitamin D for 15 days in vitamin D insufficient women. These postmenopausal women showed an increase in 25OHD of 42ng/mL and a 3.5% increase in TFCA and a trend to increase calcitriol (274). But, studies in children showed no effect of vitamin D on absorption (275;324;325). Furthermore, multiple studies have shown that an increase in 25OHD does not correlate with absorption (258;259;324). Additionally in children, serum 25OHD level greater than 20ng/mL was associated with lower fractional calcium absorption (324). Another study in black and white women (259) and children (324;325) suggests that serum 25OHD is not a predictor of absorption nor does it correlate with absorption. In conclusion, these studies show no

consistent association of serum 25OHD with absorption, although some randomized clinical controls have shown positive results. The influence of serum calcitriol on calcium absorption has been more consistent. A linear relationship has been observed between serum levels of calcitriol and absorption in both children and adults (248;258;259). Wolf et al showed that serum levels of calcitriol was an independent predictor of calcium absorption (248). Similarly in a large population calcitriol was a positive predictor of absorption in blacks and postmenopausal women (259).

It has been estimated that a 1.0 ng/mL rise in 25OHD occurs with 100 IU/d vit D intake. However, this estimate varies based on many factors such as baseline 25OHD levels, as well as obesity. Obesity attenuates the serum 25OHD response to vit D supplementation, therefore higher doses may be needed in the obese to raise the serum 25OHD levels to sufficient range (326). Some recent studies show that the rise in overweight and obese individuals is about 0.6-0.7 ng/mL for 100 IU/d vit D intake over 12 months (327;328). In addition, with increased adiposity, especially visceral adiposity serum levels of vitamin D are lower and may have a blunted response to supplementation (326) (329). Hence in this dissertation we used a dose of vitamin D (See calculations in methods section) that would increase serum level of 25OHD significantly (to above 30ng/mL, that was considered as adequate levels of 25OHD when this study was designed) in our overweight population (whom we expected will have a blunted response to supplementation) and hypothesized that this would increase TFCA.

3. RATIONALE

Several factors influence bone loss during caloric restriction such as a decrease in calcium absorption, reduced dietary calcium intake, reduced weight bearing, lower IGF-1 and hormonal changes. Calcium supplementation, exercise and mediations can attenuate the bone loss during CR. However it is not known whether an attenuation of a decrease in IGF-1 with higher protein intake during CR can prevent bone loss. Furthermore, whether or not other nutrients such as vitamin D that also has a positive impact on TFCA can attenuate loss of TFCA during CR is not understood. The influence of calcium on bone and during weight loss is well established. Dietary protein and vitamin D have a positive effect on increasing IGF-1 and calcium absorption respectively, but has never been examined during CR when calcium absorption or IGF-1 is lower. In this dissertation, the influence of vitamin D and protein supplementation on TFCA and bone respectively has been examined in two separate clinical trials.

There is some recent evidence that fracture risk is higher in obese adults at certain sites, and that the quality of bone is compromised in obese children. In a cross sectional study in this dissertation the bone quality in obese adult women has been examined and compared to those who are not obese. In addition, because the altered hormonal milieu in obesity includes a higher serum level of PTH, its role in regulating inflammatory cytokines and bone metabolism is addressed in the obese in this dissertation. The overall goal of the studies in this dissertation is to provide insight on whether bone quality is altered in obesity and with macronutrient modifications during CR. The long term goal of these studies is to help understand bone quality in obesity and nutritional interventions to attenuate bone loss during caloric restriction.

4. SPECIFIC AIMS OF THE DISSERTATION

1. To determine whether bone quality is influenced by a higher protein intake (30%) compared to normal protein intake (18%) in overweight and obese postmenopausal women during weight loss.

It is hypothesized that a higher compared to normal protein intake during caloric restriction will attenuate loss of bone density and quality.

2. To determine if a vitamin D supplementation of 2500 vs 400 IU/d can increase true fractional calcium absorption (TFCA) in postmenopausal women during short term caloric restriction compared to weight maintenance.

It is hypothesized that 2500 compared to 400 IU/d will raise serum levels of 25OHD and attenuate the decline in TFCA associated with caloric restriction.

3. To determine how body weight and/or serum parathyroid hormone (PTH) differentially regulates cortical and trabecular bone in obese vs normal weight women

It is hypothesized that similar to non-obese individuals; a higher PTH in obesity will be associated with greater trabecular and lower cortical density and geometry

A secondary aim will determine whether serum PTH influences serum levels of monocyte chemoattractant protein -1 and its influence on bone in obese compared to normal weight women.

It is hypothesized that higher PTH will upregulate MCP-1 (and not other cytokines), and be associated with lower cortical bone, that will be independent of the level of adiposity.

5. PROTOCOL AND METHODS

5.1 PROTOCOL

5.1.1 - Aim 1

To determine whether bone quality is influenced by a higher protein intake (30%) compared to normal protein intake (18%) in overweight and obese postmenopausal women during weight loss.

Subjects

Postmenopausal women were recruited by advertisements in local newspapers and through email list serves. Screening for eligibility included telephone screening, medical screening, physical and biochemical tests and bone density screening. Exclusion criteria included, but were not limited to presence of diseases or medications that are known to influence calcium or bone metabolism. Women between BMI of 25-40kg/m² were included. All eligible subjects signed the informed consent form approved by Rutgers University Institutional Review Board. The power analysis was performed with alpha set at 0.05, with the value of beta set at 0.90, using bone mineral density from sub group analysis from a previous study (178) evaluating the effect of protein intake. This analysis indicated that 15 participants per group would be necessary to avoid a type-II error. Our goal was to include at least 5 additional participants per group to account for 2 potential baseline covariates.

Study design

This is a *randomized clinical control trial*. There is no blinding in this study. A one-month stabilization period preceded intervention, during which participants were instructed to consume a 1.2g calcium and multivitamin and asked to maintain body

weight. At the end of the stabilization period, baseline measurements (See Figure 7) were performed. During this one year intervention, participants were randomized to HP (30% of calories) or NP diets (18% of calories). Participants met weekly with the dietitian the first two months and later biweekly throughout the rest of the intervention for 1 year. Weight loss diets were prescribed by using their estimated total energy requirements. An energy deficit of 600 Kcal was prescribed to lose ~ 1-2 lbs per week. A whey protein supplementation (Beneprotein, Nestle, 6g protein/scoop) was given to women in the HP groups

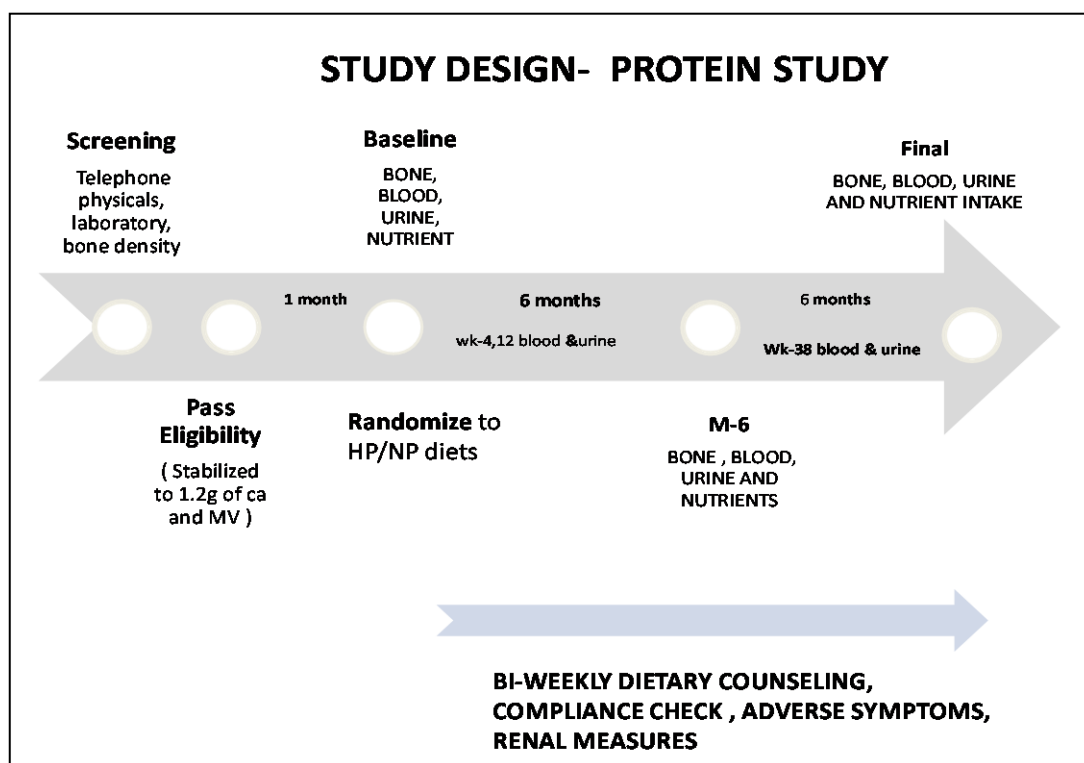


Figure 7: Study Design- Aim 1

Blood and urine was assayed for bone regulating hormones and makers of bone turnover during the study period. DXA and pQCT measurements were performed at baseline, 6mo and 12mo of intervention. (See detailed description of methods after aim 3)

5.1.2- Aim 2

To determine if a vitamin D supplementation of 2500 vs 400 IU/d can increase true fractional calcium absorption (TFCA) in postmenopausal women during short term caloric restriction compared to weight maintenance.

Subjects and protocol

Subjects: Similar to subject selection in Aim 1.

Dose calculation for vitamin D

Preliminary data from examining 25OHD levels in a subset of women in aim 1 (protein study) showed that women with BMI 25-35 kg/m², have a mean value of 23ng/mL (range: 12-33 ng/mL). The aim was to raise vitamin D level to >32ng/mL (considered as adequate when this study was designed) or higher, and an increase of ~25 ng/mL will increase serum levels to this range even in women with low vitamin D levels. It has been shown that 40 IU/d will increase serum levels by 0.4 ng/mL (306). Hence a dose of 2500 IU/d was expected to increase serum levels by 25 ng/mL. Assuming that the newly recruited women will have a mean vitamin D levels of 23 ng/mL, and that the obese have a blunted response to vitamin D supplementation (326;330), 2500 IU/d was expected to increase serum 25OHD to approximately 35-40 ng/mL (87.5-100 nmol/L), and result in an increase of TFCA. We provided a once weekly vitamin D supplement of 15,000 IU (in the form of 3 tablets of 5,000 IU each) or placebo. In addition, their daily multi-vitamin tablet had 400 IU/d of D₃.

During the 6 week intervention subjects were assigned to one of the two levels of vitamin D supplementation. All subjects were asked to consume a standard multivitamin and calcium of 1.2g. The higher vitamin D group consumed 15,000 IU vitamin D once

weekly and the placebo group consumed identical pills once weekly. Also, all participants consumed a multivitamin pill that contained 400 IU/d of vitamin D to total to ~2500 IU/d in the high D group vs 400 IU per day for the normal D group. In addition, we anticipated that vitamin D intake from the diet will account for ~80-100 IU/d.

Table 2: Vitamin D calculations from D₃ pill and multivitamins

Group	Vitamin D supplementation	Multivitamin	Diet	Total from supplements
Vitamin D	15000 IU/week	400 IU/d	80-100 IU/d	2543 IU/d
Placebo	None	400 IU/d	80-100 IU/d	400 IU/d

The power analysis was based on a study that evaluated TFCA with two levels of 25OHD resulting in a 36.5 nmol/L (14.6ng/mL) difference in 25OHD, and a 13 % difference in fractional calcium absorption (323). A minimum of 16 participants were needed to detect significant differences in TFCA between the two groups.

Protocol

Screening, eligibility and stabilization: Same as Aim 1 except that 25OHD levels were also measured at screening. If serum 25OHD levels was >30 ng/mL, participants were excluded from the study. Also participants were excluded if they planned to travel to areas of high sun exposure during the 6 week protocol. This is a *double blind randomized clinical control trial*. After one month stabilization, subjects completed baseline measurements that included a TFCA test, blood and urine collection.

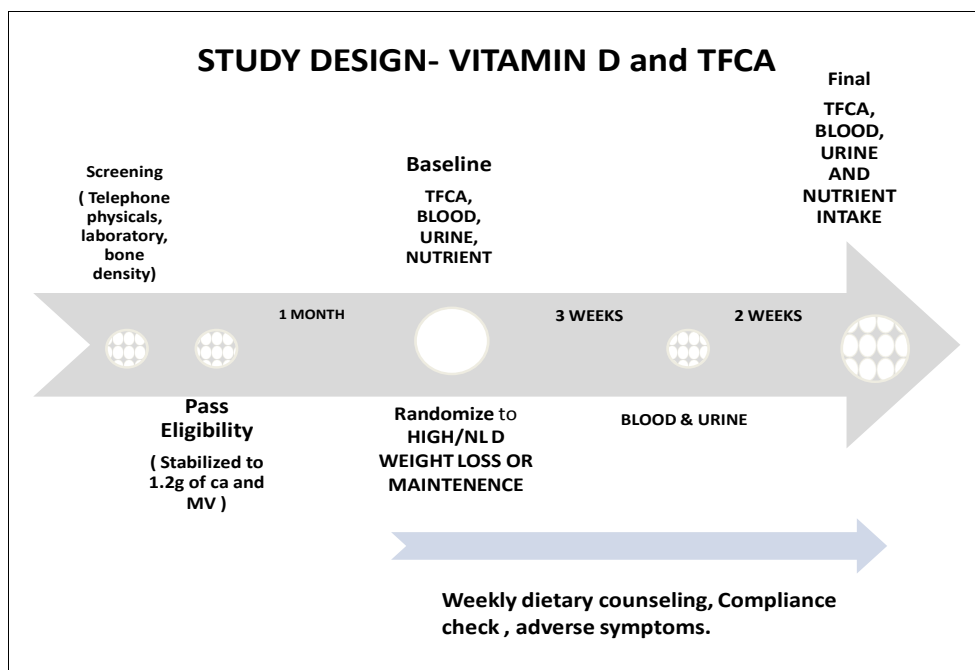


Figure 8: Study Design- Aim 2

Participants specifically recruited for weight loss or weight maintenance, were then randomized to high and normal vitamin D for 6 weeks. They were dosed once/week with vitamin D or placebo. They met with a dietitian once per week for weight loss during the 6 week period and all were encouraged to keep food diaries for at least 3 days/week. Final measurements included TFCA, blood and urine collection. Also, women were instructed to use a sunscreen during the 6 weeks to avoid influence of sunlight on vitamin D levels (331).

Methods

Bone and body composition using DXA and pQCT was measured at baseline. TFCA, weight, hormones were measured at baseline and during the 6 weeks (See detailed description of methods after aim 3)

5.1.3- Aim 3

To determine how body weight and/or serum parathyroid hormone differentially regulates cortical and trabecular bone in obese, overweight and normal weight women

Obesity is associated with a higher BMD and higher serum PTH levels. PTH has known catabolic actions specifically on the cortical bone in leaner individuals however its role in obesity where typically higher serum levels are seen is not completely understood. In this study, we aimed to understand the relationship between cortical, trabecular bone and body weight using pQCT. In addition, the effect of a higher PTH in obesity on cortical and trabecular bone was also examined to understand how adiposity influences bone quality in women.

Subjects and protocol

Women were recruited by advertisement in local newspapers and through email list serves. Screening for eligibility included telephone screenings, medical questionnaires, physical and biochemical tests and bone density screening. Exclusion criteria included presence of diseases or medications that are known to influence calcium or bone metabolism. All eligible subjects signed the informed consent form approved by Rutgers University Institutional Review Board.

Study design

This is a cross sectional study that included obese, overweight and lean women. Peripheral QCT scans of the tibia (a weight bearing site) from women who have completed previous studies in the lab (n= 81) were also included. Since no other studies have examined cortical and trabecular bone differences in women of varying BMI, power

analysis could not be based on this. Based on one previous study that examined differences in cortical and trabecular bone in pre and postmenopausal women (332), to detect differences in cortical and trabecular vBMD ($p < 0.001$) at 99% power at least 12 women in each menopausal group were needed. Based on another report (86), that examined normal postmenopausal women and women with hypoparathyroidism and hyperparathyroid patients, it was estimated that a minimum of 68 women were required to observe significant changes ($p < 0.001$) in cortical and trab BMD at 99% power with varying levels of PTH. All women were stabilized to 1.2 g of calcium and a multivitamin 1 month prior to the measurement

Methods

A fasting blood draw and urine sample was obtained for determination of hormones at baseline. Also, pQCT measurement of the distal tibia was performed on all subjects.

Bone density and content, body composition, weight and hormones were determined at baseline (See detailed description of methods after aim 3).

Secondary Aim

To determine whether serum PTH influences serum levels of monocyte chemoattractant protein -1 and whether a higher MCP-1 influences bone in obese compared to normal weight women

Subjects and protocol

In addition to the subjects that were recruited for the primary aim in this study, lean patients with primary hyperparathyroidism (defined as $PTH > 55\text{pg/mL}$) were also recruited. Based on previous studies examining the differences in another inflammatory

cytokines IL-6 and TNF- α between normal subjects and those with high serum PTH (133), 7 subjects were necessary to determine significant differences ($P < 0.001$) with a power of 99%. A limitation of this power analyses is that was not known whether the response of MCP-1 to PTH is similar to other cytokines. Hence, additional subjects were recruited for this reason and age-matched the obese and lean subjects to at least 10 subjects in each groups. We planned to enroll at least 14 subjects with normal PTH and 14 with high levels of PTH in each BMI category (Normal and obese) to total 56 women.

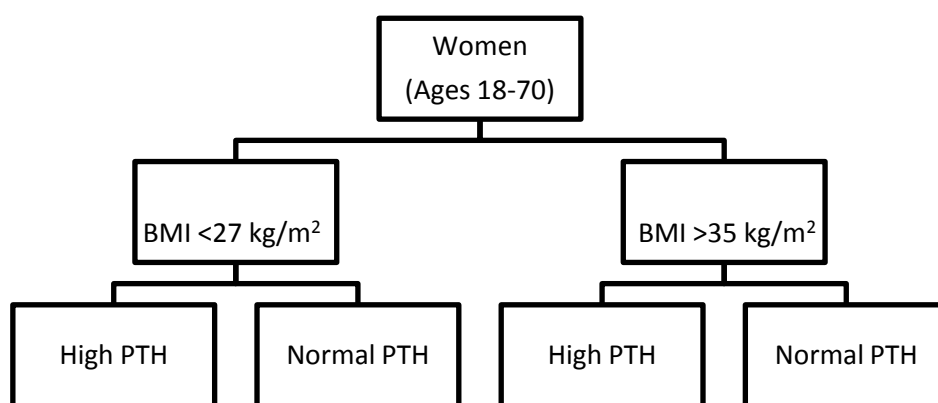


Figure 9: Study Design- Aim 3b

Women were stabilized to 1.2g/d of calcium and a multivitamin supplement 1 month prior to obtaining blood and urine sample to avoid variability in PTH due to variable calcium intake. We determined serum inflammatory MCP-1 and control cytokines CRP and adiponectin. Bone density, content and body composition using DXA and pQCT and weight and hormones were also measured (See detailed description of methods after aim 3).

5.2 METHODS

5.2.1 Dual X ray energy absorptiometry (DXA)

[Aims 1-3]

Bone mineral density, content, fat mass and lean mass were determined using DXA (Lunar Prodigy Advanced; GE- Lunar, Madison, WI; CV: < 1% for all sites, except 2% in radius) using enCORE 2004 software (version 8.10.027; GE Lunar). BMD and BMC was measured at ultradistal radius (UD Radius), 1/3rd radius (1/3 rad), femoral neck (FN), lumbar spine (L2-L4), total hip and total body. All measurements were performed at baseline, 6 and 12 months for aim 1 and at baseline for other aims in this dissertation. In addition, lumbar spine x-rays were examined for vertebral exclusion criteria specified by International Society of Clinical Densitometry - ISCD official positions 2007 (333). Vertebrae was excluded if it showed a local structural change, artifact, or evidence of anatomical abnormality with T-score difference >1.0 between the vertebra in question and adjacent vertebrae (See appendix).

5.2.2 Peripheral Quantitative computed tomography (pQCT)

[Aims 1-3]

BMD, BMC, geometric and bone strength properties were measured using pQCT - (Stratec XCT 3000, Orthometrix). Sectional images were standardized at specific sites (4%, 38% and 66%) using distal tibia as the anatomical marker and analyzed for volumetric BMD (vBMD), BMC, geometry, biomechanical property, muscle and fat area. The scans were acquired at 0.5mm voxel and a slice thickness of 2.4mm. A scout view was used to determine the positioning of the cross-sectional measurements from the tibia and was set by the integrated software (STRATEC XCT-3000, version 5.4) at 4%, 38% and 66% sites for trabecular, cortical and muscle/fat measurements respectively.

Processing of the images and calculation of the various bone indices were performed using integrated software. At the 4% site, total and trabecular volumetric bone mineral density (vBMD - mg/mm^3), BMC (mg) and total bone cross sectional area (mm^2) was calculated with the use of contour mode 2 and peel mode 2 at a threshold of $280\text{mg}/\text{cm}^3$. Cortical v BMD area, BMC, thickness, periosteal circumference (mm) and endosteal circumference (mm) was assessed in the 38% site with the use of cort mode 1 and the threshold of $710\text{ mg}/\text{cm}^3$. The polar strength-strain index (mm^3) and moment of inertia (mm^4) was calculated at the 38% site with cort mode 1 and a threshold of $280\text{ mg}/\text{cm}^3$. At the 66% site fat area, muscle area and the ratio of fat/muscle area was calculated using cort mode 1 and peel mode 1. In addition, to ensure accuracy, an in-vivo precision analysis using 23 obese subjects for 4% slice and in 18 for the 38% slice was performed. The coefficient of variation (CV) between 2 measurements was less than 1.7% for trab and cort vBMD, BMC, area and geometry.

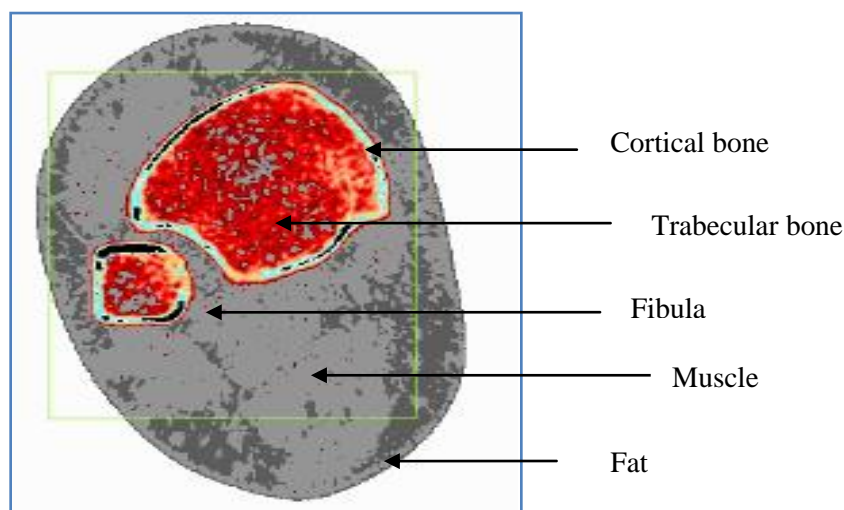


Figure 10: Cross section of the tibia as seen in pQCT

5.2.3 Hormones and Turnover markers

The following hormones were analyzed in the serum using Radioimmunoassay (RIA), Immunoradiometric assay (IRMA) or Enzyme linked immunoassay (ELISA) and the methods, manufacturer details and inter and intra assay CV is summarized in Table 3.

Extraction of serum for assays

Blood was obtained through venipuncture. Serum was extracted from whole blood that was collected in BD vacutainer SST plus Blood collection tubes and later was centrifuged at 1500g for 20 minutes. The extracted serum was then stored at -80 degrees until further analysis for the following hormones and turnover markers.

Principles of Assays:

The *RIA* involves a competition between radioactive and non radioactive antigen for a set number of binding sites and higher binding is inversely proportional to concentration of the analyte in the serum. A known amount of radiolabeled solution and antiserum and unknown amount of analyte compete for binding sites of the polyclonal antibody.

The *IRMA* also involves two antibodies, a first antibody on solid-phase coated on tubes or beads. After binding the antigen present in the sample, a second radioactively labeled antibody is added. A second monoclonal antibody will separate antibody bound substance and free substance. The bound fraction remaining in the pellet is counted in the gamma counter later and quantitated using calibration curves.

The *EIA/ELISA* also involves competitive binding for antibody sites. Briefly, diluted samples are added to microplate followed by addition of monoclonal antibody

which leads to competitive binding for antibody sites. A sandwich ELISA uses two different polyclonal antibodies; one coated onto the surface of the microtiter plate to capture the antigen in the sample and one that is conjugated to horse radish peroxidase (HRP) to bind and quantitate the captured antigen in the plate well. The competitive ELISA only uses a single polyclonal antibody that is coated onto the surface of the microtiter plate to capture the antigen in the sample. Prior to addition of the sample to the microtiter plate wells, a fixed amount of antigen conjugated to HRP is added to the sample and mixed. The antigen in the sample then competes with the antigen-HRP conjugate for binding sites in the plate well. The free unbound antigen-enzyme complex resulting from competition with measured antigen in the sample forms color-change substrate that is proportional to the concentration of antigen present in the specimen and the absorbance is measured using a spectrophotometer and the concentration is calculated.

Parathyroid hormone (PTH) [Aims 1-3]

Total intact PTH (1-84 fragments) and N-truncated PTH fragments was measured using IRMA (Scantibodies, CA). The PTH peptide (1-84) is secreted by the parathyroid glands and levels are regulated by extracellular concentration of ionized calcium, vitamin D and magnesium. The primary function of this hormone is maintenance of calcium homeostasis. In addition to its pivotal role on bone biology, PTH levels are also increased in obesity and aim 3 in this dissertation particularly addresses the role of high PTH in obesity. This IRMA kit is a total PTH coated bead kit and uses antibody for both the N and C terminal region of PTH and detects whole PTH (1-84 fragments) and N-truncated

PTH fragments. PTH was measured in all aims of this dissertation to understand its influence due to weight loss, protein and D intake and obesity.

25 Hydroxy vitamin D (25OHD) [Aims 1-3]

The major circulating unit of vitamin D is 25OHD and used as a biological indicator of vitamin D status. A RIA kit from Diasorin, MN was used that measures both circulating D₂ and D₃. Since vitamin D is a fat soluble vitamin, this assay uses a preliminary extraction procedure to extract 25OHD and other hydroxylated vitamin D metabolites in the serum. Later the amount of 25OHD in the serum was quantitated using a RIA. This hormone was measured in all aims of this dissertation to understand its influence due to weight loss, protein and D intake and obesity.

1,25 di hydroxy vitamin D₃ - 1,25(OH)₂ D₃ [Aim 2]

1,25(OH)₂ D₃ is the active form of vitamin D and primarily regulates calcium absorption in the intestine. Vitamin D derived from the sun undergoes hepatic hydroxylation and further hydroxylation at 1 position yields 1,25(OH)₂ D₃. It is tightly regulated by serum calcium, PTH and phosphorous. 1,25(OH)₂ D₃ was measured using a RIA from Diasorin MN. This assay involves a preliminary extraction and subsequent purification of vitamin D metabolites from the serum using C₁₈OH cartridges and then treated in a RIA procedure. The half life in serum of this hormone is very short and thus it is not a biological indicator of vitamin D status, but is an important regulator of calcium absorption. Hence this hormone was measured in aim 2, where the primary outcome variable is TFCA.

Estradiol [Aims 1-3]

Estradiol is a steroid hormone and is primarily synthesized in the ovary and to a lesser extent by the adipose tissue. Circulating levels are usually lower in postmenopausal women and is thought to be a major factor in the pathogenesis of postmenopausal osteoporosis. The osteoblasts contain an estrogen receptor and higher levels promote osteoprotegrin synthesis, and also osteoclast apoptosis. A RIA from Beckman Coulter, TX was used to determine estradiol. Estradiol levels decrease with weight loss and may contribute to loss of bone mass with CR. In addition estradiol is also a positive predictor of TFCA. Hence estradiol was measured in aim1 and 2 of this dissertation.

Bone Turnover markers [Aims 1-3]

The following markers of bone turnover- formation and resorption markers that are synthesized either by the osteoblast, osteoclast or collagen fragments of the bone were analyzed in serum or urine. They were measured in all aims of this dissertation to assess if bone turnover is altered due to higher protein intake or is associated with obesity and higher PTH.

Osteocalcin

Osteocalcin (OC) is a 5700 Dalton bone GLA protein and a major non collagenous bone matrix protein and has been long studied as a marker of bone formation and also in the regulation of energy metabolism. The synthesis of OC by osteoblasts and the carboxylation of its glutamyl residues are both dependent on Vitamin K. Total osteocalcin (OC) is the sum of the carboxylated and undercarboxylated forms. This was measured using a RIA from Biomedical technologies (BTI) and is based on the principle of competitive RIA.

Propeptide of type 1 collagen (PINP)

PINP in the serum is a marker of osteoblastic activity and is related to the amount of new collagen that is laid down in tissue and hence is used as a marker of bone formation in the serum. This was assessed using a quantitative radioimmunoassay from Orion Diagnostica and is based on the principle of competitive RIA.

N telopeptide of type 1 collagen (NTx)

N telopeptide of type 1 collagen (NTx) molecule is a bone specific molecule whose formation is mediated by the osteoclast. It is a type I collagen helical protein, a part of the bone tissue that is cross linked at the N terminal. It is a marker of bone resorption and can be measured both in serum and urine. It was measured in the serum using the Osteomark NTx kit (Wampole Laboratories, NJ) which is a competitive inhibition ELISA.

Pyridinium cross links (PYD and DPD)

These were measured with the use of high performance liquid chromatography (HPLC) after hydrolyzed samples were submitted to a prefractionation procedure (334). The peaks are detected with the use of fluorescence (335) and quantitated using external standards. Values were corrected for creatinine excretion to adjust for differences in the concentration of spot urine samples.

IGF-1 and IGFBP-3 (Aim1)

IGF-1 is a growth factor that is also osteotrophic. It is a 7.6KDa peptide that mediates the action of growth hormone. It is secreted by the liver and in the serum is bound to its binding protein IGFBP-3. Serum levels of IGF-1 and IGFBP-3 are positively correlated

with dietary protein intake and BMD and hence was measured in aim 1 of this protocol. They were measured using IRMA kits from DSL and both will employ a non extraction procedure.

Monocyte chemoattractant protein-1 (MCP-1) (Aim3b)

MCP-1 is 76 KDa mature protein that is a potent chemotactic factor for monocytes and is secreted by macrophages. It is a proinflammatory cytokine that is upregulated in inflammatory conditions. It has been previously shown that PTH administration increases MCP-1 levels. MCP-1 levels were measured in the serum using a quantitative sandwich enzyme assay from R and D systems that uses a sandwich enzyme immunoassay technique.

C-reactive protein (CRP) (Aim3b)

CRP is a pro inflammatory cytokine that is also synthesized in the adipose tissue. CRP was measured as a control cytokine in the protocol that aims to test whether high PTH in humans is associated with an increase in MCP-1. It was measured in the serum using the ELISA assay from R&D systems. CRP levels were measured in the serum using a quantitative sandwich enzyme assay from R and D systems that using a sandwich enzyme immunoassay technique.

Adiponectin (Aim3b)

Adiponectin is a 244 amino acid protein and is secreted by the adipocyte. It is an anti-inflammatory cytokine. Adiponectin was measured as a control cytokine in the protocol that aims to test whether high PTH in humans is associated with and increase in MCP-1.

Total adiponectin was measured in the serum using the total adiponectin multimeric assay from ALPCO diagnostics using a sandwich enzyme immunoassay technique.

Urea nitrogen (Aim1)

Urea Nitrogen was measured in the serum using the liquid urea nitrogen reagent set. It was measured using an enzymatic procedure. This was measured in Aim 1 of this dissertation to assess compliance with HP diet that usually results in rise in BUN. This was measured using the Pointe Scientific reagent.

Creatinine (Aim1-3)

Creatinine was measured in the serum and/or urine in all aims of this dissertation. The pyridinium cross links are usually obtained from spot urine sample and are normalized for creatinine excretion and thus was assessed in the urine. In Aim 1 and Aim 3b, serum creatinine was measured to assess compliance to HP diets and/or to estimate glomerular filtration rate (GFR). This was measured using the Pointe Scientific reagent based on Jaffe's reaction. Briefly, this reaction involves the action of sodium picrate to creatinine that yields a creatinine picrate complex that is yellow in color and can be detected at 510nm.

Calcium (Aim1,2,3b)

Urinary calcium was measured in a 24 hour urine collection in aim 1 and 2 of this dissertation. Serum calcium was measured in aim 3b of this dissertation. This is to understand whether urinary calcium excretion is increased in response to higher protein and D intake. Calcium was measured using the Arsenazo reagent set that employs the reaction of calcium with arsenazo III reagent in an alkaline medium to yield a calcium arsenazo complex that was measured at 650nm.

5.2.4 Nutrient Analysis

(Aim 1-3)

Subjects in all protocols were instructed to maintain food diaries at baseline and during the intervention. The food diary will include information on type of food, amount and type of cooking. These food diaries were then analyzed using software (Food Works, LongValley, NJ) that analyzed macro and micronutrient intake per day.

5.2.5 True fractional calcium absorption (TFCA)

[Aim 2]

1. Purchase and mixing of stable isotopes: The required amount of Ca^{42} and Ca^{43} depending upon sample size is purchased. Stable isotopes were supplied by Trace Sciences international (Wilmington, DE). All materials used are autoclaved and then acid washed. The isotopes were then mixed in a saline solution in a clean and sterile room at the Dept of Pharmacy, St Peters University Hospital. The isotope powder is dissolved in a few drops of 12 N HCl and then neutralized for pH with addition of 0.9% NaCl. A few drops Benzyl alcohol was added as a preservative and pH was neutralized to pH=5.5 by addition of 1N and 5N NaOH and 0.9% NaCl was added to yield the final volume. Using a sterile filter, the solution is filtered and then aliquoted to 4ml vials and immediately sealed and stored at 4⁰ C until use. The solutions were later sent for Mass spec analysis to estimate calcium concentration in the solution. The solutions were also submitted for sterility and endotoxin testing.

2. *Dose calculations for the subject:* A Ca^{42} concentration of 0.012mg/kg and 0.017mg/kg Ca^{43} is used per subject and the volume that needs to be administered is calculated based on body weight of subject prior to the test. The IV and/oral syringes for each subject are

usually dispensed a day prior to the test at the hospital and is weighed before and after administration of the isotope.

3. *On the day of the test:* Women were admitted after an overnight fast. They were encouraged to drink plenty of water before the test to facilitate easy IV placement and blood draw. A 24 hour urine collection began with a spot urine sample immediately upon arrival. A physical exam was performed and an IV catheter was placed in the arm and a 10mL blood draw is done. The subjects were then served a standard breakfast that includes 4oz of milk containing Ca^{43} . The isotope was mixed in the milk a night before the test and allowed to equilibrate for at least 12 hrs. Immediately following breakfast, Ca^{42} was injected intravenously over ~3 minutes, no more than 30 minutes after consumption of Ca^{43} . Complete urine collection was monitored in each subject throughout the following 24hrs. It has been previously shown that calcium absorption after a test load is 95.8% complete after 10 hours and fully complete by 23-26 hours (336;337).

4. *Precipitation of calcium from urine:* The next step involved oxalate precipitation of calcium from the urine that enabled samples for mass spec analysis to determine Ca^{42} and Ca^{43} concentrations. Concentrated ammonium hydroxide solution was added to 5 ml of urine and pH was adjusted to 10. Three ml of saturated ammonium oxalate solution is added and is allowed to precipitate overnight. The supernatant was decanted and transferred into test tubes and all liquid is allowed to dry on a heating plate for 8-12 hrs and later is ashed at 550°C for 14-18 hours that converts the oxalate to carbonate. The residue was mixed with few drops of nitric acid and sample was dried overnight and

washed again. The residue was then dissolved in 1ml of 3% HNO₃ and sent for analysis using ICPMS.

5. *TFCA calculations:* TFCA was estimated using dual stable isotope method. The dual-isotope method combining two stable isotopes is based on the principles of DeGrazia 1965 et al and Yergey 1987. With simultaneous oral and intravenous administration of 2 different calcium isotopes, calcium absorption can be estimated from the relative enrichment or appearance of the 2 isotopes in urine (287;288). Fractional Ca absorption (α) was calculated from the pooled 24 hour urine sample using specific equations (288).

TFCA is calculated using the following equations:

$$\alpha = \frac{\int (\text{Fraction of oral label in urine}) dt}{\int (\text{Fraction of IV label in urine}) dt}$$

$$= \frac{([\text{calcium}] (\text{vol sample})(\text{na oral}) (\Delta \% \text{ excess oral}))/\text{oral dose}}{([\text{calcium}] (\text{vol sample})(\text{na iv}) (\Delta \% \text{ excess iv}))/\text{iv dose}}$$

$$= \frac{(\text{na oral}) (\text{iv dose}) (\Delta \% \text{ excess oral})}{(\text{na iv}) (\text{oral dose}) (\Delta \% \text{ excess iv})}$$

$$\Delta \% \text{ excess} = \frac{\text{observed ratio}^* - \text{na ratio}}{\text{na ratio}} \times 100 \quad (* \text{ ratios are relative to } \text{Ca}^{44})$$

Table 3: Summary of methods and measurements

Measurements	Fluid / Site	Method	Manufacturer	CV	Aim 1	Aim 2	Aim 3a	Aim 3b
Body composition	Whole body	DXA	GE Lunar		√	√	√	√
Bone density	Hip, Spine, Radius	DXA	GE Lunar	<1 %	√	√	√	√
Bone density and geometry	Tibia	pQCT	Orthometrix	<4%	√	√	√	√
PYD and DPD	Urine	HPLC		<5%	√		√	√
NTX	Serum	ELISA	Osteomark	<14%	√		√	√
Osteocalcin	Serum	RIA	BTI Inc	<9%	√		√	√
P1NP	Serum	RIA	IDS, AZ	<10%	√		√	√
PTH	Serum	IRMA	Scantibodies, CA	<7%	√	√	√	√
Estradiol	Serum	RIA	Beckman Coulter, TX	<13%	√	√	√	
CRP, MCP-1, adiponectin	Serum	ELISA	R&D systems, MN	<9%				√
25OHD	Serum	RIA	Diasorin, MN	<13%	√	√	√	√
1,25(OH) ₂ D ₃	Serum	RIA	Diasorin, MN	<16%		√		
IGF	Serum	RIA	Beckman Coulter, TX	<8%	√			
IGFBP-3	Serum	RIA	Beckman Coulter, TX	<4%	√			
Ca isotopes for TFCA	Urine	ICPMS				√		
Creatinine	Serum & Urine	Spectrophotometric		<4%	√			
Blood urea nitrogen	Serum	Enzymatic		<2%	√			
Ca (urinary and serum)	Serum & Urine	Spectrophotometric		<2%	√	√		
Weight and height		Balance scale			√	√	√	√

6. MAIN EXPERIMENTS

6.1: Areal and volumetric bone mineral density and geometry at two levels of protein intake during caloric restriction: a randomized controlled trial.

Sukumar D, Ambia-Sobhan HA, Zurfluh R, Schlusser Y, Stahl T, Gordon CL, Shapses SA.

Journal of Bone and Mineral Research, 2011

Jun;26(6):1339-48 (338)

6.1.1. Abstract

Weight reduction induces bone loss by several factors and the effect of higher protein (HP) intake during caloric restriction on bone mineral density (BMD) is not known. Previous study designs examining the longer term effects of HP diets have not controlled for total calcium intake between groups and have not examined the relationship between bone and endocrine changes. In this randomized controlled study, we examined how BMD (areal and volumetric), turnover markers and hormones (insulin-like growth factor-1; IGF-1, IGF-binding protein-3; IGFBP-3, 25-hydroxyvitamin D, parathyroid hormone, and estradiol) respond to caloric restriction during a 1 year randomized trial using two levels of protein intake. Forty-seven postmenopausal women (58.0 ± 4.4 years; body mass index of 32.1 ± 4.6 kg/m²) completed the one year weight loss trial, and were on a higher (HP, 24%, n = 26) or normal (NP, 18%, n = 21) protein and fat intake (28%) with controlled calcium intake of 1.2 g/d. After 1 year, subjects lost 7.0 ± 4.5 % of body weight and protein intake was 86 and 60 g/d in the HP and NP groups, respectively. HP compared to NP diet attenuated loss of BMD at the ultra distal radius, lumbar spine and total hip and trabecular volumetric BMD and bone mineral content of the tibia. This is consistent with the higher final values of IGF-1 and IGFBP-3 and lower bone resorption marker (deoxypyridinoline) in the HP than NP group ($p < 0.05$). These data show that a higher dietary protein during weight reduction increases serum IGF-1 and attenuates total and trabecular bone loss at certain sites in postmenopausal women

6.1.2 Introduction

Dietary protein plays an important role in the maintenance of bone health (177). A regular supply of amino acids is required to offset losses during proteolysis and in maintenance of bone structure. Large epidemiological studies that have evaluated the relationship between dietary protein intake and bone have shown a beneficial impact of protein on bone health (178;339) similar to clinical intervention studies in patients with osteoporosis (172). There is some concern of greater acid load and increased urinary calcium excretion with higher intake of protein causing low bone mineral density (BMD) and greater fracture risk (182;183). Since the negative effects of high protein diets on BMD and fracture risk have been found to be influenced by low intakes of calcium, (187;340) controlled trials with adequate calcium intake are needed to determine the independent effect of protein on BMD.

Caloric restriction (CR) is often associated with bone mobilization and loss and this is largely observed in older women and men (19). This increase in bone mobilization may be due to a variety of reasons including a decrease in intake of calcium and/or other nutrients (148), a decrease in calcium absorption (151), reduced weight bearing (166) and/or hormonal changes (19). There is also a decrease in serum insulin-like growth factor-1 (IGF-1) levels with CR (160) and this too may negatively affect bone. A higher protein intake increases IGF-1 and calcium absorption, which may indirectly preserve bone mass (169;172;177).

Previous studies that have examined the role of dietary protein on BMD after 3-6 mo of CR have either focused on high protein and dairy intakes compared to lower protein

intakes with insufficient calcium or high calcium intakes in both groups (207;224;226).

These trials have either shown attenuated bone loss in HP group or a negative influence of HP diets on bone. Thus, the role of protein in the maintenance of bone mass during a longer period of CR is unclear, and use of a control group that is not deficient in calcium intake and with the recommended intake of calcium has not been previously examined. The goal in this study was to examine the role of high protein compared to normal protein intake on BMD during caloric restriction for 1 year in postmenopausal women with controlled and recommended calcium and vitamin D intakes between groups.

6.1.3 Subjects and Methods

Subjects:

Postmenopausal women who reported no menstruation for at least 2 years prior to the study were recruited. All participants were between ages of 50-70 yrs and free from any disease states or medications known to influence bone metabolism. Before initiation of any study procedures, subjects signed an informed consent approved by the Institutional Review Board at Rutgers University and an external advisory board. This trial was registered at clinical trials.gov (NCT00473031).

Screening for eligibility:

All participants had to pass a 3 step screening process that included a telephone, laboratory and physical screening. The telephone screening included questions about current diseases and medications, and participation in prior weight loss programs in the past few months. Participants had a comprehensive metabolic panel test and physical screening prior the study. They were screened for high fasting blood glucose, abnormal creatinine clearance, blood urea nitrogen (BUN), uric acid, calcium and phosphorus etc. For patients who were taking thyroid medications, a stable dose for the past 2 or more years was required for inclusion, and none reported any change in medication when questioned monthly during the intervention. Subjects between BMI 25-40 kg/m² were included. Participants also were screened at baseline for osteoporosis and excluded with a T score less than -2.5 at the hip or spine. Subjects who passed all the parts of screening were considered eligible for the study.

Study Design:

Subjects were recruited in 4 cohorts in the spring months between the years of 2005-2008. They were enrolled in the lifestyle behavior modification weight loss program for 1 year and randomized to a HP (30% of total calories) or NP diet (18% of total calories) using a randomization program (SAS Institute, Cary, NC, USA; Version 9.2). To determine caloric requirements, resting energy expenditure (REE) was measured at baseline using indirect calorimetry (VMax, Sensor Medics, Yorba Linda, CA). This was done after an overnight fast and about 30 minutes of rest prior to the taking a steady state measurement. Participants then received an individually tailored diet plan (about 500-600 kcal deficit per day) that included appropriate food selections and portion sizes using the diabetic exchange lists. There were 36 weight loss counseling sessions by a registered dietitian throughout the 1 year intervention. Participants also received individual counseling sessions for specific diet concerns as needed. Physical activity of the participants was monitored using a pedometer and subjects reported pedometer steps at least 3 days a month during the intervention. The average daily physical activity level was quantitated using their respective metabolic equivalent and was estimated from all active, incidental active and passive activities that was recorded periodically. These postmenopausal women were instructed to maintain the same level of physical activity throughout the intervention.

Weight and Height:

Weight and height was measured with a balance beam scale and stadiometer, respectively; (Detecto, Webb City, MO). At each morning visit, weight was recorded with minimal clothing.

Food records:

All participants were instructed to maintain food records for at least one week per month and were asked to enter details of food quantity, servings, method of preparation throughout the intervention. In addition, participants also completed a food frequency questionnaire (FFQ) and 24 hour recall with the dietitian once every 3 months, to ensure adherence to diet. Dietary intakes were analyzed using FoodWorks software (Long Valley, NJ, Version 10).

Supplements:

Subjects in the HP group were specifically counseled to increase protein intake from the diet such as lean meat, fish, legumes and dairy. In addition, subjects were also offered a whey protein supplement (Beneprotein, Nestle HealthCare Nutrition, Successor-in-interest to Novartis Medical Nutrition, Minneapolis, MN) to increase total protein intake and were asked to consume at least 1 scoop of the powder/d (1 scoop= 6g protein). Participants also completed a protein food frequency questionnaire (FFQ) with the clinical coordinator to estimate protein intake once every month. Adherence to protein intake was monitored by absolute increase in protein intake from baseline, percent protein intake, as well as urea nitrogen. All subjects completed calcium food frequency questionnaire at baseline to estimate calcium supplementation. Subjects were stabilized to calcium intake of 1.2 g/d per day, beginning 1 month prior to the intervention and throughout during the study period. If calcium intake from food and multivitamin (NatureMade Multi 50+, Mission Hills, CA) was below 1.2 g/d, subjects were given a calcium supplement without added vitamin D (Citracal, Bayer, NJ) to meet 1.2 g/d. All participants were given a multivitamin that

contained 400 IU/d of vitamin D₃. Once every month, subjects completed calcium FFQ to ensure adherence to the recommended daily calcium intake from diet and supplements.

Bone and body composition:

Bone and body composition was measured using dual-energy x-ray absorptiometry (DXA) (Lunar Prodigy Advanced; GE- Lunar, Madison, WI; CV: < 1% for all sites, except 2% in radius) using enCORE 2004 software (version 8.10.027; GE Lunar). BMD and BMC was measured at the ultra distal (UD) radius, 1/3 radius, femoral neck (FN), lumbar spine (L2-L4), total hip and total body. In addition, fat mass and lean mass were also measured. All measurements were performed at baseline, 6 and 12 months. Lumbar spine x-rays were examined for vertebral exclusion criteria specified by International Society of Clinical Densitometry - ISCD official positions 2007(333). A vertebrae was excluded if it showed a local structural change, artifact, or evidence of anatomical abnormality with T-score difference >1.0 between the vertebra in question and adjacent vertebrae.

Peripheral quantitative computed tomography:

Volumetric BMD, BMC, and geometric and bone strength properties of the tibia were measured using pQCT (Stratec XCT 3000, Orthometrix). Sectional images were standardized at specific sites (4% and 38%) using distal tibia as the anatomical marker. The scans were acquired at 0.5mm voxel and a slice thickness of 2.4mm. A scout view was used to determine the positioning of the cross-sectional measurements along the tibia and was set by the integrated software (STRATEC XCT-3000, version 5.4). Trabecular bone parameters are reported at the 4% site and cortical bone at the 38% site, as described

previously(341). The coefficient of variation (CV) was less than 1.7% for Tb and Ct vBMD, BMC, area and geometry.

Blood and urine analysis:

Fasting blood and urine samples were collected at baseline and weeks 4, 12, 24, 38 and 52. Serum 25-hydroxyvitamin D [25OHD] (DiaSorin, Stillwater, MI, CV < 12.5%) and ultra sensitive Estradiol (E2) ¹²⁵I RIA (DSL, Webster, TX, CV < 8.9%) were measured by radioimmunoassay (RIA). Our laboratory also participates in vitamin D Quality Assessment Scheme (DEQAS) that monitors the performance of our 25OHD assay. Intact PTH was determined by immunoradioassay (Scantibodies, Santee, CA, CV<6.8%). Urea nitrogen in serum was measured using the liquid urea nitrogen reagent set (Pointe Scientific, Canton, MI, CV< 4.6%). Pyridinoline (PYD, CV < 8%) and deoxypyridinoline (DPD, CV < 10%) were measured in the urine by reverse- phase HPLC and fluorescence detection and normalized for creatinine excretion. Bone formation markers, osteocalcin (OC) (BTI; Stoughton, MA, CV < 9%) and propeptide of type 1 collagen (PINP) (UNIQ PINP RIA, Orion Diagnostica, Espoo, Finland CV< 10.2%) were measured by RIA. Serum N-telopeptide of type I collagen (NTx) was measured by ELISA (Osteomark; Princeton, NJ, CV <4.6%). IGF-1 and IGFBP-3 were measured using immunoradiometric assay (DSL, Webster, TX, CV < 7.4 % and <3.9% respectively)

Statistical Analysis:

The influence of diet (HP vs NP) and time (0, 6, 12 months) on BMD, BMC, fat and lean tissue, hormones, bone turnover markers and nutrient intake was measured using two-factor repeated measures ANOVA. Tukey's post hoc analysis was performed when model

F ratio was significant. One-way ANOVA was used to examine differences between the percent changes in BMD from baseline between the two groups. Paired t-test for comparison of means was used to determine changes in outcome variables within a group compared to baseline. Values are expressed as mean \pm SD. P values ≤ 0.05 were considered significant. A power analysis was performed with alpha set at 0.05, with the value of beta set at 0.90, using bone mineral density from sub group analysis from a previous study (178) evaluating the effect of protein intake. This analysis indicated that 15 participants per group would be necessary to avoid a type-II error. Our goal was to include at least 5 additional participants per group to account for 2 potential baseline covariates.

6.1.4 Results

Participants

We screened a total of 182 women over a period of 4 years of which sixty subjects were enrolled for the study (Figure 11). Women were randomized to either the HP group (n=29) or NP group (n=31). Three subjects from the HP group and 4 in the NP group were dropped from the study due to non-compliance with weight loss, i.e., lost less than 2.5% of their initial body weight over the first 4 months. Six subjects who were randomized to the NP group dropped from the study due to a lack of time commitment and inability to attend bi-weekly sessions. Subjects were primarily Caucasians (N = 53), African Americans (N = 6) and there was 1 Asian. Forty-seven subjects with 5 African Americans and 1 Asian completed the 1 year trial with 26 subjects completing the HP diet and 21 to the NP diet. In the HP group there were 3 African Americans, 22 Caucasians and 1 Asian; and in the NP group, there were 2 African American and 19 Caucasian women.

Weight, Bone and body composition changes

The mean age (58 ± 4 years) and REE (1365 ± 177 Kcal/d) at baseline were not significantly different between the groups. Subjects in the HP group lost $6.6 \pm 4.0\%$ and NP group lost $7.4 \pm 5.2\%$ of their initial body weight with no difference between groups at baseline or during the intervention (Table 4). Subjects lost $11.7 \pm 10.1\%$ of fat mass and $2.7 \pm 4.0\%$ of lean mass during the intervention with no difference between groups. In addition, there was a decrease in BMD at ultra distal radius, 1/3 radius, hip and TB and BMC at lumbar spine and femoral neck ($p < 0.05$) at 1 year compared to baseline. There

was an interaction between diet and time observed for BMD at UD radius, lumbar spine and total hip ($p < 0.05$, Table 4). Subjects in the NP compared with the HP group lost significantly more BMD at the UD radius ($-3.3 \pm 4.2\%$ vs $-0.9 \pm 3.2\%$) lumbar spine ($-1.4 \pm 3.6\%$ vs $0.2 \pm 3.4\%$) and total hip ($-1.2 \pm 1.8\%$ vs $-0.4 \pm 1.3\%$) (Figure 12). For the entire group of overweight/obese women at baseline, osteopenia at the lumbar spine and femoral neck was 23% and 43%, respectively, and 2-4% had osteoporosis. At the end of the study, the total cases of osteopenia remained the same, and osteoporosis increased to 6% at the lumbar spine and femoral neck.

Changes in trabecular and cortical bone

The effect of protein intake on trabecular and cortical bone and geometry is presented in Table 5. There was a decrease in tibial total vBMD accompanied by an increase in total area over a 1 year period ($p < 0.05$). There was a greater decrease in total vBMD and trabecular vBMD and BMC in the NP compared to the HP group over time ($p < 0.05$). In addition, there was also a trend to increase cortical vBMD and decrease in cortical area over time ($p \leq 0.09$), without differences between the HP compared to the NP diets. Tibia muscle area did not significantly decrease over time or differ between groups. Fat area around the tibia decreased over time in both groups ($p < 0.001$) and this decrease was greater in the NP compared to the HP group ($p < 0.05$).

Nutrient intake during the intervention

Nutrient intake from food and supplements is presented in Table 6. Intake of total calories, carbohydrates and fat was significantly lower ($p < 0.01$) during the intervention. As expected, subjects in the HP group consumed significantly higher protein (86 vs. 60

g/d) compared to NP group ($p < 0.001$). The percent of protein calories was 24 % as compared to 18% in the HP and NP group, respectively. Women on the HP diet had a greater intake of dairy and meat ($p < 0.01$) and tended to have higher intake of eggs ($p < 0.08$) compared to those on a NP diet. The whey protein supplement contributed to < 4% of total daily protein intake. Differences in protein intake between groups were also supported by findings that BUN was higher in the HP than NP group ($p < 0.01$). Subjects consumed 1128 ± 202 mg of calcium/d from diet, multivitamin and calcium supplements with no differences between the groups during the intervention.

Changes in hormones and binding proteins during intervention

There was a significant effect of weight loss independent of diet on serum levels of PTH, 25OHD and IGF-1 during the intervention ($p < 0.05$) (Figure 13). PTH decreased during the 1 year period ($p < 0.05$), while there was a rise in 25OHD ($p < 0.001$). Those in the NP group showed a trend for higher values for 25OHD ($p < 0.08$) compared to HP group. In addition, there was a tendency for estradiol to increase over time in HP compared to the group ($p < 0.06$). PTH and 25OHD levels did not differ significantly between groups during the intervention. Serum levels of IGF-1 increased significantly over time in the HP group ($p < 0.05$) compared to NP group. Serum IGF-1 increased by 20 ± 37 % during intervention compared to baseline ($p < 0.01$) in HP group compared to an insignificant rise of 3 ± 18 % ($p = 0.92$) in the NP group. Serum IGFBP-3 remained unchanged in the HP group, however decreased significantly in the NP group ($p < 0.05$).

Changes in bone turnover during intervention

The change in bone turnover markers during the intervention period is shown in Table 7. PYD increased during the 1 year of CR ($p < 0.05$), and this increase was independent of diet. Those in the NP group showed a trend for higher values PINP ($P < 0.06$) compared to the HP group. Resorption marker DPD significantly increased in the NP compared to the HP group over time ($p < 0.05$) and a similar tendency ($p < 0.08$) was also observed for PYD. There were no significant differences in the bone formation markers OC and PINP between the groups during intervention.

6.1.5 Discussion

High protein intake is a popular means of weight reduction because in the short term it has shown to result in greater weight loss, fat loss and preservation of lean mass compared to high carbohydrate low fat diets (193). However longer term studies show that a greater weight loss and/or fat loss on a higher protein diet is not sustained and is similar to that seen with a standard high carbohydrate diet after 1-2 years (209;210), as observed in this study as well. This is the first study that delineates the influence of protein intake during weight reduction on bone that controls for dietary intake of calcium and other micronutrients and more importantly compares the effect of a HP diet to a control group with sufficient and recommended calcium intake. We show that CR for 1 year with a protein intake of 24% of total calories attenuates loss of bone at the radius, hip and spine and increases IGF-1 compared to a diet with 18% protein intake.

During weight stable conditions, an increase in dietary protein intake has a positive influence on the bone. A HP diet has been shown to increase serum levels of IGF-1 (171;172) which promotes osteoblast proliferation and matrix formation (163) and may in turn increase bone mass and reduce fracture risk (172). A higher protein (2.1 g/kg) compared to moderate (1.0 g/kg) intake has also been shown to increase intestinal calcium absorption that leads to a parallel increase in urinary calcium and decrease in bone turnover markers (173). In this 10 day study (173) and another short term (8 week isotopic tracer) study examining protein intake (174) there were trends towards better calcium retention during high protein intakes. It is interesting that with higher calcium intake (~1500mg), there was no protein-related increase in calcium absorption (175) .

Because a higher protein intake is associated with an increase in muscle mass and promotes collagen synthesis, this too may contribute to acquisition of bone mass (177).

Similar to previous findings from our laboratory and others (148;151;168) we observed a loss of bone mass at a few sites with CR. In rodents, protein calorie malnutrition has been shown to decrease trabecular and cortical BMD (342) the effect of high protein and CR on these compartments has not been examined in humans. We show that higher protein intake attenuated the loss of BMD at the lumbar spine, ultra distal radius, total hip and trabecular bone at the tibia compared to the NP diet. Multiple clinical intervention trials have examined the role of higher dietary protein on bone mass and turnover during CR, although none of these trials control for calcium intake between groups and findings is not consistent (207;223;224;226;343). The importance of dietary calcium on bone and during weight loss is well established (148;168;227). These previous studies that address the role of higher dietary protein intake during dieting have accomplished this by increasing both dairy and calcium intake and were compared to calcium insufficient (~600 mg/d) high carbohydrate diets. Some of these high calcium and protein trials (207;224) have shown a positive impact of the diet on maintenance of bone mass during CR, similar to calcium supplementation studies without higher protein intake (148;168;227). In contrast, a recent CR study with very high calcium intake in all women resulted in a greater total body BMD loss in the higher protein group, but the study is limited by its short duration (12 weeks) and an absence of measuring specific bone sites (226). The current study is the first to examine the effect of 1 year of CR on bone and to demonstrate that dietary protein alone, with calcium and vitamin D at recommended intakes will attenuate bone loss during CR.

The increase in IGF-1 levels during the intervention in HP compared to the NP group may have mediated a greater preservation of bone in the HP group. There is a well known anabolic effect of IGF-1 on bone (163;344) protein and calorie deprivation lowers IGF-1 (160) and is associated with lower bone density (344). We observed a 20% increase in IGF-1 in the HP group, while it remained unchanged in the NP group. Many studies have evaluated the effect of total protein intake using animal (meat, milk) or plant foods rich in protein such as soy on the serum levels of IGF-1 (172;345-348). These studies suggest both animal and plant protein intakes contribute to increase in IGF-1. In the current study the women consumed a mixture of both animal and plant protein. The increase in IGF-1 levels in response to protein intake in some (172;345), but not all studies (346) also shows a positive effect of IGF-1 on bone turnover and density. In one such study, supplementation with three servings of milk per day increased IGF-I by 10% and decreased bone resorption in adult men and women (345). Similarly meat supplementation to up to 24% of total calories increased IGF-1 by 25% and also decreased bone turnover (347). In another trial, elderly malnourished patients that received a whey protein supplementation (20 g/d) for 6 months following hip fracture (172) had a 35 ug/L (62 %) increase in IGF-1 and attenuated bone loss compared to baseline. The greater increase in IGF-1 following protein supplementation in the Schurch study compared to the current study, is likely due to their older and malnourished population who started out with lower baseline IGF-1 levels. In addition, we observed a decrease in IGFBP-3 over time with weight loss in the NP group that is consistent with the decrease found with fasting (349), but no decrease in the HP group. The relationship between IGFBP-3 and BMD is a weakly positive one and serum levels are low in

osteoporosis (350) . Overall, the absence of a change in IGFBP-3 and rise in IGF-1 supports the notion that IGF-1 contributes to BMD preservation in HP group. However we did not find an increase in bone turnover markers in the HP group. We suggest that a modest 20% increase in serum IGF-1 in response to HP diet is too small to have a measurable effect on bone formation markers. This is in contrast to the dramatic increases with exogenous treatment of IGF-1 that increase both formation and resorption markers (351). In addition, these findings are consistent with findings in other dietary protein studies showing no rise in bone formation and a decrease in bone resorption due to higher protein intake (172;347). It is possible that the small decline in bone resorption markers can explain the attenuated bone loss. A trend for a higher estradiol, as in the current study, may be another mechanism besides IGF1 that is attenuating bone loss on a HP diet. In addition, greater calcium absorption with higher protein intake (173) may explain the decline in bone resorption markers.

Women in the HP group tended to increase serum estradiol over time more compared to the NP group. The slight decrease in estradiol in the NP group due to CR is consistent with previous findings during weight loss as observed in our laboratory and by others (151;292) . It is interesting to note that in a rodent model, a lower compared to higher protein diet is not only associated with lower IGF-1 levels but also estrogen deficiency, and may contribute to the loss of bone mineral and strength (344). To our knowledge, there has been no study to specifically examine the effect of a HP diet on serum levels of estradiol, and the absence of a decrease in estradiol supports the attenuated bone loss and resorption in the HP group. Other trials that have evaluated small physiological differences in estradiol in older individuals have showed that

differences as low as 3.7 pg/mL (13.6 pmol/L) (352;353) are associated with higher BMD. It is thus possible that while the ~7 pmol higher estradiol in the HP compared to NP group is a small difference, it may have acted in concert with other factors, such as higher IGF-1 to attenuate bone loss in the HP group.

Caloric restriction is associated with changes in several other hormones such as an increase in cortisol (168) and PTH in the absence of calcium supplementation, all of which may mediate the bone loss during this process. A rise in PTH due to CR may be due to the decrease in calcium absorption (151) and/or a reduced intake of calcium. Similar to previous findings in our laboratory and others (168;354) we observed a rise in 25OHD and decrease in PTH over a one year period of CR. It is possible that a loss of fat mass is associated with release of vitamin D from the adipose tissue (108) resulting in an increase in serum levels of 25OHD and thereby suppressing PTH levels.

Some limitations of this study include the following. There is a concern for artifacts associated with projection based axial DXA measurements due to excess fat tissue surrounding the bone in an obese individual and changes in the surrounding soft tissue due to weight reduction (89). However, the cross-sectional measurements of peripheral sites (i.e tibia, radius) with less fat thickness than at central sites, show similar bone changes. Also, we use a control group with similar weight loss to help address this issue. In addition, subjects in the HP group increased their protein intake to only 24% of the calories, although the goal was higher. However, we did achieve a 26 g/d difference in protein intake between groups that was a more attainable intake for older individuals, so it may have more practical implications. Also we did not examine other sex steroids that may have also been influenced by weight loss and affect bone. There are also several

strengths to this study. Careful monitoring of calcium and protein intake during the intervention included biweekly checks of compliance, and periodic assessment of biomarkers. This is also the first one-year randomized weight loss trial that evaluates protein intake on bone and controls for calcium intake between groups. In contrast, previous studies consisted of low calcium intake in the normal protein groups and 3-6 months of weight loss, (207;223;224;343) and some were followed longer by a weight stable period (223;224;343) In addition, ISCD vertebral exclusion criteria were applied, which can be even more important in an obese population due to their higher incidence of osteoarthritis. Furthermore, this study examines the bone response in a variety of approaches by including measurements of both areal and volumetric bone and geometry and examining potential endocrine regulators.

In summary, a higher protein diet that increases serum IGF-1 attenuates bone loss during CR at certain sites in postmenopausal women over a one year period. With a significant percentage of the older population being on weight loss diets, bone loss has become a major concern. A higher protein diet at 24% of total calories with recommended calcium and vitamin D intake preserves BMD and can be recommended to postmenopausal women during caloric restriction.

6.1.6 Acknowledgments

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Table 4: Body composition, bone mineral density and content over 12 months in the two treatment groups¹⁻²

	HP (n=26)		NP (n=21)		P value²		
					Diet	Time	Diet*Time
	Baseline	12 months	Baseline	12 months			
Age (yrs)	58.5 ± 4.1		57.4 ± 4.7				
Weight (kg)	88.5 ± 15.1	82.8 ± 15.4	82.7 ± 12.2	76.6 ± 11.7	0.151	<0.001	0.755
Fat mass (kg)	39.6 ± 9.5	35.4 ± 10.6	36.9 ± 8.4	32.4 ± 8.3	0.321	<0.001	0.834
Lean mass (kg)	45.2 ± 6.4	44.0 ± 5.8	41.7 ± 5.5	40.3 ± 4.6	0.034	<0.001	0.504
BMD (g/cm²)							
UD radius	0.37 ± 0.05	0.37 ± 0.05	0.37 ± 0.07	0.36 ± 0.06	0.837	0.0017	0.032
1/3 Radius	0.69 ± 0.06	0.68 ± 0.06	0.68 ± 0.08	0.67 ± 0.08	0.419	0.012	0.490
Lumbar spine ¹	1.24 ± 0.17	1.25 ± 0.18	1.13 ± 0.17	1.12 ± 0.17	0.063	0.193	0.028
Trochanter	0.82 ± 0.11	0.82 ± 0.12	0.75 ± 0.09	0.74 ± 0.10	0.032	0.287	0.512
Femoral Neck	0.94 ± 0.10	0.94 ± 0.11	0.89 ± 0.10	0.89 ± 0.10	0.085	0.887	0.884
Total Hip	1.02 ± 0.12	1.01 ± 0.12	0.94 ± 0.01	0.93 ± 0.10	0.020	0.004	0.050
Total Body	1.20 ± 0.09	1.19 ± 0.09	1.14 ± 0.10	1.12 ± 0.10	0.023	0.007	0.951
BMC (g)							
UD radius	1.31 ± 0.19	1.28 ± 0.19	1.28 ± 0.21	1.28 ± 0.20	0.846	0.246	0.119
1/3 Radius	1.67 ± 0.20	1.69 ± 0.23	1.61 ± 0.26	1.61 ± 0.26	0.289	0.553	0.591
Lumbar spine	54.0 ± 11.3	54.3 ± 12.1	46.5 ± 9.7	46.1 ± 9.6	0.047	0.009	0.469
Trochanter	10.1 ± 2.1	10.3 ± 2.1	9.0 ± 1.6	9.3 ± 1.9	0.047	0.354	0.739
Femoral Neck	5.0 ± 0.7	4.9 ± 0.7	4.4 ± 0.6	4.4 ± 0.5	0.003	0.042	0.706
Total Hip	32.1 ± 3.9	32.0 ± 4.0	29.1 ± 3.3	29 ± 3.5	0.007	0.661	0.930
Total Body	2523 ± 299	2520 ± 338	2352 ± 408	2340 ± 357	0.058	0.258	0.177

Values are Mean ± SD (all variables); UD, ultra distal radius.

¹ 16 subjects had their lumbar spine BMD scans reanalyzed according to ISCD exclusion criteria

² A two factor repeated measures ANOVA was performed with time (0, 6, 12 months) and Diet (HP or NP) as independent variables

Table 5: Trabecular and cortical vBMD, BMC and geometry over 12 months in the two treatment groups ^{1,2}

	HP (n=26)		NP (n=21)		P value ²		
					Diet	Time	Diet*Time
	Baseline	12 months	Baseline	12 months			
Total BMC (mg)	301.3 ± 40.5	304.8 ± 46.4	280.1 ± 36.1	276.2 ± 32.5	0.036	0.718	0.061
Total vBMD (mg/cm ³)	298.8 ± 33.3	296.9 ± 34.3	306.8 ± 45.7	295.8 ± 40.2	0.679	0.031	0.049
Trab BMC (mg)	104.0 ± 18.8	110.9 ± 26.6	97.1 ± 20.3	95.3 ± 16.8	0.083	0.581	0.043
Trab BMD(mg/cm ³)	228.6 ± 31.7	232.9 ± 33.8	237.6 ± 58.7	225.2 ± 32.4	0.831	0.207	0.041
Total area (mm ²)	1012.3 ± 117.0	1036.3 ± 161.9	926.4 ± 134.2	945.2 ± 128.6	0.021	0.026	0.700
Cort BMC (mg)	325.5 ± 31.6	325.6 ± 31.2	294.7 ± 41.2	291.9 ± 40.8	0.005	0.423	0.173
Cort vBMD(mg/cm ³)	1147.1 ± 27.3	1148.6 ± 24.0	1126.7 ± 42.6	1131.8 ± 43.8	0.014	0.093	0.697
Cort Area (mm ²)	284.0 ± 27.0	282.7 ± 27.5	261.1 ± 31.3	257.3 ± 29.5	0.008	0.076	0.237
Cort thk (mm)	5.2 ± 0.5	5.1 ± 0.5	4.7 ± 0.6	4.7 ± 0.6	0.011	0.169	0.475
Peri circ (mm)	71.5 ± 4.0	71.6 ± 3.6	70.3 ± 4.1	70.1 ± 3.9	0.228	0.663	0.419
Ip (mm ⁴)	24442 ± 5039	24212 ± 4910	22231 ± 4544	21925 ± 3992	0.110	0.127	0.929
SSI (mm ³)	1590 ± 525	1600 ± 239	1423 ± 253	1425 ± 239	0.025	0.774	0.412
Muscle Area (mm ²)	6894 ± 1093	6707 ± 1025	6523 ± 1125	6438 ± 1010	0.112	0.098	0.300
Fat Area (mm ²)	5218 ± 1439	4856 ± 1606	5663 ± 1750	4892 ± 1454	0.544	<0.001	0.034

¹ Mean ± SD; Trab, Trabecular; vBMD, Volumetric BMD; Cort, Cortical; Ip, Polar moment of inertia; SSI, Stress strain index;

² A two factor repeated measures ANOVA was performed with time (0, 6, 12 months) and Diet (HP or NP) as independent variables.

Table 6: Nutrient intake at baseline and over 12 months in the two treatment groups ¹⁻⁴

	HP (n=26)	Months 1-12 of intake ¹	NP (n=21)	Months 1-12 of intake	P Value ²		
					Diet	Time	Diet *Time
	Baseline		Baseline				
% Protein	18.4 ± 4.2	23.6 ± 4.6	16.7 ± 4.5	17.8 ± 2.8	<0.001	<0.001	0.021
Protein (g)	79.6 ± 20.2	86.3 ± 11.3	70.0 ± 24.3	60.1 ± 10.2	<0.001	0.955	0.045
Carb (g)	199.4 ± 62.7	153.1 ± 48.3	199.4 ± 65.6	157.1 ± 33.8	0.983	0.002	0.336
Fat (g)	74.3 ± 34.2	59.5 ± 13.7	68.9 ± 36.9	56.9 ± 21.7	0.755	0.013	0.937
Energy (Kcal)	1733 ± 505	1480 ± 270	1672 ± 577	1375 ± 283	0.765	<0.001	0.474
Calcium (mg) ⁴	1226 ± 286	1127 ± 240	1204 ± 174	1129 ± 164	0.538	0.281	0.980
Vit D (µg)	10.4 ± 0.7	10.6 ± 0.7	10.5 ± 0.9	10.4 ± 0.5	0.868	0.517	0.877
Mg (mg)	361 ± 131	352 ± 67	343 ± 112	348 ± 73	0.322	<0.001	0.640
Phos (mg)	1270 ± 432	1238 ± 238	1182 ± 313	1063 ± 218	0.009	0.766	0.225
Na (mg)	3190 ± 1129	2475 ± 437	2938 ± 859	2166 ± 298	0.026	<0.001	0.574
Vitamin K (µg)	126 ± 128	137 ± 91	88 ± 86	115 ± 50	0.139	0.142	0.374

¹ Mean ± SD; Nutrient intake is an average over 12 months (3 day food diaries from each month). Intake includes multivitamins (200 mg calcium, 10ug of vitamin D, 48 ug of Phosphorous, 100 mg magnesium, 10ug vitamin K), individualized calcium supplement and whey protein powder.

² A two-factor repeated measures ANOVA was performed with time (0, 6, 12 months) and diet (HP or NP) as independent variables.

³ Nutrient intake did not differ between the groups at baseline.

⁴ Prestabilisation intake of calcium was 802 ± 351 and 730 ± 272mg in HP and NP groups respectively

Table 7: Bone turnover markers at baseline, 6 months and after 12 months in the two treatment¹⁻²

							P value ²		
	HP (n=26)			NP (n=21)			Diet	Time	Diet *Time
Turnover markers	Baseline	6 months	12 months	Baseline	6 months	12 months			
Osteocalcin (ng/mL)	9.2 ± 1.7	9.5 ± 2.5	9.5 ± 2.5	10.0 ± 2.4	10.0 ± 2.4	10.0 ± 2.5	0.342	0.925	0.405
PINP (ug/L)	48.2 ± 16.3	44.7 ± 16.7	46.1 ± 16.4	56.6 ± 15.4	53.4 ± 16.1	56.6 ± 17.6	0.058	0.215	0.910
NTx(BCE)	12.5 ± 3.7	12.3 ± 3.7	12.1 ± 3.5	13.0 ± 6.4	14.6 ± 7.8	13.1 ± 4.8	0.268	0.571	0.319
PYD/C(nmol/mmol)	22.6 ± 8.9 ^a	24.8 ± 8.6 ^a	24.9 ± 9.5 ^a	24.9 ± 16.2 ^a	24.8 ± 11.0	28.3 ± 18.7 ^b	0.332	0.018	0.084
DPD/C (nmol/mmol)	10.5 ± 6.3 ^a	9.8 ± 4.4 ^a	8.9 ± 3.9 ^b	9.3 ± 5.5 ^a	9.6 ± 3.9 ^a	10.8 ± 6.2 ^a	0.906	0.399	0.049

¹ Mean ± SD; Mean ± SD; PINP: Propeptide of type 1 collagen, NTx; N-telopeptide of type 1 collagen, PYD/C; pyridinoline/creatinine, DPD/C; Deoxypyridinoline/creatinine.

²A two factor repeated measures ANOVA showing effect of diet (NP or HP), time (0, 6, 12 months) and diet*time interaction. Values with different superscript letters are significantly different.

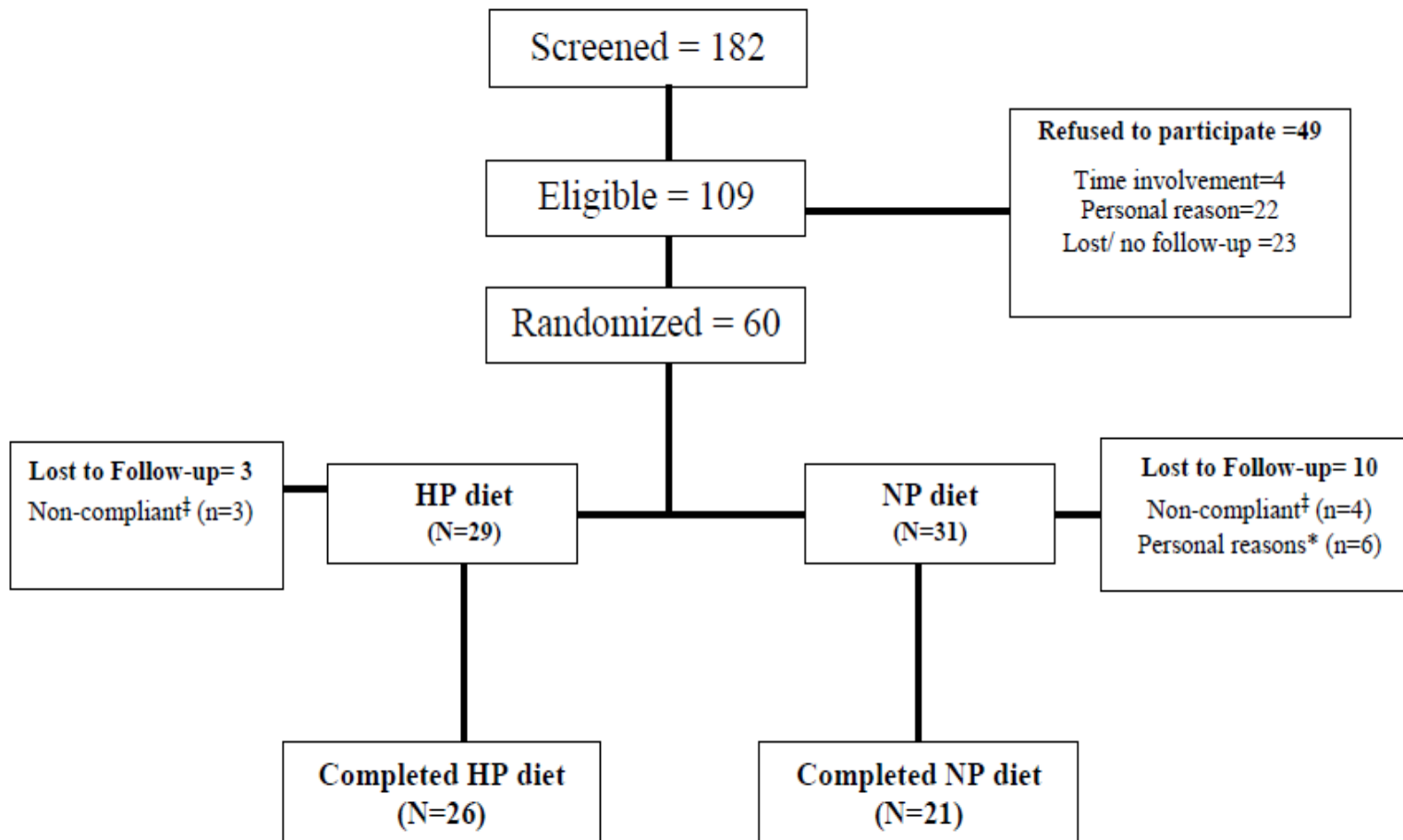


Figure 11: Flowchart of study participants for Aim 1

‡ Non compliant if weight loss <2.5%, * Dropped due to personal reasons-distance to travel, time involvement etc.

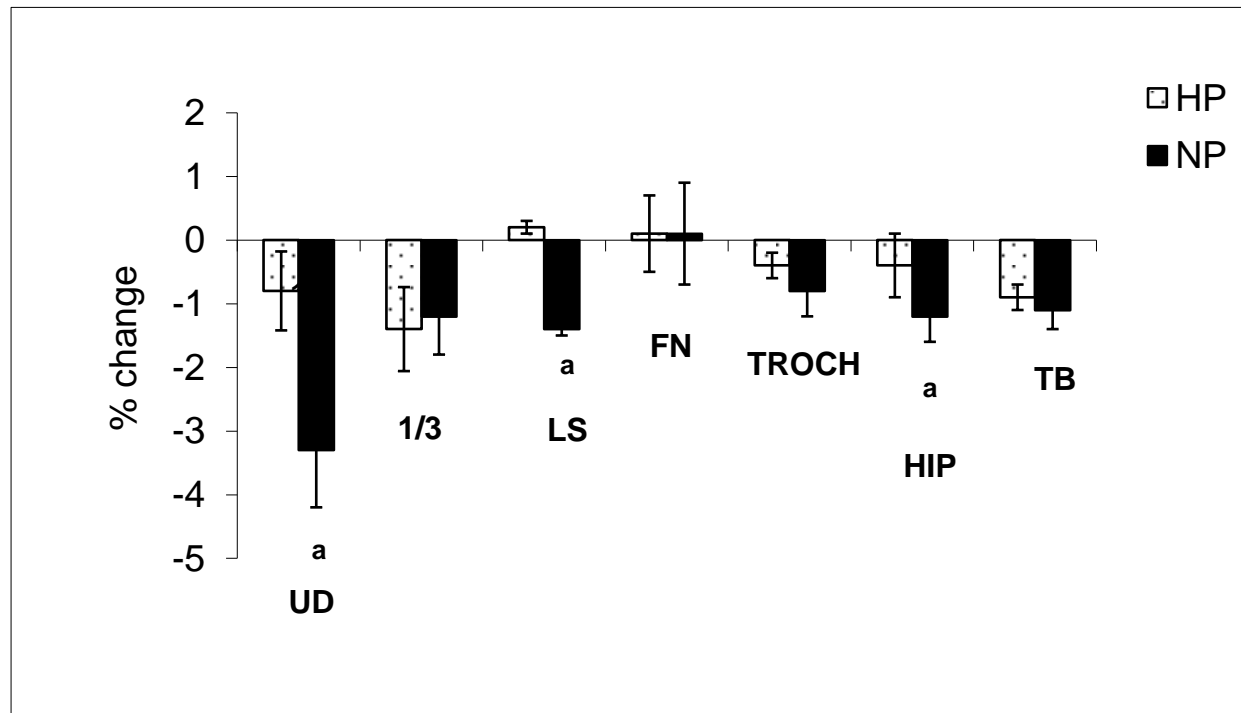


Figure 12: The percent change in bone mineral density (BMD) compared to baseline at all sites in the HP and NP groups at 1 year

Comparison of percent change between two groups was done by one-way ANOVA. ^a $p < 0.05$

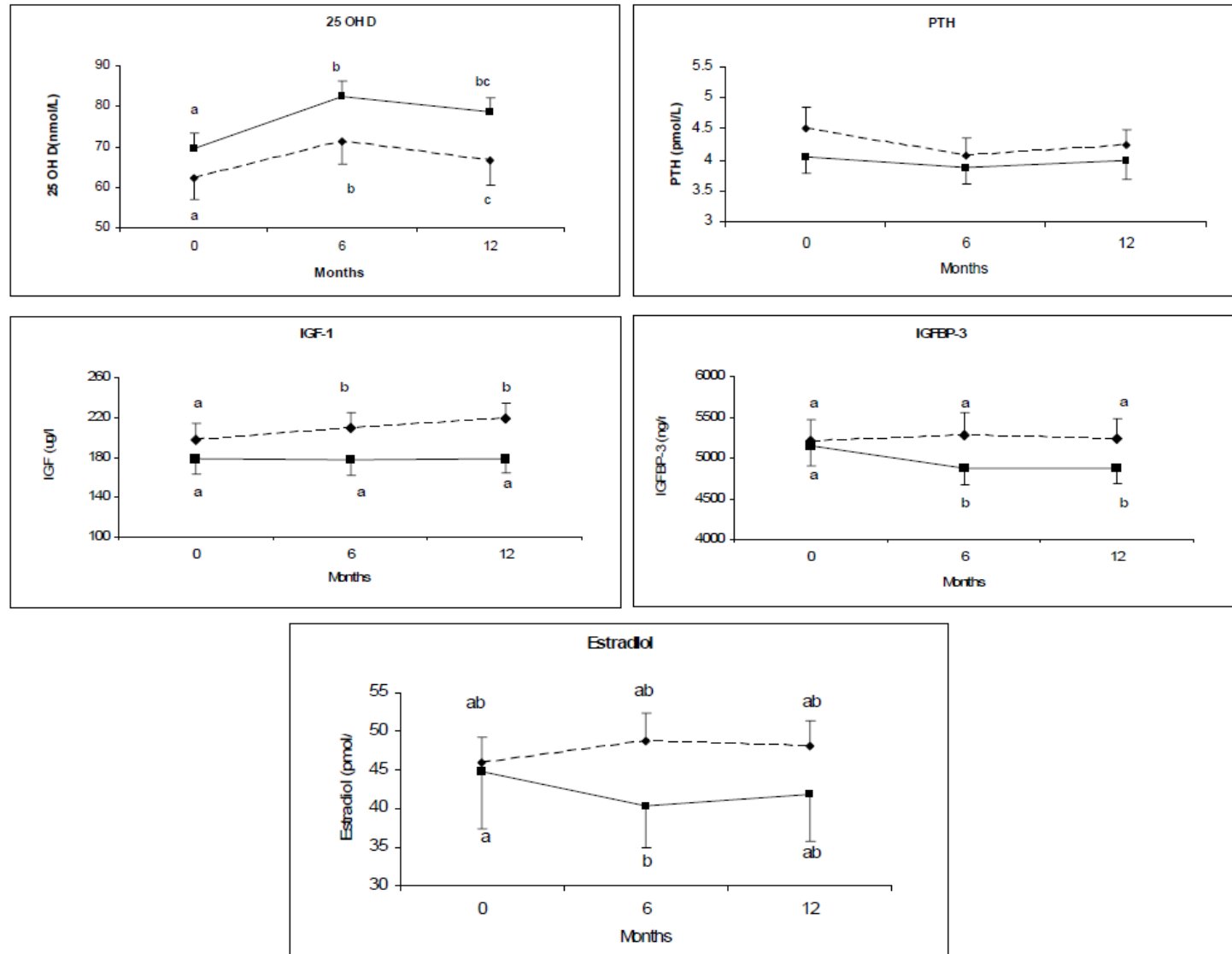


Figure 13: Changes in 25 hydroxy vitamin D (25 OH D), parathyroid hormone (PTH), estradiol, insulin like growth factor (IGF-1) and insulin like growth factor binding protein -3 (IGFBP-3) during the intervention in two groups.

The influence of diet over time on these hormones was analyzed by two-factor repeated measures ANOVA (Diet*Time: D*T). Values with different superscript letters are significantly different. *Diamond and dotted line represents HP (n=26); Square and solid line represent NP (n=21).*

6.2. Vitamin D supplementation does not attenuate caloric restriction induced reduction in calcium absorption in postmenopausal women.

6.2.1 Abstract

The classical effect of vitamin D on calcium absorption is mediated by calcitriol ($1,25(\text{OH})_2\text{D}_3$) that increases true fractional calcium absorption (TFCA) in the intestine.. Weight loss (WL) is associated with a decrease in TFCA and may be one mechanism inducing bone loss with weight reduction. Since vitamin D supplementation has shown to have a positive influence on TFCA, the goal in this study was to examine whether vitamin D supplementation during WL as compared to weight maintenance (WM) will attenuate the decrease in TFCA that is associated with weight reduction. Seventy seven women (58 ± 5.9 years of age; BMI of $30.2 \pm 3.6 \text{ kg/m}^2$; $25\text{OHD} = 24.8 \pm 5.7 \text{ ng/mL}$) were randomized into 4 groups to receive either vitamin D supplementation (D) or placebo (Pl). . The women completed the 6 week study [WL-D (n=19), WL- Pl (n=20), WM-D (n=19) and WM-D (n=18)] and received either 2500 IU/d [2100 IU D_3 + 400 IU from multivitamin (MV)] or 400 IU/d [Placebo + 400 IU from MV] of vitamin D_3 with 1.2g of calcium intake in all groups. Subjects in the WL group lost $-3.8 \pm 1.2 \%$ of initial body weight with no differences between treatment groups. The greatest increase in 25OHD was in WL-D group followed by WM-D group and PTH decreased with D treatment ($p < 0.05$). TFCA increased due to treatment with vitamin D ($p < 0.01$) and decreased with weight loss ($p < 0.01$) with the greatest increase in WM-D group ($3.7 \pm 5.2\%$), however vitamin D supplementation did not attenuate the CR induced decrease in TFCA. There was no change in serum levels of $1,25(\text{OH})_2\text{D}_3$ or estradiol with weight loss or with treatment. These results suggest that vitamin D supplementation for 6 weeks increases TFCA in overweight-obese women; however this does not attenuate the decrease in TFCA associated with caloric restriction.

6.2.2 Introduction

Caloric restriction influences calcium absorption by reducing its efficiency (151) , and may be one mechanism by which weight reduction induces bone loss. Several endocrine changes occurring during caloric restriction may be involved in the decrease in calcium absorption including a decrease in estrogen levels (151;260;292;355) and glucocorticoids (151;168;356). There is a classical effect of vitamin D on calcium absorption. The active form 1, 25 dihydroxy vitamin D ($1,25(\text{OH})_2\text{D}_3$) facilitates the intestinal absorption of calcium by mediating active calcium transport across the intestinal mucosal brush border across to the basolateral side of the mucosal cell (319). Vitamin D supplementation raises serum levels of 25OHD and some but not all studies have shown a positive effect of increasing serum 25OHD on true fractional calcium absorption (TFCA) (274;275;323)(276).

Since weight reduction is associated with a decrease in TFCA, the goal in this study was to determine whether 2500 IU/d vs 400 IU/d of vitamin D will attenuate the decline in TFCA during weight reduction compared to weight maintenance in postmenopausal women.

6.2.3 Subjects and Methods

Subjects:

Postmenopausal women who reported no menstruation for at least 2 years prior to the study were recruited. All participants were between ages of 50-70 yrs and free from any disease states or medications known to influence bone metabolism. Before initiation of any study procedures, subjects signed an informed consent approved by the Institutional Review Board at Rutgers University and an external advisory board. This trial was registered at clinical trials.gov (NCT00473031).

Screening for eligibility:

All participants had to pass a 3 step screening process that included a telephone, laboratory and physical screening. The telephone screening included questions about current diseases and medications, and participation in prior weight loss programs in the past few months. Participants had a comprehensive metabolic panel test and physical screening prior the study. They were screened for serum 25OHD levels and were excluded if levels were $> 30\text{ng/mL}$, high fasting blood glucose, abnormal creatinine clearance, blood urea nitrogen (BUN), uric acid, calcium and phosphorus etc. For patients who were taking thyroid medications, a stable dose for the past 2 or more years was required for inclusion, and none reported any change in medication when questioned monthly during the intervention. Subjects between BMI $25\text{-}40\text{ kg/m}^2$ were included. Participants also were screened at baseline for osteoporosis and excluded if they had a T score less than -2.5 at the hip or spine. Subjects who passed all the parts of screening were considered eligible for the study.

Weight and Height:

Weight and height was measured with a balance beam scale and stadiometer, respectively; (Detecto, Webb City, MO). At each morning visit, weight was recorded with minimal clothing.

Food records:

All participants were instructed to maintain food records for at least three days per week. Dietary intakes were analyzed using Food Works software (Long Valley, NJ, Version 10).

Supplements:

All subjects completed calcium food frequency questionnaire at baseline to estimate calcium supplementation. Subjects were stabilized to calcium intake of 1.2 g/d per day, beginning 1 month prior to the intervention and throughout during the study period. If calcium intake from food and multivitamin (NatureMade Multi 50+, Mission Hills, CA) was below 1.2 g/d, subjects were given a calcium supplement without added vitamin D (Citracal, Bayer, NJ) to meet 1.2 g/d. Vitamin D supplements and matching placebos were obtained from Bio Tech Pharmacal, AR, USA. The vitamin D supplements consisted of 5000 IU of vitamin D₃ and women were asked to consume 3 tablets (15,000 IU) on every Monday during the intervention. All participants were given a multivitamin that contained 400 IU/d of vitamin D₃ and were asked to take these supplements with the largest meal of the day. Subjects in the vitamin D group received 2500 IU/d of vitamin D (2100 IU/d from D supplement + 400 IU/d from multivitamin) and those in the placebo received 400 IU/d of vitamin D (placebo + 400 IU/d from multivitamin).

Blood and urine analysis:

Fasting blood and urine samples were collected at baseline, wk-3 and wk5. Serum 25-hydroxyvitamin D [25OHD], serum 1,25 dihydroxy vitamin D [1,25(OH)₂ D₃] (DiaSorin, Stillwater, MI, CV < 12.5%, CV < 16% respectively) and ultra sensitive Estradiol (E2) ¹²⁵I RIA (DSL, Webster, TX, CV < 8.9%) were measured by radioimmunoassay (RIA). Our laboratory also participates in vitamin D external quality assessment scheme (DEQAS) that monitors the performance of our 25OHD assay. Intact PTH was determined by immunoradioassay (Scantibodies, Santee, CA, CV<6.8%). Urinary calcium was measured in a 24 hour urine sample using the arsenazo reagent set.

True Fractional calcium absorption

True fractional calcium absorption was determined before and 6 weeks after the intervention using dual stable calcium isotope methods and inductively coupled plasma mass spectrometry, and TFCA calculated, as described previously (151;357). Briefly, on the day of the Ca absorption test, women were admitted between 7 -9 AM after an overnight fast. After blood collection (10 mL), subjects were asked to void and were then served a standard breakfast consisting of milk, toast, fruit and/orange juice. The total calcium load of this meal was 200 mg and included ⁴³Ca that had been mixed in half cup of skim milk (153 mg Ca),that had been equilibrated overnight (~12 hours). The milk was consumed in its entirety under supervision, and the cup was rinsed with deionized water three times. All of the rinsed water was also consumed by patients. Immediately after breakfast, an intravenous injection of ⁴²Ca was administered over ~3 minutes. Syringes containing the isotopes (that were mixed with the milk or infused intravenously) were

weighed before and after administration on a precision balance scale. The inductively coupled plasma mass spectrometry instrument precision and accuracy for this method is ~1% and the day-to-day coefficient of variation (CV) for 6 women measured twice was 1.2%. Complete urine collection was carefully monitored in each subject throughout the following 24 h period, and the ratio of each isotope to ^{44}Ca was determined in oxalate-precipitated aliquots of the pooled 24-h urine by using high-resolution, inductively coupled plasma mass spectrometry. TFCA was then estimated using equations reported previously (151).

Statistical Analysis:

The influence of treatment (Vitamin D or placebo) and weight (WL or WM) on TFCA, hormones, bone turnover markers was measured using two- factor factorial ANOVA. Tukey's post hoc analysis was performed when model F ratio was significant. One-way ANOVA was used to examine differences between the percent changes in TFCA from baseline between the two groups. Values are expressed as mean \pm SD. P values ≤ 0.05 were considered significant. A power analysis was performed with alpha set at 0.05, with the value of beta set at 0.90, using TFCA changes from a previous study (323) evaluating the effect of vitamin D supplementation on TFCA. This analysis indicated that 15 participants per group would be necessary to avoid a type-II error. Our goal was to include at least 5 additional participants per group to account for dropouts.

6.2.4 Results

Two hundred fourteen women were screened and 98 were found to be eligible and passed all parts of screening (Figure 14). Women were recruited for WM or WL separately and were then randomly assigned to receive 2500 IU/d vitamin D (400 IU/d from multivitamin + 2100 from vitamin D supplement) or placebo (400 IU/d from multivitamin + placebo). Eighty-three women were randomized into vitamin D (n=40) and placebo (n=43), of which eighty two women completed the study. Women were excluded from data analysis if their change in TFCA was abnormally high (i.e greater than 2.5 standard deviations from the mean). Seventy seven women were eligible for analysis in WL-D (n= 19), WM-D (n=19), WL-Pl (n=20), WM-Pl (n=19). Women were primarily Caucasians (n=64) with 9 African Americans and 2 Hispanics, 1 Asian and 1 from mixed ethnic background. The mean age of the women was 57.7 ± 5.9 years and BMI was 30.2 ± 3.6 kg/m² with no differences between the 4 groups at baseline. Fat, lean mass and BMD at femoral neck and total hip was also not different between the 4 groups (Table 8).

Changes in hormones during intervention

Baseline values for hormones and change during the intervention is presented in Table 9. At baseline there were no differences between the 4 groups for both hormones and urinary calcium excretion. The average weight loss in the WL-D group was -3.0 ± 1.2 kg and in the WL-Pl group was -3.3 ± 1.2 kg and was not different between the groups. 25OHD increased with 2500IU/d vitamin D supplementation with a 7.9 ± 5.8 ng/mL increase in the WL-D and 3.3 ± 4.0 ng/mL in the WM-D group ($p < 0.05$). PTH was influenced by treatment ($p < 0.02$) with greater decrease in the D supplemented groups compared to placebo, whereas estradiol and $1,25(\text{OH})_2\text{D}_3$ was not influenced by weight loss

or vitamin D supplementation. The change in 24 hour urinary calcium excretion was not different between the four groups.

Nutrient intake during the intervention

The intake of macro and micronutrients at baseline and intervention is presented in Table 10. Total caloric intake was on an average 200 kcal lower in the WL groups compared to baseline ($p < 0.05$). Carbohydrate intake also lower in the WL-D groups compared to baseline and fat intake was lower in the WL-Pl group compared to baseline ($p < 0.05$). Mean calcium intake from diet, multivitamin and calcium supplement in the 4 groups during the intervention was 1183 ± 217 mg and vitamin D intake was 410 ± 56 IU/d and was not different between the 4 groups. Other macro and micronutrient intake were not influenced due to intervention in any of the 4 groups.

Changes in TFCA during the intervention

The absolute change in TFCA from baseline during the intervention in the four groups is shown in Figure 15. TFCA increased due to treatment ($p = 0.001$) and decreased due to weight loss ($p = 0.004$), however there was no weight*treatment effect ($p = 0.159$).

There was a reduction in TFCA due to weight loss compared to WM ($p < 0.05$). In addition, TFCA increased by 3.7 ± 5.0 % in the weight maintenance vitamin D supplemented group and was significantly higher ($p < 0.01$) from other 3 groups. Vitamin D supplementation did not attenuate the decrease in TFCA that is associated with CR.

6.2.5 Discussion

Vitamin D has a classical effect on calcium absorption. In this 6 week study, postmenopausal women were supplemented with a higher (2500IU/d) vs. 400 IU/d dose of vitamin D to understand whether the higher dose will attenuate the decrease in calcium absorption that is associated with caloric restriction. As expected (151), we showed that caloric restriction reduced TFCA in this study. We also show that despite a 7.8 ng/mL increase in serum 25OHD with weight loss and 2500 IU/d Vitamin D supplementation, the decrease in TFCA associated with caloric restriction was not significantly attenuated. It is interesting that only 3.9 ng/mL rise in 25OHD significantly increased TFCA by 3.7% in the weight maintenance women. This disparity in the rise in 25OHD with the same dose of vitamin D intake in the WL and WM group suggests that release from adipose tissue is likely the explanation, yet the absence of a significant influence on TFCA suggests a smaller role of 25OHD specifically in regulating absorption.

Caloric restriction is associated with a decrease in TFCA (151). A reduced TFCA may be one mechanism by which weight reduction is associated with a loss of bone mass. Several factors contribute to a reduction in TFCA with caloric restriction. A decrease in estradiol with fat loss during weight reduction may be an important contributor to decreased TFCA, however the current study does not show a change in estradiol with CR, possibly due to shorter duration of the intervention that does not lead to enough loss of fat mass to cause a decrease in estradiol. We controlled for calcium and other micronutrients, so this should not been a factor in attenuating TFCA during CR. Dietary protein intake is known to positively affect TFCA, and because protein intake was reduced with CR and it

is possible that it plays a role. Vitamin D supplementation had a positive effect on calcium absorption in the current trial (treatment effect), however it was surprising that the effect was not significant for CR group. Although serum 25OHD levels rose even more in the CR than WM group, it is possible that it does not reflect the mechanism regulating TFCA under these conditions.

Several studies have shown both positive and no effect of D supplementation on TFCA. Serum 25OHD is a biological marker of vitamin D status; however the influence of serum 25OHD and vitamin D supplementation on calcium absorption has been controversial. In a study that examined the serum level of 25OHD in reference to calcium absorption using area under the curve analysis, showed that absorption was significantly lower when serum levels of 25OHD were 50nmol/L (20 ng/mL) as compared to 86nmol/L (34ng/mL) (323). In another study TFCA was examined after supplementation of 50,000 IU of vitamin D₃ for 15 days in vitamin D insufficient women. These postmenopausal women showed an increase in 25OHD of 42 ng/mL and a 3.5% increase in TFCA (274). In contrast, some recent reports suggest that serum 25OHD and higher vitamin D supplementation does not influence .Calcium absorption (259;275). Adolescent girls who were supplemented with 1000 IU/d of vitamin D₃ showed no increase in calcium absorption or skeletal retention of calcium over 3 weeks of supplementation (275). Similarly in 492 black and white healthy women (age range: 20–80 y) calcium absorption was measured using that single isotope method with ⁴⁵Ca. In these women, there was no relationship between serum 25OHD and calcium absorption efficiency (259). Out of the 3 RCT's that have evaluated to understand this relationship, 2

of them have shown positive results in adult postmenopausal women, while in children this relationship is not seen.

It is interesting that in the current study, a higher 25OHD was not associated with an increase in TFCA, however D supplementation with 2500 IU/d was associated with an increase in TFCA. The weight loss women supplemented with vitamin D showed a higher increase in serum 25OHD, however did not show an increase in TFCA. The higher 25OHD may be most likely a weight loss effect. Several factors associated with weight loss such as an increase in stress hormone cortisol may have attenuated the effect of vitamin D on increasing TFCA (151). Nevertheless, a small increase in 25OHD in the WM-D supplemented group was associated with an increase in TFCA. It is interesting that in the WL-D group an increase of 25OHD from 26ng/mL to 33 ng/mL was not associated with an increase in TFCA, however in the WM-D group, an increase in 25OHD from 25ng/mL to 28ng/mL was associated with an increase in TFCA. Thus, the absence of an effect on TFCA despite a higher 25OHD in the WL group, suggests that factors other than the level of serum 25OHD influence TFCA or it is possible that the 25OHD is counterbalanced by factors during weight loss that suppress a rise in TFCA. Thus the increase in 25OHD does not explain TFCA differences between the 4 groups, and mechanisms regulating the suppression in TFCA during caloric restriction cannot be explain by serum 25OHD.

Our previous study showed that serum PTH is the major predictor, explaining 22% of the variance, for calcium absorption during caloric restriction in women receiving 400 IU/d vitamin D with serum 25OHD levels of 28 ± 5.6 ng/mL. In the current study, we saw a decrease in PTH, but no change in $1,25(\text{OH})_2\text{D}_3$ with vitamin D supplementation or

weight loss. Thus PTH or $1,25(\text{OH})_2\text{D}_3$ does not explain the differences in TFCA with vitamin D supplementation. There was an increase in $1,25(\text{OH})_2\text{D}_3$ with 2500 IU/d in women maintaining weight at the end of the study compared to baseline (t test, $p < 0.05$), but this increase was not significantly higher compared to the other 3 groups. The ability of hormones to predict the change in TFCA was also analyzed in a multiple regression model, however findings suggest that there are no discernable predictors of TFCA including 25OHD, $1,25(\text{OH})_2\text{D}_3$, PTH or estradiol that can explain the change in TFCA with vitamin D supplementation in the entire set up women.

There are several strengths and limitations to this study. The dual isotope method to is considered the gold standard to estimate absorption and was employed in this study. Also, the double blind placebo controlled design is strength to this study. Compliance was monitored throughout the 6 weeks with weekly pill distribution and pill count to increase compliance. This is the first study to address the influence of vitamin D supplementation during CR. Our laboratory also participates in DEQAS (Vitamin D External Quality Assessment Scheme), an international quality assurance group that monitors the performance of the 25OHD assay in the laboratory. These studies were carried out in the spring time to avoid the influence of sun on serum 25OHD measures. Some limitations of this study include the potential generalizability of the data, since women who were enrolled in the study were primarily Caucasians and thus results may not apply to other ethnic groups. Secondly, women in our studies were younger postmenopausal women and it is possible that there is a differential response of vitamin D on TFCA in older women during CR due to declining renal function (conversion to calcitriol), or other factors. Finally, it is possible that a higher intake of vitamin D and a corresponding higher serum level may have

a different response on TFCA during moderate CR and because this was not a dose response study, this relationship could not be determined.

In summary, postmenopausal women who are undergoing short term caloric restriction decrease TFCA and supplementation with vitamin D (2500 IU/d) does not increase TFCA despite a 8 ng/mL rise in serum 25OHD. During weight maintenance however, an increase of 3.3ng/mL of 25OHD with 2500IU/d of vitamin D increases TFCA by 3.7% in these women. Future studies are needed to identify whether other factors and nutrients are regulating the TFCA decline during CR. In summary, short term vitamin D supplementation increases TFCA in postmenopausal women, but not during weight reduction.

6.2.6 Acknowledgments

We thank the laboratory and clinical staff for their invaluable technical and clinical assistance. We appreciate the commitment of the volunteers in this study. This work was supported by grants from the National Institutes of Health (RO1-AG12161) and a Busch Biomedical Award to SAS.

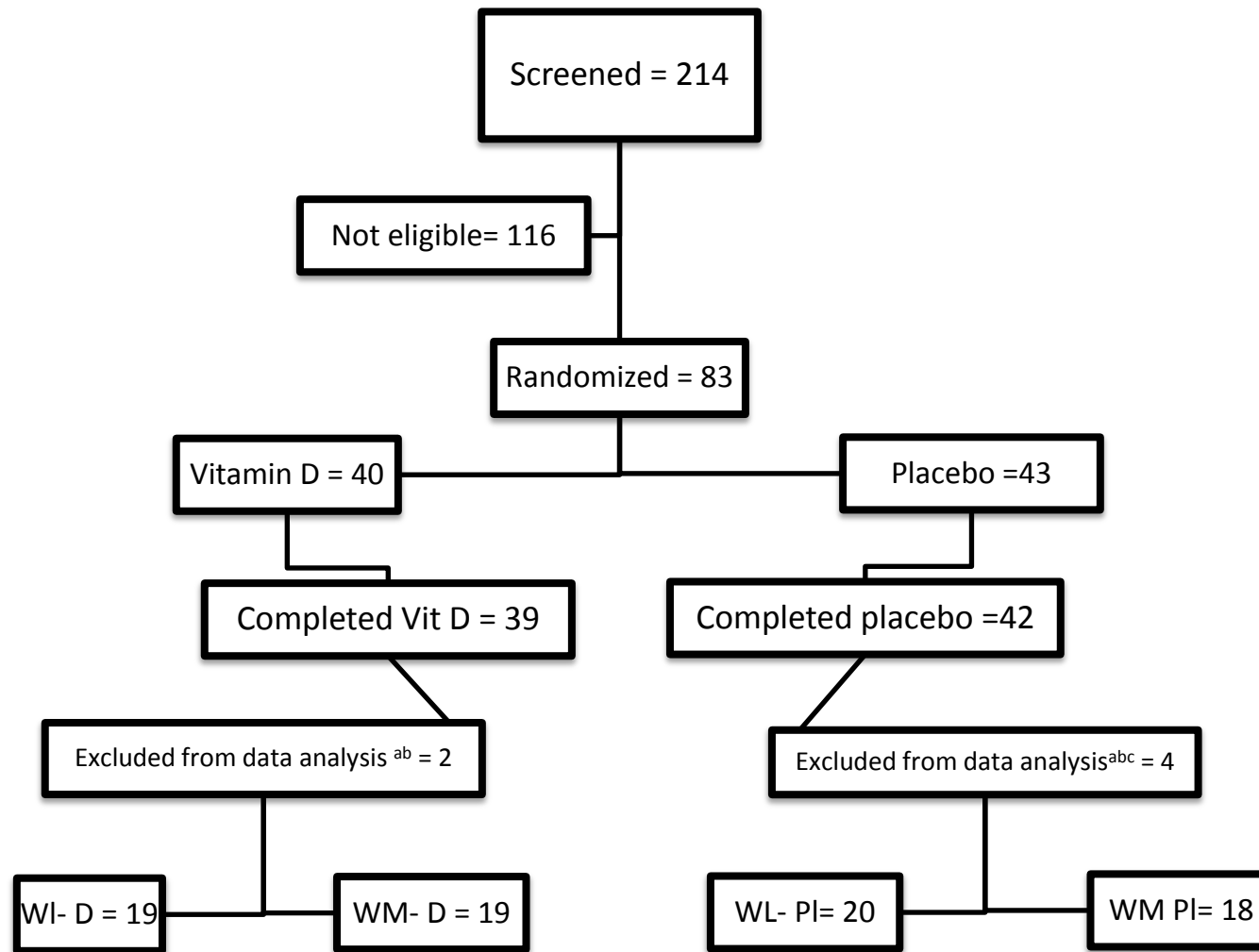


Figure 14: Flow diagram of study participants for Aim 2

^a Outliers for change in TFCA (N= 1 in D group and N=2 in pl group)

^b Urine spillage (N=1 in D group and N=1 in pl group), C = dropped from study at wk-4 due to personal reasons (N=1)

Table 8: Baseline characteristics of participants who completed the study¹⁻²

Variable	WL-D (N=19)	WM-D (N=19)	WL-PI (N= 20)	WM-PI (N= 18)	Vit D	Wt	Vit D*Wt
Age (Yrs)	58.1 ± 5.7	58.1 ± 6.9	56.2 ± 5.7	58.4 ± 5.3	0.597	0.424	0.424
Weight (Kg)	79.1 ± 12.8	79.2 ± 9.6	84.4 ± 14.7	74.2 ± 10.4	0.944	0.073	0.064
BMI (Kg/m ²)	30.1 ± 3.2	30.4 ± 3.8	31.2 ± 4.3	29.1 ± 2.9	0.922	0.289	0.130
Fat mass (Kg)	35.1 ± 8.2	35.3 ± 7.0	37.8 ± 8.6	31.7 ± 9.5	0.813	0.130	0.104
Lean mass (Kg)	40.5 ± 5.0	41.3 ± 4.8	41.8 ± 6.4	38.5 ± 4.7	0.549	0.279	0.087
Total Hip BMD (g/cm ²)	1.011 ± 0.120	0.962 ± 0.091	0.997 ± 0.135	0.922 ± 0.097	0.301	0.020	0.606
Total body BMD(g/cm ²)	1.176 ± 0.097	1.155 ± 0.077	1.169 ± 0.090	1.124 ± 0.078	0.336	0.101	0.530

¹A two way factorial ANOVA (Wt * Vit D) was used. Wt factor includes WL and WM groups. Vit D includes 2 levels of vit D intake (400 and 2500 IU/d)

²No posthoc analysis was done since F ratio was greater than 0.05 for all variables

Table 9: Baseline and changes in body weight, urinary calcium and hormones during the intervention ¹⁻²

Variable	WL-D (N= 19)		WM-D (N= 19)		WL-PI (N=20)		WM-PI (N= 18)		Vit D	Wt	Wt * Vit D
	Baseline	delta	Baseline	delta	Baseline	delta	Baseline	delta			
Weight (kg)		-3.0 ± 1.2		-0.1 ± 1.5		-3.3 ± 1.2		-0.1 ± 1.1	0.513	<0.01	0.600
25OHD (ng/mL)	25.8 ± 5.8	7.9 ± 5.8 ^a	24.7 ± 5.6	3.3 ± 4.0 ^b	24.7 ± 6.9	0.6 ± 4.9 ^b	25.2 ± 5.6	0.2 ± 3.1 ^b	<0.01	0.017	0.052
PTH (pg/mL)	39.2 ± 19.1	-3.4 ± 9.2	45.7 ± 28.0	- 5.5 ± 15.1	40.7 ± 14.6	1.1 ± 16.2	39.8 ± 12.7	4.7 ± 11.5	0.019	0.824	0.360
Estradiol (pg/mL)	12.9 ± 3.8	-1.1 ± 4.5	11.2 ± 3.9	-0.8 ± 3.7	11.3 ± 4.9	1.0 ± 2.5	13.7 ± 4.7	0.3 ± 5.2	0.101	0.807	0.588
1,25(OH) ₂ D ₃ (pg/mL)	52.9 ± 19.5	-0.6 ± 17.7	57.1 ± 19.3	5.1 ± 14.1	62.2 ± 16.0	-1.6 ± 13.9	53.9 ± 17.9	-2.3 ± 18.9	0.267	0.509	0.391
Ur calcium (mg/d)	172.4 ± 115.5	-37.6 ± 72.2	159.4 ± 73.2	-4.5 ± 49.1	155.0 ± 69.4	-7.4 ± 53.1	159.1 ± 98.9	-13.0 ± 46.8	0.699	0.478	0.123

¹ A two way factorial ANOVA (Wt * Vit D) was used. Wt factor includes WL and WM groups. Vit D includes 2 levels of vit D intake (400 and 2500 IU/d)

² Post-hoc analysis was performed when model F ratio was less than 0.05. Different superscripts indicate that values are significantly different.

Table 10: Dietary intake at baseline and during the intervention¹⁻²

Variable	WL-D		WM-D		WL-PI		WM-PI	
	Baseline	Intervention	Baseline	Intervention	Baseline	Intervention	Baseline	Intervention
Energy (Kcal)	1516 ± 602	1391 ± 669*	1332 ± 347	1328 ± 347	1611 ± 432	1354 ± 470*	1335 ± 379	1266 ± 310
Protein (g)	66.4 ± 29.8	65.3 ± 28.3	60.4 ± 16.7	62.4 ± 17.6	79.1 ± 21.5	63.8 ± 15.8	67.0 ± 22.5	59.7 ± 13.0
Fat (g)	61.9 ± 31.2	59.1 ± 37.5	44.1 ± 16.8	46.8 ± 15.9	67.2 ± 36.0	45.6 ± 26.9*	54.3 ± 25.0	45.8 ± 16.3
Carbohydrates(g)	180.4 ± 66.2	149.3 ± 62.7*	176.9 ± 57.8	164.7 ± 43.8	176.8 ± 51.9	176.0 ± 60.4	145.6 ± 51.8	159.5 ± 58.5
Calcium (mg) ²	1195± 98	1195± 104	1206± 258	1208 ± 187	1190 ± 161	1183 ± 217	1195 ± 84	1182 ± 73
Vit D (µg)	10.9 ±1.3	10.5 ± 0.8	10.9 ± 1.2	10.8 ± 0.9	10.6 ± 1.1	10.2 ± 1.3	11.0 ± 1.3	10.8 ± 1.6
Phosphorous (mg)	970 ± 420	973 ± 352	997 ± 325	963 ± 310	1173 ± 379	1053 ± 407	999 ± 336	854 ± 297
Sodium (mg)	2433 ± 1236	2054 ± 1242	2692 ± 868	2702± 949	2789±1198	2533± 1267	1938±814	2083±571
Vit K (µg)	113±1511	110±104	119±220	99±124	119±168	85±81	106±180	131±183

*P <0.05 compared to baseline in same group using t test

¹Factorial ANOVA with WL/WM and treatment showed no significant interaction or significant posthoc analysis for any nutrients

²All Subjects were stabilized for one month before baseline measurements to receive a total 1200 mg of calcium. If intake was below 1200 mg from diet and MV, a calcium supplement was provided. Include individualized calcium supplementation, 48 mg phosphorus, 10 µg vitamin D, 100 mg magnesium, 10 µg vitamin K from multi-vitamin/mineral. Does not include 62.5 µg of vitamin D supplement in the vit D group.

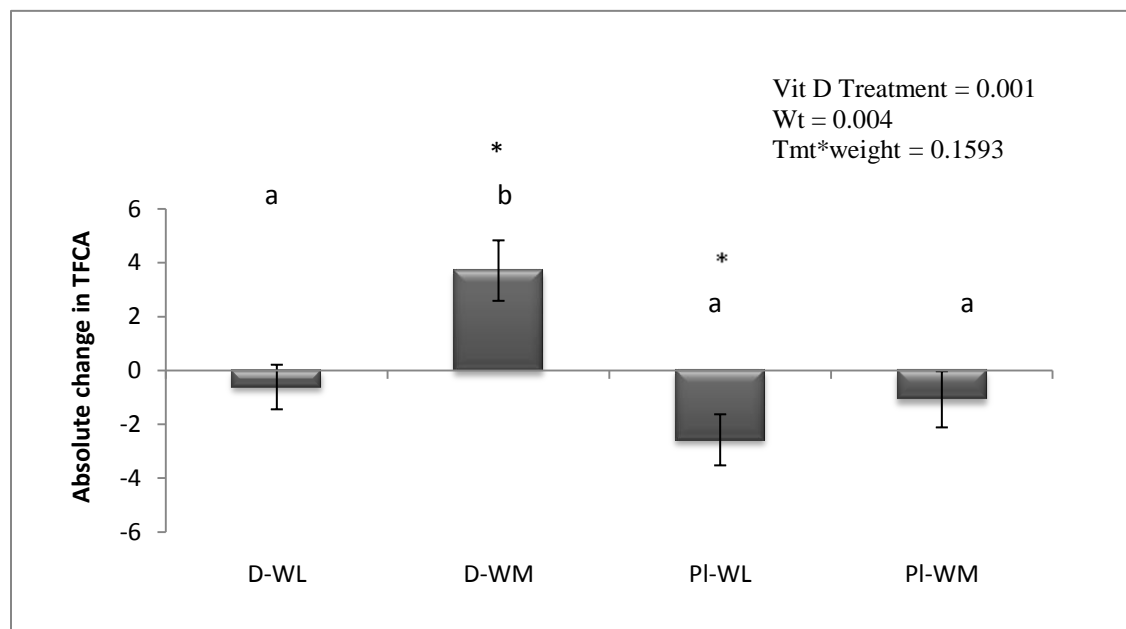


Figure 15: Changes in TFCA with 2500 vs. 400 IU/d in weight loss (WL) and weight maintenance (WM) groups¹⁻²

*Significantly different from baseline (t test)

¹ A two way factorial ANOVA (Wt * Vit D) was used. Wt factor includes WL and WM groups. Vit D includes 2 levels of vit D intake (400 and 2500 IU/d)

² Post-hoc analysis was performed when model F ratio was less than 0.05. Different superscripts indicate that values are significantly different.

6.3: Bone quality and role of Parathyroid hormone in obesity

6.3.1 Obesity alters cortical and trabecular bone density and geometry in women

*Sukumar D, Schluskel Y, Riedt CS, Gordon C, Stahl T, Shapses SA
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6.3.1.1 Abstract

The influence of adult obesity on the trabecular and cortical bone, geometry and strength has not been fully addressed. The goal in this study was to determine the relationship between body mass index (BMI) and cortical and trabecular bone mass and geometry, over a wide range of body weights. We examined 211 women (25-71 years; BMI 18-57 kg/m²) who were classified into 3 categories of BMI (kg/m²) including normal-weight (BMI<25), overweight and obese-class I (BMI 25-35) and obese-class II-III (BMI>35), and also by menopausal status. Volumetric bone mineral density (vBMD, mg/cm³), trabecular and cortical components, as well as geometric characteristics at the 4, 38 and 66% from the distal tibia were measured by peripheral quantitative computed tomography, and serum was analyzed for parathyroid hormone (PTH) and 25-hydroxy-vitamin D (25OHD). Higher BMI was associated with greater values of trabecular bone and cortical BMC and area and PTH ($r > 0.39$, $p < 0.001$), but lower cortical vBMD and 25OHD ($r > -0.27$, $p < 0.001$). When controlling for lower leg muscle area, fat area was inversely associated with cortical vBMD ($r = -0.16$, $p < 0.05$). Premenopausal obese women with both higher BMI and PTH had lower cortical vBMD ($r < -0.40$, $p < 0.001$). While age is a predictor for most bone variables, fat mass explains more variance for vBMD, and lean mass and 25OHD explain greater variance in geometric and strength indices ($p < 0.05$). Severe obesity (BMI>35) increases trabecular vBMD and in the presence of a higher PTH is associated with a lower cortical vBMD without compromising bone geometry and strength. Whether or not a lower cortical vBMD in obesity influences fracture risk over time needs to be further explored.

6.3.1.2 Introduction

Obesity is associated with a higher bone mass (13) but more recently it has been suggested that bone mineral density (BMD) is proportionally lower than would be expected in obesity based on body size, and that fracture risk may be increased despite normal BMD (71;358-360). The influence of obesity on trabecular and cortical bone has not been examined and could explain whether it is influenced with excess body weight.

Although BMD is the most common clinical measurement to diagnose osteoporosis, there is a growing awareness that a reduction in BMD is not the sole pathology underlying fracture risk, nor does a high BMD completely protect an individual from fractures. Risk factors for fracture are also influenced by bone quality that has been defined as a composite of bone structure, composition, microarchitecture and microdamage that contribute to bone strength independently of BMD (73-75). Indeed, the FRAX model addresses clinical risk factors along with BMD to better predict fracture risk (361). In addition, BMI has been used as a surrogate for BMD in instances where BMD cannot be measured, although it may have a limited ability to predict fractures at high body weights (67).

Peripheral quantitative computed tomography (pQCT) allows for separate yet simultaneous assessment of the trabecular and cortical compartments of the bone. It includes measurement of volumetric BMD (vBMD), bone geometry, bone mineral content (BMC) and bone strength indices, and is considered a useful method to assess bone quality and strength (92). It has also been reported that bone variables measured by pQCT such as trabecular and cortical vBMD may be good predictors of fracture risk and

in certain cases better than DXA (362). The sex and age effects on trabecular and cortical bone have been well established using pQCT (363-365), however, their relationship with BMI or adiposity has not been addressed in an adult population.

The positive influence of body weight on bone is primarily attributed to a greater lean mass but not always in older populations (366-368). In addition, there is an altered hormonal milieu associated with obesity, including higher serum concentrations of parathyroid hormone (PTH) and lower circulating 25-hydroxy-vitamin D (25OHD) (83;108). Serum PTH has a negative effect on cortical bone, while 25OHD is positively associated with trabecular bone, but this has not been studied in obese women. (85;86;369). In addition, the influence of both fat and muscle mass on the trabecular and cortical relationship in obesity has been examined in young men and children (81;82), but to our knowledge no report to date has examined this in adults. By simultaneously examining hormones and muscle and fat mass separately in pre- and post-menopausal women, this study clarifies factors that may be influencing trabecular and cortical bone due to excess body weight, especially in the higher range of body weight where geometry may be altered and may increase fracture (71;358-360).

6.3.1.3 Subjects and methods

Subjects

Two hundred and eleven women aged 25-71 years were recruited by advertising in local newspapers and email list-serves. Some of these women later took part in other intervention trials in the laboratory. Women were excluded if they were osteoporotic, (defined as a T-score < -2.5 at the hip and spine), taking medications known to influence bone or mineral metabolism in the past year, or had evidence of metabolic bone disease, thyroid disorders, diabetes, immune disease, heart attack or stroke in the past 6 months, kidney stones, diabetes, active cancers or cancer therapy within the past 12 months or were pregnant or lactating within the past year or used hormone replacement therapy. Premenopausal women who were menstruating monthly were included, and postmenopausal women were required to be at least 2 years since their last menstruation. This study was approved by the Rutgers University Institutional Review Board and all participants signed an informed consent form.

Methods

Weight and height were measured with a balance beam scale and stadiometer, respectively; (Detecto, Webb City, MO). Dual-energy x-ray absorptiometry (DXA) (Lunar Prodigy Advanced; GE- Lunar, Madison, WI; CV: $< 1\%$ for all sites) scans were performed by using enCORE 2004 software (version 8.10.027; GE Lunar) was used to assess, total hip, lumbar spine, femoral neck and total body BMD and BMC, as well as total fat and lean mass. Calcium and vitamin D intake was estimated using 3 day food records and analyzed using the Food Works Software, version 10.1 (Long Valley, NJ).

Physical activity level (PAL) was assessed using general practice physical activity questionnaire. Women were advised to consume a multi-vitamin/mineral tablet beginning, approximately 4-6 weeks prior baseline measurements to equal a total intake of 1000-1200 mg Ca/d (diet and supplement) . This uniform intake of calcium avoids variability in serum PTH levels that occurs with a wide range of dietary calcium intake.

pQCT

Volumetric BMD, BMC, and geometric and bone strength properties were measured using pQCT (Stratec XCT 3000, Orthometrix). Sectional images were standardized at specific sites (4%, 38% and 66%) using distal tibia as the anatomical marker and analyzed for density, content, geometry and strength indices and we report vBMD, BMC, geometry, biomechanical property, muscle and fat area. The scans were acquired at 0.5mm voxel and a slice thickness of 2.4mm. A scout view was used to determine the positioning of the cross-sectional measurements from the tibia and was set by the integrated software (STRATEC XCT-3000, version 5.4) for trabecular (Tb), cortical (Ct) and muscle-fat measurements respectively. Processing of the images and calculation of the various bone indices were performed using integrated software. At the 4% site, total and Tb vBMD (mg/mm^3), Tb BMC (mg) and total bone cross sectional area (mm^2) were calculated with the use of contour mode 2 and peel mode 2 at a threshold of $280\text{mg}/\text{cm}^3$. Ct vBMD, area, BMC, thickness, periosteal circumference (mm) and endosteal circumference (mm) were assessed in the 38% site with the use of cortical mode 1 and the threshold of $710\text{ mg}/\text{cm}^3$. The polar strength-strain index (mm^3) and moment of inertia (mm^4) were also calculated at the 38% site with cort mode 1 and a threshold of $280\text{ mg}/\text{cm}^3$. At the 66% site fat area, muscle area and the ratio of fat/muscle area were

calculated using cort mode 1 and peel mode 1. We also performed an in-vivo precision analysis using 18 subjects. The coefficient of variation (CV) between the 2 measurements was less than 1.7% for Tb and Ct vBMD, BMC, area and geometry.

Serum analysis

Blood samples were collected after an overnight fast and serum was extracted using centrifugation and stored at -80 degree Celsius until further analysis. Concentrations of PTH and 25OHD were analyzed by radioimmunoassay (RIA) in batch analysis, using both internal and external standards. The CV was <6.8% for PTH (Scantibodies laboratory, CA) and <12.5% for 25OHD (Diasorin MN; DSL corp).

Statistical Analysis

Women were separated into 3 categories based on their BMI (kg/m^2): Normal-weight (BMI<25), overweight and obese class I (BMI 25-35) and obese class II and III (BMI >35). Women were also grouped according to menopausal status since estrogen has an independent effect on trabecular and cortical bone. Two-way ANOVA was used to analyze the differences in bone variables across BMI categories and menopausal status. Tukey's post-hoc analysis was performed when the model F ratio was significant ($p<0.05$). Analysis was controlled for physical activity level, nutrient intake and lean mass in linear models. Pearson correlation coefficients were used to assess the relationships between bone variables, to assess the independent influence of adiposity. Multiple regression analysis was used to assess the relative influence of independent variables (age, fat and lean mass, PTH and 25OHD) on bone. Analyses were conducted using the SAS statistical package (SAS Institute, Cary, NC; version 9.1.3). P values < 0.05 were considered significant. Data are presented as means \pm SD.

6.3.1.4 Results

The baseline characteristics of the 211 women are presented in Table 11. Study participants were primarily Caucasian (183 out of 211) with 90% and 86% in the pre- and postmenopausal population, respectively. There were 7% and 11% African Americans in the pre- and postmenopausal groups respectively, with 3% Asian and Hispanic in the remaining population. Participants were between 25-71 years of age, with no age differences between the various weight categories within the same menopausal status. The percent body fat was higher ($p<0.0001$) in the more obese women, and was higher ($p<0.001$) in the post compared to pre menopausal women with BMI between 25-35 kg/m^2 (Table 11). Calcium (Ca) intake was not significantly different in the pre- (1379 ± 521 mg) compared to postmenopausal women (1148 ± 579 mg). However, postmenopausal women with BMI >35 had a lower Ca intake ($p<0.02$) compared to normal weight women. There were small differences in total vitamin D intake between post- (7.6 ± 4.8 $\mu\text{g/d}$) compared to premenopausal (9.2 ± 4.1 $\mu\text{g/d}$) women ($p=0.019$) and was lowest in the heavier postmenopausal women (Table 11). Serum PTH levels were higher in women with BMI >35 compared to leaner women and were highest in this group of premenopausal ($p<0.0001$). Serum levels of 25OHD were lower ($p<0.0001$) in the heaviest (BMI >35) compared to normal-weight women. Areal BMD (g/m^2) at most sites was significantly lower in the post- compared to the premenopausal women ($p<0.01$). The heavier women had significantly higher BMD ($p<0.05$) at the hip, but not other sites compared to normal weight women. Also, among postmenopausal women, femoral neck BMD was also higher in the heavier (BMI > 25) compared to normal weight (BMI < 25) women ($p<0.01$).

Relationship with Body mass index

The relationship between BMI and bone parameters was examined in the entire group of women by correlation analysis (Figure 16). A higher BMI was positively associated ($r > 0.35$, $p < 0.001$) with Tb vBMD and Tb BMC, Ct BMC, area, thickness, periosteal and endosteal circumference and Stress Strain Index (SSI). However, a significant inverse association ($r = -0.27$, $p < 0.001$) was found between BMI and Ct vBMD in all women. In addition, BMI was positively associated with serum PTH ($r = 0.48$) and negatively associated with 25OHD ($r = -0.45$) (Figure 16). Interestingly, the negative association between BMI and Ct vBMD was significant only in the pre- ($r = 0.54$, $P < 0.0001$) and not in the postmenopausal ($r = 0.14$, $p = 0.1$) women (Figure 2). Also, the negative association between Ct vBMD and PTH ($r = -0.4$, $p < 0.001$) was only observed in the premenopausal women (Figure 17), who had a higher BMI ($p < 0.05$) and correspondingly higher PTH ($p < 0.05$) as compared to the postmenopausal women.

Adjustment for potential confounders

We also analyzed this relationship separately in pre and postmenopausal women with BMI and menopausal status as independent variables and controlling for lean mass, physical activity level and nutrient intake such as carbohydrates, protein, fat, calcium and sodium (Table 12). A significant BMI* menopause interaction was observed for total BMC, trab BMC and periosteal circumference ($p < 0.05$). Similar to correlation analysis, there was a significant effect of BMI on total and trab vBMD, BMC and cort area and cort vBMD. Similarly there was a significant effect of menopause on most trab and cort variables except circumference. When analyzing the pre and postmenopausal women

separately, the post hoc analysis showed that the heavier pre and postmenopausal women mostly had higher trab vBMD and BMC compared to leaner women. On the other hand Ct vBMD, BMC, geometry and strength indices were not significantly higher in the pre and postmenopausal heavier women. In addition Ct vBMD was lower in the heavier premenopausal women compared to normal weight women ($p < 0.05$).

Muscle and fat area, and bone

Partial correlation analysis shows a positive relationship ($r > 0.2$, $p < 0.05$) between tibia muscle area and trabecular and cortical bone variables, except Ct vBMD. Similarly, a positive association was observed for most trabecular and cortical variables and fat area. However, when controlling for muscle area, the positive association of all variables with fat area was no longer observed ($r < 0.1$, $p = \text{NS}$), but the negative association with Ct vBMD still remained ($r = -0.2$, $p < 0.05$) (Table 13).

Predictors of trabecular and cortical bone

In order to identify how BMI influences trabecular and cortical bone parameters, we examined whether whole body fat or lean tissue was more important in predicting the relationship using multiple regression analyses (Table 14). Age, body composition (total fat or lean mass), serum PTH and 25OHD served as explanatory variables for each of the dependent variables. As expected, age was a primary predictor of Ct vBMD (Table 4). Fat mass explained 9% of the negative and positive influence on Ct and Tb vBMD respectively, whereas lean mass only explained about half as much (~5%). In contrast, lean mass explained 17% of the geometric and 33% of the strength indices, whereas fat mass only explained about a third of the variance at 6 and 13%, respectively (Table 14).

Not surprisingly, when BMI was in the model, it also was a significant predictor ($p < 0.001$) of Tb and Ct vBMD, Ct thickness and SSI (8-16%). When pre- and postmenopausal women were analyzed separately without age in the model, fat and lean tissue mass explained greater variance for all bone variables in the premenopausal women ($\leq 62\%$) than in postmenopausal women ($< 35\%$). We also examined calcium and vitamin D intake as a covariate in the model and found that it was not a significant predictor, explaining less than 0.05% of the bone variables.

6.3.1.5 Discussion

While the influence of age, PTH and/or menopausal status on trabecular and cortical bone and geometric properties in normal weight and overweight population has been reported (85;86;332;363;370;371) the aim of this study was to determine how obesity influences these parameters in women with a wide range of body weights (BMI 18-57 kg/m²). Our results indicate that obesity is associated with higher trabecular and cortical bone parameters, except Ct vBMD. While age is a predictor for most bone variables, fat mass explains more variance for vBMD, and lean mass and 25OHD explain greater variance in geometric and strength indices. These findings may be important in explaining the apparent discrepancy in studies examining the effect of fat mass on total body BMD, where some show a positive association (13;366;367) and other reports show an inverse or less important role (16;372).

Interestingly, there were differences in trabecular and cortical bone that were detected using pQCT that were not observed with areal BMD analysis. For example, DXA analysis showed no effect of menopause on BMD and BMC in the obese population, whereas Tb and Ct vBMD and BMC differed between the obese pre- and postmenopausal women. Although a higher BMI is associated with a greater BMD, it is interesting to note that the early protective effect of a higher BMI on maintenance of Tb and Ct BMC may be lost with aging in heavier women.

Trabecular bone is influenced by several factors including BMI, age, estradiol, and physical activity (363;370;373). Consistent with previous reports (363;373) lower estradiol and/or aging is associated with lower trabecular bone, as observed in the post-

compared to premenopausal women in this study. Our results also show that trabecular bone is positively influenced by body weight and is not associated with serum PTH and 25OHD. Cortical bone is similarly influenced by BMI, age, estradiol and also by PTH and 25OHD (332;369;371;373). A decrease in cortical BMD begins in mid-life and is continues after menopause (332;371;373). The lower Ct vBMD seen in our post- compared to the premenopausal women supports this finding. Our findings also suggest that Ct vBMD is negatively influenced by BMI, but the major contribution of this effect comes from adipose rather than lean tissue, as shown with multiple regression. The more marked negative relationship in the pre- compared to postmenopausal women is probably due to higher PTH and body weight in the premenopausal group. A previous study showed that Ct vBMD was lower in normal (BMI<25) compared to overweight postmenopausal women (374) at the radius, but similar at the tibia. We also found no difference for Ct BMD between these 2 lower weight categories (BMI < 25 compared to BMI 25-35). It appears that BMI may have a differential effect on different bone sites, as has been found in children (375), and the lower Ct density with higher BMI is only present in the extremely obese women with BMI > 35. In addition, because the effect of high BMI on Ct BMD was more pronounced in the obese premenopausal women, it is possible that cortical bone does not decline as dramatically as in leaner women with aging. However a low cortical bone density over a longer duration may compromise bone quality and explain recent findings of increased fracture risk at peripheral sites (71;358-360), possibly due to less soft tissue padding as compared to axial sites. In addition our results also suggest that BMI does not have an additional positive influence on strength

indices which may further help explain increased fracture risk at certain sites in this population.

Adiposity is associated with increased PTH levels, possibly due to lower circulating concentrations of 25OHD attributed to increased sequestration in the adipose tissue (108). Our data also show a higher PTH and a lower 25OHD in the heavier compared to leaner women. Patients with primary hyperparathyroidism have a compromised Ct vBMD and a normal Tb vBMD (85). The catabolic effect of excess PTH on cortical bone is typically seen in hyperparathyroid patients, and our results show that these extend to obese populations as well. The absence of a relationship between PTH and Ct vBMD in the postmenopausal women may be due to the more dramatic effects of estrogen depletion in early postmenopausal years, which independently has a marked negative effect on cortical bone (376). Alternatively, it is possible that even a slightly higher estradiol or other hormones associated with excess adiposity contribute to maintenance of cortical bone. A lower Ct vBMD due to high BMI, as found in the obese in this study may be due to factors other than PTH. For example, obesity is associated with higher inflammatory cytokines and lower physical activity and sleep (371), all of which may negatively influence cortical bone.

Consistent with reports that fat mass has a negative influence on BMD (17;69) one short-term weight reduction study found a moderate increase in Ct vBMD with fat loss in obese premenopausal women. Although a higher body weight is associated with higher trabecular bone, it provides no additional benefit to Ct vBMD in overweight children (81;93;375). In late adolescence, a higher body fat has been shown to be negatively correlated with Ct BMD in the tibia and radius (81), and is consistent with our

new findings in adults. Although a lower Ct BMD is typically associated with fracture risk, in the obese population, this higher risk is possibly compensated by increased Ct area, BMC, thickness and periosteal circumference in the obese individual, similar to that observed due to aging, to increase bone size and strength (93;377). In addition, obesity is also associated with higher trabecular bone which may further offset fracture risk. Nevertheless, as in aging, one would expect that this compensation to be limited and result in a higher risk of fracture, in the obese (71;359). In addition, our study shows that greater adiposity per se in the obese does not have a positive influence on bone. Specifically the partial correlation analysis, corrected for muscle area, shows that there is indeed a negative relationship of fat mass with Ct bone and no association with strength indices. Taken together, these data help to explain why a BMD in the normal range in obesity is associated with an increased peripheral fracture risk in women (71;358-360), but it is interesting that this is not observed in men (359).

There are some limitations of this study including the cross sectional design which does not address a cause/effect relationship. Also the results are not generalizable since we report on a single bone site, the weight bearing tibia. However, the influence of excess weight in children at different sites (tibia and radius) have been found to be similar (81), yet one study showed the negative effect of higher weight may be more exaggerated at the radius(93). BMD measurement artifacts are an ongoing concern in the obese population due to excess fat tissue (89;378). However, previous studies with this concern have examined axial sites with a greater fat thickness compared to the peripheral site used in the current study. Also, true volumetric BMD (mg/cm^3) that uses fat tissue at

a density of zero should also eliminate the concern about artifacts due to excess fat tissue, as occurs with areal BMD.

These findings suggest that obesity has a positive and negative effect on Tb and Ct vBMD, respectively, and serum PTH and 25OHD at least partially explain these findings. The mechanisms that mediate the long term effects of a higher PTH and lower 25OHD associated with obesity on trabecular and cortical bone need to be further explored in longitudinal studies. The role of other hormones and adipokines that are altered in obesity and their specific effect on trabecular and cortical bone also need to be addressed. In summary, this study provides novel insight into the trabecular-cortical bone relationships altered by BMI and menopause, and should aid in our understanding of how excess adiposity influences fracture in obesity.

6.3.1.6 Acknowledgments

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Table 11: Characteristics of 211 women by menopausal status and BMI category

BMI Category	Premenopausal women (N=73)			Postmenopausal women (N=138)			BMI	Menop	BMI* menop
	BMI<25	BMI 25-35	BMI>35	BMI<25	BMI 25-35	BMI>35			
Age (years)	44 ± 7 ^a	40 ± 8 ^a	40 ± 9 ^a	58 ± 5 ^b	58 ± 5 ^b	59 ± 6 ^b	0.240	<0.001	0.059
Weight (kg)	59.1 ± 6.4 ^a	75.4 ± 5.7 ^b	129.5 ± 25.7 ^c	57.1 ± 6.5 ^a	79.5 ± 9.4 ^b	109.4 ± 16.6 ^d	<0.001	0.002	<0.001
BMI (kg/m ²)	22.1 ± 1.7 ^a	28.5 ± 2.1 ^b	45.6 ± 6.6 ^c	22.5 ± 2.3 ^a	30.1 ± 2.6 ^d	40.9 ± 4.9 ^e	<0.001	0.078	<0.001
DXA									
Lean mass (kg) [†]	37.8 ± 4.7 ^a	41.8 ± 3.4 ^b	49.8 ± 4.9 ^c	36.2 ± 4.6 ^a	41.1 ± 4.6 ^a	50.9 ± 7.8 ^c	<0.001	0.552	0.581
Fat mass (kg) [†]	18.6 ± 5.2 ^a	29.1 ± 4.8 ^b	50.9 ± 17.7 ^c	18.3 ± 5.0 ^a	35.2 ± 6.8 ^d	44.9 ± 18.2 ^c	<0.001	0.942	0.008
Fat (%)	32.8 ± 7.4 ^a	40.9 ± 4.7 ^b	52.9 ± 2.7 ^c	33.3 ± 7.7 ^a	45.9 ± 4.6 ^d	49.8 ± 3.7 ^c	<0.0001	0.870	<0.001
BMD (g/m²)									
Femoral neck	0.942 ± 0.131 ^a	0.984 ± 0.126 ^{ab}	1.057 ± 0.184 ^a	0.795 ± 0.105 ^c	0.915 ± 0.112 ^b	0.977 ± 0.143 ^{ab}	<0.001	<0.001	0.244
Total Hip	0.896 ± 0.127 ^a	1.039 ± 0.123 ^b	1.157 ± 0.185 ^c	0.809 ± 0.119 ^a	0.973 ± 0.119 ^b	1.058 ± 0.152 ^c	<0.001	<0.001	0.815
Lumbar spine	1.127 ± 0.058	1.243 ± 0.156	1.306 ± 0.184	1.113 ± 0.289	1.179 ± 0.162	1.264 ± 0.225	0.006	0.097	0.960
Total Body	1.169 ± 0.120	1.204 ± 0.063	1.256 ± 0.095	1.047 ± 0.114	1.164 ± 0.089	1.245 ± 0.113	<0.001	0.006	0.269
Hormones									
PTH (pg/ml)	32.9 ± 11.2 ^a	40.2 ± 16.3 ^a	74.9 ± 31.3 ^b	33.7 ± 11.8 ^a	43.2 ± 22.4 ^a	60.5 ± 30.9 ^c	<0.001	0.330	0.107
25OHD (ng/ml)	28.3 ± 8.8 ^a	28.8 ± 7.9 ^a	19.5 ± 6.4 ^c	27.9 ± 7.9 ^a	23.6 ± 6.1 ^b	17.6 ± 8.1 ^c	<0.001	0.064	0.300
Nutrient Intake*									
Calcium (mg/d)	1415 ± 543 ^a	1435 ± 512 ^a	1192 ± 499 ^{ab}	1501 ± 548 ^a	1132 ± 586 ^{ab}	995 ± 513 ^b	0.010	0.129	0.178
Vit D (ug/d)	9.9 ± 1.8 ^a	8.5 ± 4.5 ^{ab}	9.8 ± 5.3 ^a	8.6 ± 4.9 ^{ab}	7.7 ± 4.7 ^b	6.6 ± 5.2 ^b	0.318	0.019	0.378

Values are mean ± SD. Values with different superscripts within a row are significantly different (Tukey's post- hoc test p<0.05)

The number of women in the BMI<25, BMI 25-35, and BMI>35 groups were n=23, n=34 and n=16 for the premenopausal group, and n=19, n=85 and n=34 for the postmenopausal group, respectively.

*Includes intake of calcium and D from diet and supplements.

[†] total body fat and lean mass was not available in 10 women

BMI = body mass index, PTH= parathyroid hormone, 25 OH D= 25 hydroxy vitamin D, vit= vitamin

Table 12: Volumetric Bone mineral density, content, geometry and strength indices in 211 women

BMI Category	Premenopausal women (N=73)			Postmenopausal women (N=138)			BMI	Menop	BMI*meno P
	BMI<25	BMI 25-35	BMI>35	BMI<25	BMI 25-35	BMI>35			
Total vBMD (mg/m ³)	286 ± 31.7 ^a	310 ± 39.6 ^b	328 ± 38.2 ^b	252 ± 38.7 ^c	293 ± 37.5 ^{ab}	307 ± 36.9 ^{ab}	0.0002	0.0022	0.5751
Total BMC(mg)	290 ± 35.7 ^a	304 ± 36.7 ^{ab}	334 ± 55.1 ^b	251 ± 40.2 ^c	288 ± 37.2 ^a	270 ± 37.9 ^{ac}	0.0048	<.0001	0.0180
Total area (mm ²)	1021 ± 122 ^a	993 ± 140 ^a	1010 ± 191 ^a	995 ± 134 ^a	991 ± 108 ^a	885 ± 127 ^b	0.3022	0.0226	0.0669
Trabecular site (4%)									
BMC (mg)	94.8 ± 16.2 ^a	102.4 ± 17.5 ^a	120.6 ± 26.1 ^c	82.1 ± 15.8 ^b	98.4 ± 18.6 ^a	90.5 ± 17.4 ^{ab}	0.0042	<.0001	0.0086
vBMD(mg/m ³)	203 ± 28 ^{ab}	226 ± 37 ^b	260 ± 32 ^c	183 ± 35 ^a	220 ± 32 ^b	226 ± 28.3 ^b	<.0001	0.0028	0.1735
Cortial site (38%)									
BMC (mg)	321 ± 44 ^{ac}	338 ± 44 ^a	350 ± 41 ^a	290 ± 45 ^b	315 ± 38 ^c	293 ± 43.6 ^b	0.0209	<.0001	0.1172
vBMD (mg/m ³)	1185 ± 18 ^a	1175 ± 21 ^a	1159 ± 17 ^b	1140 ± 30 ^b	1144 ± 35 ^b	1137 ± 30 ^b	0.0383	<.0001	0.3227
Area (mm ²)	272 ± 39 ^{ac}	287 ± 36 ^{ab}	302 ± 37 ^{ab}	256 ± 39 ^c	275 ± 30 ^{ac}	257 ± 38 ^c	0.0186	<.0001	0.0563
Thickness (mm)	5.1 ± 0.5 ^a	5.2 ± 0.6 ^a	5.5 ± 0.9 ^a	4.6 ± 0.7 ^b	5.1 ± 0.5 ^a	4.9 ± 0.6 ^{ab}	0.0706	<.0001	0.1045
Peri circ (mm)	69.3 ± 4.9 ^{ac}	71.2 ± 4.1 ^b	71.8 ± 4.9 ^{ab}	70.3 ± 5.3 ^{abc}	70.7 ± 4 ^{ab}	68.2 ± 4.5 ^c	0.0806	0.1033	0.0487
Endo circ (mm)	37.1 ± 3.6 ^a	39.1 ± 4.1 ^{ab}	37.4 ± 4.1 ^{ab}	41.4 ± 6.4 ^b	39.2 ± 5.0 ^{ab}	37.8 ± 4.9 ^{ab}	0.4908	0.0907	0.0752
Ip (mm ⁴)	22530 ± 6067 ^{ac}	24941 ± 5214 ^{bc}	27093 ± 7554 ^{bc}	22504 ± 5165 ^{ac}	23280 ± 4613 ^c	20198 ± 6078 ^a	0.2363	0.0019	0.0134
SSI (mm ³)	1505 ± 274 ^{ac}	1640 ± 266 ^b	1679 ± 294 ^{bc}	1446 ± 267 ^{ac}	1511 ± 240 ^c	1366 ± 296 ^a	0.0780	0.0003	0.0930

Values are mean ± SD. Values with different superscripts within a row are significantly different (Tukey's post- hoc test p<0.05).

Controlled for lean mass, physical activity level, and dietary intake of carbohydrate, protein, fat and sodium intake.

The number of women in the BMI<25, BMI 25-35, and BMI>35 groups were n=23, n=34 and n=16 for the premenopausal group, and n=19, n=85 and n=34 for the postmenopausal group, respectively.

vBMD: volumetric bone mineral density. Circ: circumference; Endo: Endosteal; Peri: Periosteal; Ip: polar moment of inertia, SSI: Stress Strain Index

Table 13: Partial correlation analysis examining relationship between bone variables and tibia fat and muscle area

	Tibia Fat area	Tibia Muscle area	Tibia Fat area¹
Tb BMC	0.10	0.27**	0.009
Tb BMD	0.15	0.21*	0.09
Ct BMC	0.18*	0.38**	0.04
Ct BMD	-0.18*	-0.08	-0.16*
Ct Ar	0.21*	0.41**	0.07
Ct thk	0.14	0.31**	0.05
Peri C	0.19*	0.39**	0.05
Endo C	0.07	0.13	0.14
SSI	0.19*	0.38**	0.04
Ip	0.18*	0.37**	0.06

¹Controlled for muscle area, *p<0.05, **p<0.01, Ct: cortical, Tb: trabecular, vBMD: volumetric bone mineral density. Circ: circumference; Endo: Endosteal; Peri: Periosteal; Ip: Polar moment of inertia, SSI: Stress Strain Index

Table 14: Multiple regression analysis of relative contribution of age, body composition (fat and lean mass) and hormones on vBMD and geometry

	<i>Total Fat mass</i>				<i>Total Lean mass</i>			
	Beta Coefficient¹	P value	R² %	Model r²	Beta coefficient	P value	R² %	Model r²
Tb vBMD								
Fat or Lean	0.41	<0.001	9.1	10.2	0.23	0.0025	5.4	6.6
Age	-0.15	0.028	1.1		-0.10	0.163	1.1	
PTH	-0.09	0.259	0.03		0.009	0.909	0.03	
25OHD	0.09	0.229	0.08		0.007	0.928	0.08	
Ct vBMD								
Age	-0.43	<0.001	23.6	37.8	-0.46	<0.001	23.6	33.0
Fat or Lean	-0.20	0.007	9.1		-0.17	<0.001	4.4	
25OHD	0.10	0.139	5.0		0.13	0.048	5.0	
PTH	0.06	0.358	0.06		0.03	0.641	0.06	
Ct Thk								
Fat or Lean	0.34	<0.001	5.8		0.41	<0.001	16.6	
Age	-0.33	<0.001	8.3	16.5	-0.28	<0.001	8.3	27.3
25OHD	0.15	0.044	0.08		0.11	0.094	0.08	
PTH	0.06	0.395	2.4		0.10	0.128	2.4	
SSI								
Fat or Lean	0.44	<0.001	12.9	20.4	0.57	<0.001	33.4	41.0
Age	-0.24	<0.001	3.1		-0.17	0.002	3.2	
25OHD	0.16	0.028	0.04		0.12	0.052	0.04	
PTH	0.07	0.337	4.4		0.11	0.067	4.4	

¹ Independent Predictors with greatest beta coefficient is listed first.

vBMD: volumetric bone mineral density, PTH: parathyroid hormone, 25OHD : 25 hydroxy vitamin D.
Tb : trabecular, Ct: cortical, Thk: thickness, SSI: stress Strain Index

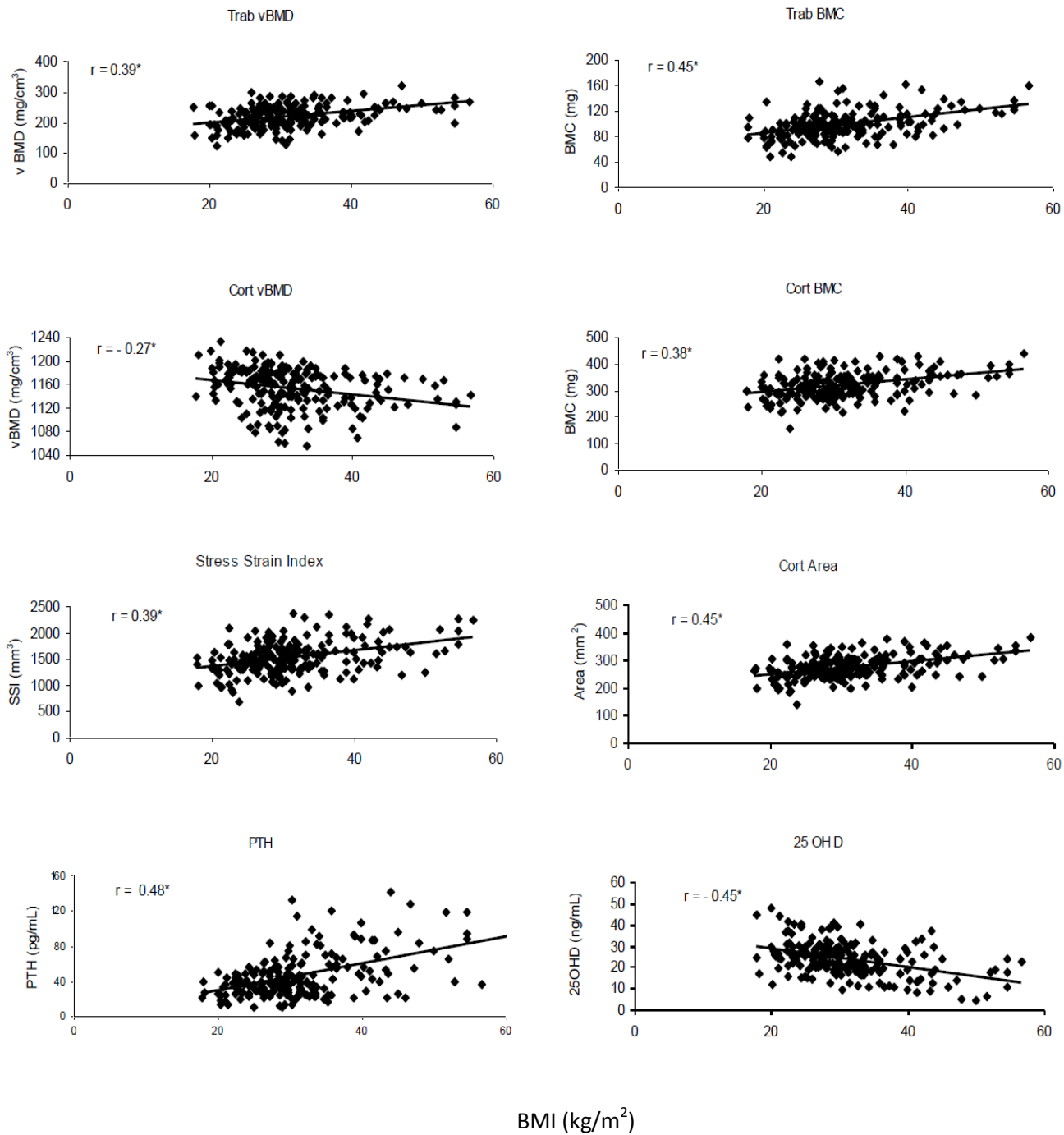


Figure 16: Relationship between body mass index (BMI), cortical and trabecular bone parameters and hormones

(Volumetric bone mineral density (vBMD) and content (BMC), cortical area and stress: strain index), and serum parathyroid hormone (PTH) and 25-dihydroxy-vitamin D (25OHD) $*p < 0.001$.

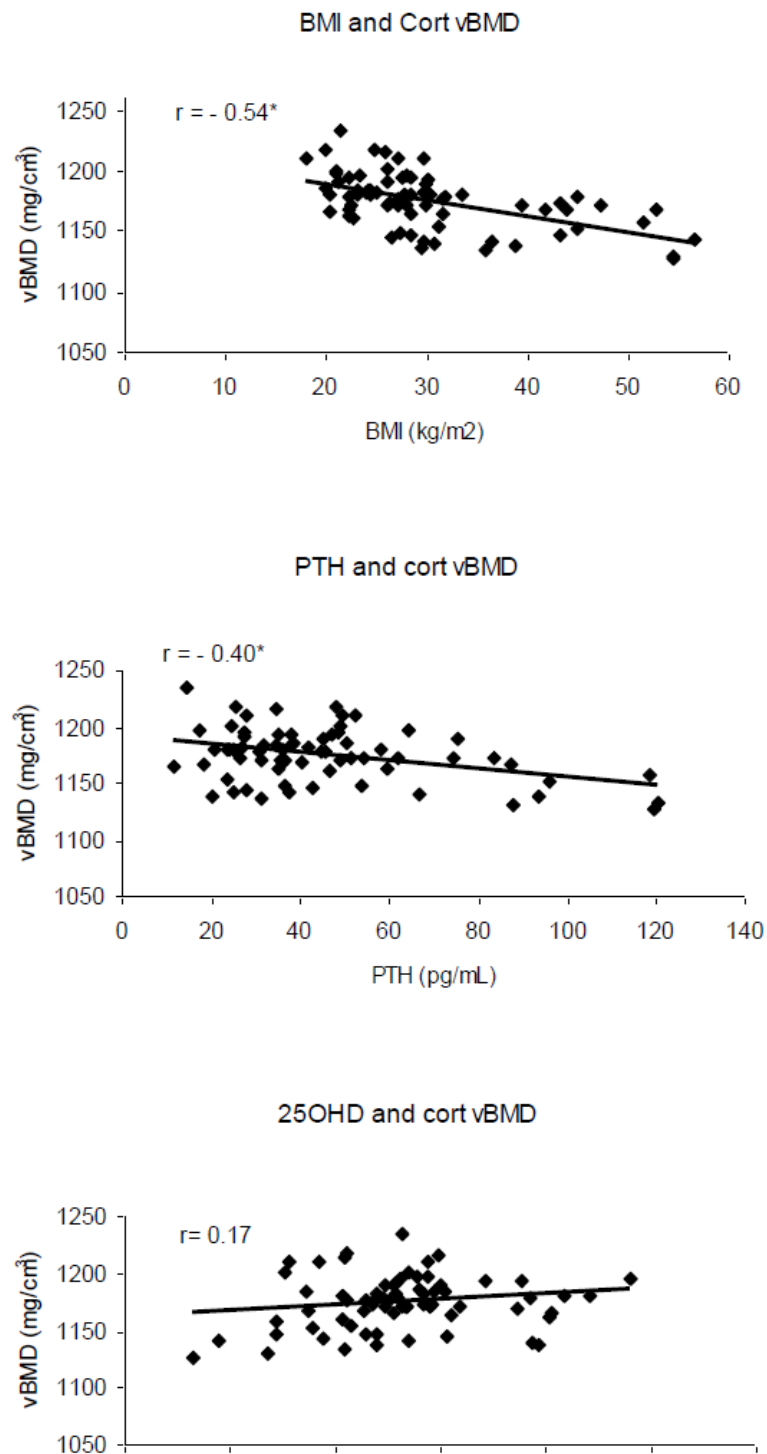


Figure 17 : Relationship between cortical volumetric bone mineral density (vBMD), body mass index (BMI) and hormones

Serum parathyroid hormone (PTH) and 25-dihydroxy-vitamin D (25OHD) in premenopausal women (n=73)

* $p < 0.001$.

**6.3.2 The high serum monocyte chemoattractant protein-1 in
obesity is influenced by high parathyroid hormone and not
adiposity**

Sukumar D, Partridge NC, Wang X, Shapses SA

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6.3.2.1 Abstract

Chronic high levels of parathyroid hormone (PTH) may be associated with up-regulation of proteases and cytokines. Monocyte chemoattractant protein-1 (MCP-1) is an inflammatory cytokine, produced predominantly by macrophages, endothelial cells and is expressed in adipose tissue. More recently it has been shown that PTH administration increases MCP-1 expression in osteoblasts. Objectives: Since both PTH and MCP-1 levels are higher in obesity, the goal was to determine whether the high MCP-1 occurs only in the presence of high serum PTH and is independent of adiposity and examine its relationship with bone mineral density (BMD) and turnover. In this case control clinical design, 111 eligible women were categorized into 4 groups: leaner women (body mass index, BMI: 23 ± 2 kg/m²) with normal or higher PTH and obese (BMI: 44 ± 7 kg/m²) with normal or higher PTH. Serum MCP-1 levels were higher ($p < 0.01$) in the high (421.5 ± 157.0 pg/ml) compared to normal PTH (322.5 ± 97.8 pg/ml) group, independent of BMI. C- reactive protein and adiponectin were only influenced by BMI, and not PTH. MCP-1 was positively associated with osteocalcin and propeptide of type 1 collagen in the leaner ($r > 0.3$, $p < 0.05$), but not the obese women and was not associated with BMD in either group. Together these data suggest that MCP-1 is only higher in the presence of increased PTH, and that adiposity alone cannot explain the higher MCP-1 levels in obesity.

6.3.2.2 Introduction

Serum parathyroid hormone (PTH) plays a central role in the regulation of calcium homeostasis. In addition to its well established role in the skeleton, reports suggest an association between high PTH and risk of hypertension, insulin resistance, dyslipidemia, cardiovascular mortality, and cancer (380), but is not a consistent finding in all studies (114) . Chronic high levels of PTH in patients with hyperparathyroidism are associated with the up-regulation of proteases, cytokines and also increased bone turnover (133). More recently, it has been shown that treatment of osteoblasts with PTH increases expression of monocyte chemoattractant protein-1 (MCP-1) (124).

MCP-1 is a proinflammatory cytokine that is produced predominantly by macrophages and endothelial cells (116). Serum MCP-1 levels are higher in patients with atherosclerosis (117) and both protein and mRNA levels of MCP-1 are higher in atherosclerotic lesions (118). In addition, MCP-1 levels are increased in obesity (119;121) showing higher mRNA expression and protein levels in the adipose tissue, and MCP-1 levels decrease with weight loss (122;123). MCP-1 is also expressed by the osteoblast and both mRNA and protein expression in bone as well as serum levels increase with PTH administration in a rodent model (124;126). The relationship between the elevated PTH and MCP-1 in obesity has not been linked, although both high PTH and MCP-1 have similar effects on cardiovascular disease (381), insulin resistance (119;382) and mortality (383) .

Obesity is associated with an increase in serum PTH and a positive relationship is observed between fat mass and serum levels of PTH (83;84;341) . In addition, patients

with hyperparathyroidism are often heavier than their peers (107). The higher PTH may be a result of the lower serum level of 25-hydroxyvitamin D (25OHD), possibly due to increased sequestration of vitamin D into adipose tissue or decreased exposure to sunlight. Low 25OHD levels may thus promote secondary hyperparathyroidism and may contribute to the increase in serum PTH in this population. However, higher serum levels of PTH levels are also associated with lower whole body fat oxidation and suppresses insulin signaling in the adipocyte and may thus promote adiposity and insulin resistance (382;384-386).

Obesity is described as a state of low grade inflammation due to the presence of elevated levels of pro-inflammatory cytokines such as MCP-1, tumor necrosis factor- α (TNF- α), interleukin-6 (IL-6), and C reactive protein (CRP) together with lower adiponectin. Both serum levels of PTH and MCP-1 are higher in obese adults, yet whether the higher PTH increases MCP-1 is not known, and it is not clear if this occurs independent of adiposity. We examined whether PTH influences MCP-1 in obese and lean women with normal and higher levels of PTH in a case control design. In addition, because our laboratories have found that PTH increases MCP-1 in osteoblasts, we also examine the relationship with bone turnover and bone mineral density (BMD) in this study.

6.3.2.3 Subjects and Methods

Subjects

Women aged 25- 71 years who were either leaner (BMI < 27 kg/m²) or obese (BMI > 35 kg/m²) were recruited by advertising in local newspapers, email list-serves and in the medical outpatient clinics at Robert Wood Johnson University Hospital. Clinic patients who were diagnosed with hyperparathyroidism and were eligible for this study (n=14) were referred by the endocrinologist. Those patients interested in participating in the study completed blood work and body composition analysis before treatment. Volunteers were also recruited for this study (n=23) to serve as controls without diagnosis of hyperparathyroidism, however some of them had higher levels of PTH. Others included in this study had been enrolled to participate in weight loss trials in the laboratory previously (n= 74) and their baseline data was used for this study. Women were excluded if they were osteoporotic (T-score < -2.5), taking osteoporosis medications known to influence bone or mineral metabolism including use of hormone replacement therapy in the past year, had evidence of metabolic bone disease, thyroid disorders, immune disease, heart attack or stroke in the past 6 months, currently undergoing treatment for hyperparathyroidism, kidney stones, diabetes, active cancers or cancer therapy within the past 12 months. Premenopausal women who were menstruating monthly were included, and were excluded if they were pregnant or lactating within the past year. Postmenopausal women were required to be at least 2 years since their last menstruation. Women were advised to consume 1-1.2g/d of calcium (Citracal, Bayer, NJ) depending on the recommended intake for their age and a daily multivitamin that contained 400 IU/d of vitamin D for at least 4-6 weeks before all the measurements were performed. This

was done to attenuate variability in serum PTH due to potential large differences in dietary intake of Ca or vitamin D. This study was approved by the Institutional Review Boards at Rutgers University and University of Medicine and Dentistry of New Jersey, and participants signed an informed consent form before initiation of study procedures.

Methods

Weight and height were measured with a balance beam scale and stadiometer, respectively; (Detecto, Webb City, MO). Nutrient intake was estimated using 3-day food records and analyzed using nutrient software (Food Works, version 10.1, Longvalley, NJ). Bone mineral density, content and body composition were measured using dual-energy x-ray absorptiometry (DXA) (Lunar Prodigy Advanced; GE- Lunar, Madison, WI; CV: < 1% for all sites using enCORE 2004 software (version 8.10.027; GE Lunar). Volumetric BMD (vBMD) was measured using peripheral quantitative computed tomography (pQCT - Stratec XCT 3000, Orthometrix). Sectional images were standardized at specific sites (4% and 38%) using distal tibia as the anatomical marker and analyzed for volumetric BMD as has been described previously (341)

Laboratory Methods

Blood was aliquoted and centrifuged to separate serum that was stored at -80° C until further analysis. Serum was analyzed for calcium (Arsenazo III, Endpoint, Ponte Scientific, Canton, MI) and other markers. Radioimmunoassays were used to analyze serum levels of PTH (Scantibodies laboratory, CA, CV <6.8%), 25OHD (Diasorin, MN, CV <12.5%), bone formation markers such as osteocalcin (BTI, Sloughton, MA, CV < 9%) and pro-peptide of type 1 collagen (PINP, Orion Diagnostica, Finland, CV < 10.2%).

Our laboratory also participates in the international Vitamin D External Quality Assessment Scheme (DEQAS) to ensure quality and accuracy of 25OHD analysis. Enzyme linked immunoassays were used to analyze N-telopeptide of type 1 collagen (NTx, Wampole Laboratories, Princeton, NJ, CV<14%), MCP-1 (R&D systems, Minneapolis, MN, CV< 7.8%), CRP (R&D systems, Minneapolis, MN, CV< 8.3%) and total adiponectin (Alpco Diagnostics, Salem, NH, CV< 9.2%). Urinary pyridinium crosslinks, pyridinoline (PYD) and deoxypyridinoline (DPD), were measured by HPLC (CV< 6%) and corrected for creatinine. Serum creatinine was measured using pointe Scientific reagent (Canton, MI, CV <3.6%). Estimated GFR (eGFR) was calculated based on serum creatinine and calculated body surface area ($eGFR = GFR \text{ (MDRD equation)} * BSA / 1.73 \text{ m}^2$)

Statistical Analysis

We separated women into 4 groups based on BMI and median of PTH. The categories included normal BMI (leaner) and high (obese) BMI, and normal and high PTH. A two-factor ANOVA was used to analyze the differences in the 4 groups (Leaner and Obese, Hi and NL-PTH). Differences among the four groups were tested by Tukey's post-hoc analysis when the model F ratio was significant. Pearson's correlation coefficients (r) were used to assess relationship between MCP-1 and PTH. In addition, linear regression models were used to explain relative contributions of independent variables such as age, PTH, BMI and 25OHD levels on MCP-1 and other cytokines. A P value ≤ 0.05 was considered significant. Data are presented as means \pm SD unless otherwise indicated. All analyses were conducted using the SAS statistical package (SAS Institute, Cary, NC; version 9.2).

6.3.2.4 Results

One hundred and eleven women who were either leaner with BMI < 27 kg/m² (mean 23.4 ± 2.4 kg/m²) or obese with BMI > 35 kg/m² (mean 44.3 ± 7.0 kg/m²), were further classified as having higher (Hi) or normal (NL) serum PTH levels, according to the median PTH value (40 pg/mL for leaner and 59 pg/mL for obese) in each group. The mean age was 52 ± 10 years (range 25-71 years) and did not differ between groups (Table 1). Women were primarily Caucasians (N = 95) with 11 African Americans, 3 Hispanics and 2 Asians. Women in the higher PTH groups had higher fat mass (P < 0.01) and body weight was greater (P < 0.08) than those in normal PTH groups. There was no significant interaction between BMI and serum PTH for weight, body composition or age. Mean calcium intake was 1208 ± 360 mg/d and vitamin D intake was 464 ± 416 IU/d and was similar between the groups (Table 15). Areal BMD at femoral neck, spine (L2-L4) and hip and tibial trabecular vBMD was higher and cortical vBMD was lower in the obese women (P < 0.02) and were not affected by PTH levels. The eGFR was low in one obese subject in the Hi PTH group at 27.8 mL/min, and in 13 leaner subjects (6 with NL PTH and 7 with Hi PTH) with eGFR in the range of 42.9-59.8 mL/min.

Bone regulatory hormones and turnover markers

As expected, PTH was higher and 25OHD was lower in the Hi than NL-PTH groups (P < 0.01) (Table 16). In addition, serum levels of PTH were higher and 25OHD was lower in the obese compared to leaner women (P < 0.001) and there was a significant interaction between PTH and BMI for 25OHD (P = 0.008). The levels of PTH were 40-45 pg/mL higher in both leaner and obese Hi than NL-PTH groups, but only the obese

group showed a lower serum 25OHD in the Hi-PTH group ($P < 0.01$). Bone formation markers (OC and P1NP) were both lower in the obese than leaner women ($P < 0.01$) whereas the bone resorption marker, NTx, tended ($P < 0.08$) to be higher in the leaner population. Serum Ca and PYD was higher ($p < 0.02$) in the Hi than NL-PTH groups ($p < 0.01$).

MCP-1 and control cytokines

The serum levels of MCP-1 and control cytokines in the 4 groups are shown in Figure 18. In the leaner women, serum levels of MCP-1 were higher in the Hi-PTH (408.3 ± 121.6 pg/ml) compared with the NL-PTH groups (300.7 ± 82.0 pg/ml) ($p < 0.01$). Similarly in the obese category, MCP-1 levels were higher ($P = 0.049$) in the Hi-PTH (436.3 ± 191.7 pg/ml) as compared with the NL-PTH groups (348.5 ± 109.9 pg/mL). Interestingly, MCP-1 levels did not differ between the BMI categories ($P = 0.13$) and was also similar between Hi-PTH groups independent of BMI. In contrast, CRP levels were significantly higher and adiponectin levels lower in the obese ($P < 0.01$) compared with leaner women, but were not different between the PTH categories ($P > 0.4$).

Independent predictors of MCP-1 and control cytokines

The relative contribution of independent variables such as BMI, age, PTH and 25OHD on serum levels of MCP-1 was examined using multiple regression analysis (Table 17). PTH was the most significant predictor of MCP-1 ($P < 0.02$) and the β -coefficient for PTH shows that a one standard deviation increase in PTH leads to a 0.26 standard deviation increase in MCP-1, when the other variables are held constant. Interestingly BMI was not a significant predictor of MCP-1 ($P > 0.4$) in the multiple regression model. However,

BMI explains a significant portion of the variance ($P < 0.01$) for control cytokines i.e. CRP and adiponectin, followed by 25OHD, while PTH and other variables were not significant predictors of both these control cytokines.

Cytokines and bone turnover

The relationship of MCP-1 with bone formation and resorption markers using Pearson's correlations is presented in Table 18. Serum MCP-1 was not associated with bone turnover in the entire cohort of women or in the obese women, however in the leaner group, MCP-1 was positively associated ($r > 0.3$, $P < 0.05$) with bone formation markers OC and P1NP and resorption marker DPD. The positive associations of MCP-1 with bone turnover in the leaner women remained even after adjusting for PTH levels.

Similarly, in all women, CRP was negatively associated with osteocalcin and P1NP ($r > -0.2$, $P < 0.03$) and adiponectin was positively with these bone formation markers and BMD ($r > 0.3$, $P < 0.01$) in all women, but these relationships disappeared after controlling for BMI. MCP-1 tended to be weakly associated with FN and hip BMD ($r > 0.17$, $P < 0.09$) in all women.

6.3.2.5 Discussion

It is well established that MCP-1 levels are elevated in obesity, and it is attributed to its synthesis in adipose tissue. Studies from our laboratories (124) have shown that PTH induces osteoblastic expression and increases serum levels of MCP-1 in rodents (126). A major role of MCP-1 in bone is to facilitate bone resorption by recruiting pre-osteoclasts to the remodeling sites and then promoting osteoclast differentiation. Whether or not the higher PTH in obesity increases MCP-1 expression and serum levels is not known. In this study we examine how two levels of serum PTH in obese and leaner individuals influence MCP-1 levels, and show that MCP-1 is not high in all obese individuals and only in those with high PTH. In addition, we found that in leaner individuals with high PTH, there is a similar high level of MCP-1 that cannot be attributed to their adiposity. Unlike MCP-1, there was no positive association between PTH and other cytokines, such as CRP or adiponectin in weight-matched obese and leaner women, suggesting a unique relationship between PTH and MCP-1 that is independent of adiposity.

Obesity is associated with higher levels of serum PTH and multiple studies have shown this relationship in both healthy individuals and in patients with primary hyperparathyroidism (83;84;341). Several mechanisms have been proposed for the higher PTH seen in obesity including lower 25OHD levels (108;385;387) and may influence their altered BMD, as reported by our laboratory previously (341). Serum levels of 25OHD are key regulators of serum PTH levels, and are negatively correlated with BMI and body fat mass (109;388;389). This relationship may occur because 25OHD is fat

soluble, leading to increased sequestration into excess adipose tissue and possibly other tissues in obese individuals (108;109) . It is also possible that obese people have decreased exposure to sunlight due to lower physical and outdoor activity resulting in lower 25OHD and higher PTH. High PTH levels may exacerbate obesity and its associated insulin resistance, since an increase in PTH has been shown to decrease whole body fat oxidation (385) and suppresses insulin signaling in adipocytes (382). The cause-effect relationship between excess body weight and PTH is unclear with reports showing that weight loss decreases PTH (390;391) , while some show that PTH lowering via parathyroidectomy does not decrease body weight. (114). Given that higher PTH exists with excess adiposity, we suggest that chronically elevated PTH levels contributes to higher MCP-1 levels. Higher serum MCP-1 has been previously shown to exacerbate inflammation and comorbidities associated with obesity.

The predominant source of MCP-1 in obesity is believed to be from the macrophages of the white adipose tissue (119;392). However, the source of MCP-1 in response to high serum levels of PTH is unclear and indeed our laboratories have shown that osteoblasts secrete MCP-1 in response to PTH administration. Because PTH suppresses insulin signaling in the adipocyte (382), and MCP-1 has been shown to have a similar action (393) ;Kamei, 2006 5773 /id}, it is not clear if PTH and MCP-1 have independent effects on the adipocyte or if PTH acts through MCP-1, on the adipocyte. PTH stimulates MCP-1 in another cell; the osteoblast, so we hypothesize that it may also stimulate MCP-1 in the adipocyte, since both arise from the same progenitor cell. It may be that the osteoblast is a more important source of MCP-1 in lean individuals, given the positive associations of osteoblast specific bone formation markers (OC and P1NP) in the

leaner population, whereas excess adipose tissue may be the primary source of MCP-1 in obesity, although future research is needed to confirm the potential role of MCP-1 in bone turnover.

Hyperparathyroid patients have a low BMD, particularly cortical bone density, compared to healthy controls (394-396). One possible mechanism that may reduce BMD and increase bone turnover in patients with hyperparathyroidism, includes upregulation of osteoblastic expression of cytokines (397). Since PTH influences BMD one would expect MCP-1 to also show a similar relationship with bone; however neither MCP-1 nor PTH were associated with BMD. This is possibly because our high PTH groups only had mean PTH levels of 64 and 86 pg/mL in leaner and obese women, respectively, and are much lower than those of hyperparathyroid patients low BMD (85;133;398).

The strengths and limitations of the study include the following. One strength is that patients that present with primary hyperparathyroidism are usually overweight or obese (107) and recruitment of a leaner population of women with higher levels of PTH was an important aspect of the current study to understand the influence of PTH in the absence of obesity. In addition, women were also advised to consume adequate calcium intake of 1-1.2 g of calcium before biochemical assessment to ensure that a low calcium intake did not transiently increase PTH levels in these women. Also, all subjects were recruited in the winter and early spring months to avoid the influence of sun exposure on 25OHD levels. Some limitations to this study include the following. Since this is a retrospective case control design, a cause-effect relationship cannot be determined and this may also be subject to selection bias. In addition, in the obese group, the fat and lean mass was not available in 25% of the obese women, because some of the severely obese

subjects could not fit on the DXA table for body composition analysis. It is likely that fat mass is higher than reported in the obese groups, but because we are missing approximately the same number of women with similar BMI in both groups, fat mass is unlikely to differ between the 2 obese groups. In addition, we did not have any data on the duration of high PTH in our subjects, and this may be important in the interpretation of these results, especially with respect to BMD. In addition, although the relationship of MCP-1 with bone turnover showed weak associations with formation markers in the leaner population, there was no relationship found in the obese women, and clearly further research is required to address the potential role of MCP-1 in bone turnover. Also, the small model r^2 for MCP-1 suggests that there are other factors influencing MCP-1 that explain a large part of its variance. However, we know from this data that PTH, and not BMI, explains a significant amount of the variance for serum MCP-1. Together these data suggest that high PTH levels, irrespective of adiposity, are associated with higher MCP-1 levels. These findings may contribute to a better understanding of the association between high PTH levels and greater CVD mortality and insulin resistance, since previous studies have shown that MCP-1 plays an important role in etiology of both these diseases. Future studies should aim to understand whether traditional treatments to reduce PTH will decrease levels of MCP-1 and thereby reduce progression of disease.

6.3.2.6 Acknowledgments

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Table 15: Age, body composition and nutrient intake of study participants (n=111) ¹⁻³

	Leaner		Obese		<i>P</i> ²		
	NL-PTH (n=31)	Hi-PTH (n=28)	NL-PTH (n=26)	Hi-PTH (n=26)	BMI	PTH	BMI*PTH
Age(yrs)	49.5 ± 8.3	51.8 ± 8.8	53.2 ± 12.2	52.2 ± 11.3	0.250	0.780	0.450
Weight(kg)	60.1 ± 7.4 ^a	63.0 ± 7.7 ^a	109.4 ± 18.9 ^b	117.5 ± 22.9 ^b	<0.001	0.054	0.397
BMI(kg/m²)	23.0 ± 2.3 ^a	23.8 ± 2.5 ^a	44.1 ± 6.5 ^b	44.6 ± 7.4 ^b	<0.001	0.075	0.275
Fat mass (kg)¹	19.7 ± 6.6 ^a	22.1 ± 6.4 ^a	48.5 ± 5.5 ^b	50.4 ± 7.6 ^b	<0.001	0.004	0.116
Lean mass (kg) ¹	36.6 ± 4.8 ^a	38.0 ± 4.1 ^a	49.3 ± 6.4 ^b	49.2 ± 6.9 ^b	<0.001	0.540	0.490
FN BMD (g/cm²)	0.886 ± 0.135 ^a	0.876 ± 0.108 ^a	0.974 ± 0.128 ^b	1.010 ± 0.170 ^b	0.001	0.649	0.408
Total Hip BMD (g/cm²)	0.923 ± 0.134 ^a	0.907 ± 0.118 ^a	1.062 ± 0.139 ^b	1.077 ± 0.175 ^b	<0.001	0.979	0.621
L2-L4 BMD (g/cm²)	1.155 ± 0.153	1.139 ± 0.230	1.252 ± 0.208	1.254 ± 0.185	0.014	0.875	0.836
Trab vBMD (mg/cm³)	197.8 ± 28.0 ^a	190.9 ± 32.1 ^a	240.6 ± 29.6 ^b	252.1 ± 35.8 ^b	<0.001	0.709	0.181
Cort vBMD (mg/cm³)	1177.9 ± 28.5 ^a	1171.9 ± 28.6 ^a	1142.1 ± 24.7 ^b	1144.2 ± 28.1 ^b	<0.001	0.744	0.513
Calcium intake (mg/d)	1216 ± 359	1285 ± 413	1194 ± 188	1131 ± 429	0.160	0.850	0.280
Vit D intake (ug/d)	11.3 ± 2.1	10.9 ± 1.3	10.6 ± 2.5	9.8 ± 4.9	0.119	0.307	0.716

¹Fat and lean mass is reported for a subset n= 18 and n=21 for Hi and NL-PTH, respectively in the Obese group.

² A two factor ANOVA with BMI and PTH as independent variables was performed

³ Values with different superscript letters are significantly different using Tukey's post-hoc testing.

Table 16: Bone regulating hormones, calcium and turnover markers (n=111)¹⁻²

	Leaner		Obese		<i>P</i> ²		
					BMI	PTH	BMI*PTH
	NL-PTH (n=31)	Hi-PTH (n=28)	NL-PTH (n=26)	Hi-PTH (n=26)			
PTH (pg/mL)	25.6 ± 6.9 ^b	64.0 ± 31.8 ^a	39.3 ± 13.8 ^d	85.7 ± 22.1 ^c	<0.001	<0.001	0.305
25OHD (ng/ml)	29.0 ± 7.0 ^a	28.8 ± 7.5 ^a	23.3 ± 8.4 ^c	15.5 ± 5.9 ^b	<0.001	0.005	0.008
Serum Ca (mg/dL)	9.2 ± 0.6 ^a	9.6 ± 0.8 ^{ab}	9.5 ± 0.4 ^{ab}	9.8 ± 0.8 ^b	0.128	0.015	0.957
Osteocalcin (ng/mL)	10.9 ± 4.9 ^a	11.5 ± 2.8 ^a	8.8 ± 3.1 ^b	9.4 ± 3.2 ^b	0.005	0.426	0.986
PINP (ug/L)	52.6 ± 20.8 ^a	57.0 ± 21.7 ^a	39.6 ± 12.7 ^b	48.3 ± 15.6 ^{ab}	0.008	0.112	0.594
NTx (nMBCE)	16.0 ± 7.2 ^a	15.7 ± 4.4 ^a	12.1 ± 4.8 ^b	15.4 ± 6.4 ^a	0.080	0.234	0.142
PYD (nmol/mmol)	26.5 ± 7.4 ^a	31.4 ± 12.5 ^{ab}	28.1 ± 17.9 ^a	36.6 ± 15.8 ^b	0.208	0.013	0.492
DPD (nmol/mmol)	10.2 ± 3.4	11.1 ± 4.6	9.9 ± 6.5	10.7 ± 3.8	0.724	0.365	0.905
Serum Creatinine	0.90 ± 1.14 ^a	0.95 ± 0.15 ^a	1.09 ± 0.30 ^c	0.94 ± 0.15 ^a	0.023	0.231	0.015
eGFR	69.3 ± 11.2 ^{ab}	66.7 ± 11.6 ^a	76.2 ± 17.6 ^b	93.2 ± 21.3 ^c	<0.0001	0.025	0.003

PTH, parathyroid hormone; 25OHD, 25-hydroxyvitamin D; NTx, N-telopeptide of type-1 collagen; PYD, pyridinium cross links; DPD, deoxypyridinium crosslinks; PINP, propeptide of type 1 collagen

¹ Values with different superscript letters are significantly different.

² A two factor ANOVA with BMI and PTH as independent variables was performed

Table 17: Multiple regression model for the relative influence of age, BMI, PTH, and 25OHD on cytokines

	MCP-1 (Model R²= 10.3)			CRP (Model R²= 48.2)			Adiponectin (Model R²= 21.3)		
	R²	Std β	P	R²	Std β	P	R²	Std β	P
PTH	7.3	0.26	0.01	0.03	0.03	0.773	0.01	-0.03	0.718
Age	2.5	0.15	0.10	0.82	-0.11	0.220	6.91	0.26	0.004
BMI	0.3	0.08	0.45	46.4	0.61	<0.001	12.56	-0.27	0.009
25OHD	0.2	0.06	0.61	0.91	-0.09	0.186	1.72	0.14	0.183

MCP-1, Monocyte chemoattractant protein-1; CRP, C-reactive protein; BMI, body mass index; PTH, parathyroid hormone; 25OHD, 25-hydroxyvitamin D.

Table 18: Relationship of Serum Monocyte Chemoattractant protein (MCP-1) with bone turnover¹⁻²

MCP-1	All women (n=111)		Leaner (n=59)		Obese (n=52)	
	r	P	r	P	R	P
OC²	0.111	0.251	0.331	0.012	0.023	0.873
NTx	-0.090	0.375	0.121	0.383	-0.236	0.118
PYD²	0.209	0.031	0.216	0.106	0.184	0.205
DPD	0.132	0.175	0.267	0.044	0.064	0.66
PINP²	0.078	0.495	0.315	0.043	-0.021	0.896

OC, osteocalcin; MCP-1, monocyte chemoattractant protein-1; NTx, N-telopeptide of type-1 collagen; PYD, pyridinium crosslinks; DPD, deoxypyridinium crosslinks; PINP, propeptide of type 1 collagen.

¹ Pearson's correlations (r) was performed between MCP-1 and bone turnover

² Relationship exists even after controlling for PTH.

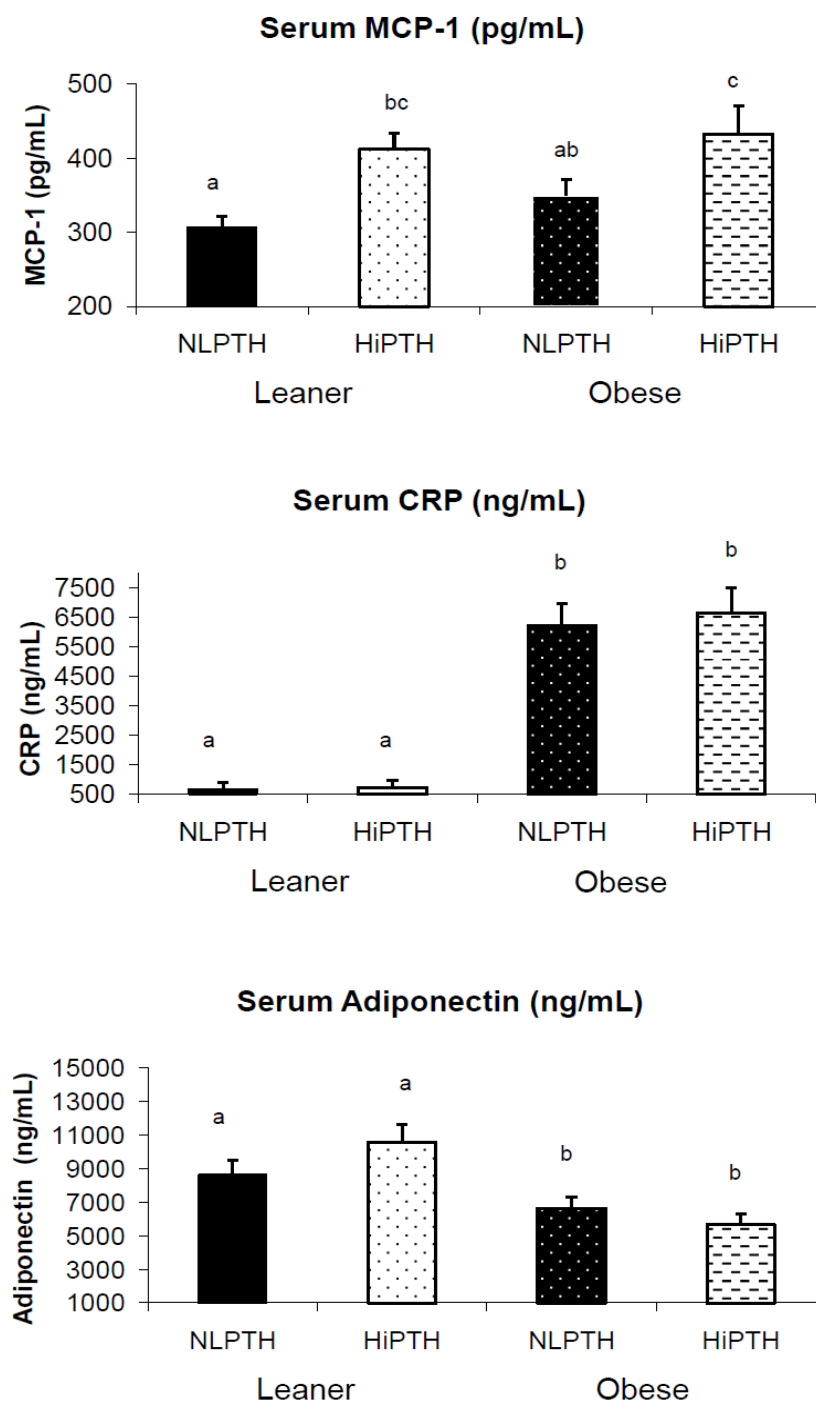


Figure 18: Serum levels of inflammatory cytokines (MCP-1, CRP and adiponectin) in leaner and obese women

Values with different superscript letters are significantly different, using Tukey's post-hoc testing

7. CONCLUSIONS

Weight reduction is advocated to reduce the comorbidities associated with obesity, however it is also associated with bone loss. The two randomized clinical trials in this dissertation address the influence of nutrient supplementation on bone loss and calcium absorption during caloric restriction. The other two studies examined adult women to better elucidate the relationship between body weight, hormones and bone quality.

7.1 NUTRIENTS AND BONE DURING CALORIC RESTRICTION

Role of dietary calcium on bone during weight reduction

Previous trials from our laboratory and others have shown that short term CR and weight reduction is associated with higher bone turnover and bone loss (19;135-142). Following this observation, other trials in our laboratory evaluated the influence of calcium to understand whether a higher intake of calcium can attenuate the bone loss associated with weight reduction. Calcium supplementation has shown to suppress bone turnover and attenuate loss of BMD in obese and overweight women (137;168) however bone loss is not entirely attenuated with additional calcium intake. In addition, there is weak evidence in recent reports that show that a higher intake of calcium (399;400) is associated with a higher incidence of cardiovascular events. Furthermore, compliance to calcium supplements due to their large pill size is always a concern. It is thus important to identify whether or not other nutrient modifications can also attenuate bone loss. The primary aim of this dissertation was to understand whether or not a higher intake of

dietary protein and vitamin D can attenuate bone loss during CR. Weight reduction induces bone loss by several factors, including a reduction in calcium absorption or IGF-1 and was primarily addressed in this dissertation.

Role of dietary protein on bone during weight reduction

Dietary protein is important for maintenance of bone mass. Importantly, dietary protein increases IGF-1 that is anabolic to the osteoblast and can thus prevent bone loss (163). In addition a higher intake of protein also increases calcium absorption(173;401) . Although there is some concern for the negative effects of high meat diets on bone, recent epidemiological trials show that a higher intake of dietary protein and /or animal protein intake in the presence of adequate calcium has a positive influence on BMD (187). Whether or not a higher protein intake will attenuate the decrease in IGF-1 and calcium absorption associated with CR and thus attenuate bone loss during CR is not known. We hypothesized that if a higher intake of dietary protein attenuates the decrease in TFCA and IGF-1 associated with CR, then it would also attenuate bone loss associated with CR. The first experiment in this dissertation addressed whether or not a higher intake of dietary protein (24%) vs a normal intake (18%) attenuates bone loss associated with weight reduction. Our results in 47 women showed that a higher protein intake attenuated bone loss at the trabecular rich sites such as ultradistal radius, hip and lumbar spine. This was accompanied by 20% increase in IGF-1 and a tendency to increase estradiol in the HP group, that was also supported by reduction in bone resorption markers. We did not address whether the CR induced reduction in TFCA was attenuated with higher protein intake due to very high cost of stable isotopes and given the solid evidence of effect of protein on TFCA in weight stable women (173). However this study did measure

important biochemical markers including changes in bone regulating hormones and IGF-1 and IGFBP-3. This study suggests that during weight reduction, a higher protein intake preserves BMD possibly mediated by increases in IGF-1, a slight increase in estradiol that has an anabolic effect on bone, and also by attenuating bone resorption. This suggests that postmenopausal women should consume a higher protein intake in the presence of adequate calcium (1200mg) and this would prevent bone loss associated with CR. Future studies should assess whether compliance to higher protein intake can be maintained over a longer term and whether such long term intake continues to exert a positive effect on bone. These findings confirm the hypothesis that higher compared to normal protein intake will preserve bone mass during CR.

Role of higher vitamin D intake on calcium absorption during caloric restriction

Vitamin D has a physiologically important effect on calcium absorption primarily mediated by its active form $1,25(\text{OH})_2\text{D}_3$. In the second aim of this dissertation, we addressed whether or not a higher intake of vitamin D of 2500 IU/d vs a lower intake of 400 IU/d attenuates CR mediated decrease in TFCA. Since vitamin D supplementation has shown to have a positive influence on TFCA, the goal in this study was to examine whether vit D supplementation during WL as compared to weight maintenance (WM) will attenuate the decrease in TFCA that is associated with weight reduction. The results from our 6 week trial showed that 2500 IU/d did not attenuate the decline in TFCA in women during CR, but did increase TFCA by 3.7% in women who were not losing weight. Thus a higher vitamin D intake of 2500 IU/d does not attenuate the decrease in TFCA associated with CR. It is interesting that during WM, TFCA increased by 3.7% with 2500IU/d of vitamin D. This is similar to findings from others who show that

supplementation with D₃ results in an increase in absorption (274;323) , however many recent studies do not suggest a positive effect (259;275;324). The WL group showed the greatest increase in 25OHD levels of 7.8ng/mL. It is interesting that a rise in 25OHD is not positively associated with higher absorption, suggesting that during CR, other physiological changes and/or hormonal changes may also influence absorption. For example CR is associated with increases in cortisol and decreases in estradiol, both of which inhibit calcium absorption (151;261;402). Although PTH decreased with vitamin D supplementation, other bone regulating hormones such as 1,25(OH)₂D₃ or estradiol also did not change with vitamin D supplementation or during weight loss. The unchanged estradiol may have been due to the short duration of the study with only a small amount fat loss. Serum PTH decreased with vitamin D supplementation, but other hormones including 1,25(OH)₂D₃ were not affected. This study shows that a higher vitamin D supplementation will not attenuate decrease in TFCA during CR. Because of the controversial normal range of serum 25OHD levels and because at the time of designing this study the normal levels of 25OHD was considered >30ng/mL, we included individuals with levels below 30 ng/mL for this study. The new IOM 2011 recommendation considers serum 25OHD levels < 20ng/mL as inadequate, and thus it would be especially interesting to determine if the effect of vitamin D supplementation on TFCA during CR is different in this population. Also whether a higher serum 25OHD level should be achieved with higher vitamin D supplementation during CR to attenuate bone loss also needs to be understood. These findings do not confirm the hypothesis that 2500IU/d compared to 400 IU/d vitamin D supplementation will attenuate the decrease in TFCA during CR.

Overall, following interventions with higher calcium intake during CR previously in our laboratory, in the current trials the effect of other nutrients in addition to calcium intake was also evaluated. Our results suggest that a higher intake of protein upto 24% of total calories, but not vitamin D of 2500 IU/d will attenuate bone loss or TFCA associated with caloric restriction.

7.2 CHANGES IN BONE REGULATING HORMONES AND BIOCHEMICAL MARKERS OF BONE TURNOVER DURING CALORIC RESTRICTION

Hormonal changes with higher protein and vitamin D intake and/or body weight

Changes in estradiol, PTH, 25OHD were evaluated in response to protein intake and in addition, 1,25(OH)₂D₃ change was evaluated in response to vitamin D supplementation. Interestingly estradiol levels responded to a higher intake of protein during one year, with a slight increase in estradiol over a one year period in the HP compared to the NP group. The rise in estradiol with higher protein intake has not been reported previously and it was interesting to examine if other sex steroids also respond in a similar fashion. A positive effect of estradiol on bone has been observed in many studies (265;403-405). Trials that have evaluated small physiological differences in estradiol in older individuals have showed that differences as low as 3.7 pg/mL (13.6 pmol/L) (352;353) are associated with higher BMD. We observed a 7 pmol/L higher estradiol in the HP compared to NP group and thus we cannot exclude the possibility that the slightly higher estradiol may have acted in concert with other factors, such as higher IGF-1 to attenuate bone loss in the HP group. Estradiol levels were unchanged in response to vitamin D supplementation.

Interestingly PTH levels were not influenced by a higher protein intake, although in the short term it has been shown that higher protein intake leading to increase in calcium absorption causes a suppression of PTH (406-408). It is possible that in the long term, the protein related increase in calcium absorption reaches a plateau, above which there is no protein induced increase in calcium absorption, and thus a further suppression in PTH levels is not observed. PTH levels responded to vitamin D supplementation with a significant decrease in PTH in the 2500 IU/d group. The decrease in PTH with vitamin D supplementation has also been shown in previous studies and the physiological mechanism mediating this suppression is due to an increase in $1,25(\text{OH})_2\text{D}_3$ that increases calcium absorption, leading to suppression of PTH. Although this is the known physiological mechanism, we did not observe changes in $1,25(\text{OH})_2\text{D}_3$ in this study and this may be due to the short half life of this hormone (~15hours) that does not allow detection of this hormone in the serum and it is unclear whether other factors are mediating a suppression in PTH.

Serum levels of 25OHD did not respond to higher protein intake however did increase in both groups with weight reduction in this study. This is possibly due to release of vitamin D from the adipose with fat loss and since both groups showed a loss of fat mass during the 1 year intervention (108;168;328;354). In the vitamin D supplementation study, the change in serum 25OHD was an important outcome in the study. The group that was supplemented with higher vitamin D of 2500 IU/d, showed a significant increase in serum levels of 25OHD compared to the placebo both during weight loss and during weight maintenance. However it is interesting that the women in this trial showed a much lower rise in 25OHD levels compared to normal weight women. It has been estimated

that a 1.0 ng/mL (2.5 nmol/L) rise in 25OHD occurs with 100 IU/d (2.5 ug) vit D intake. Thus with a 2500 IU/d supplementation, we expected a 25ng/mL increase in TFCA. However it has been shown previously that obese women have a lower rise in 25OHD with supplementation (326). Based on previous findings, this blunted response was not unexpected, nevertheless this was a much lower increase than expected. Interestingly, a combination of weight loss and higher D supplementation had the greatest increase in 25OHD compared to the group that maintained weight and had a higher D supplementation, that supports the hypothesis that weight loss increases serum levels of 25OHD. However the higher 25OHD was not associated with TFCA in these women. The active form of vitamin D, 1,25(OH)₂D₃ was measured only in aim 2 in response to vitamin D supplementation, however did not change in response to higher vitamin D intake during CR. Overall changes in these bone regulating hormones, help explain changes in primary outcome variables in both the protein and vitamin D supplementation studies

Changes in bone turnover with higher protein and vitamin D intake and/or body weight

Selected bone formation and resorption markers were analyzed to understand the response of dietary protein and vitamin D on bone turnover. PINP, a bone formation marker was not influenced by 1 year higher protein intake or with higher vitamin D supplementation for 6 weeks. Infact this marker was not even influenced by short or long term CR. Perhaps this marker is not sensitive to changes in body weight and/or changes in protein and vitamin D intake. Osteocalcin, also a bone formation marker was not influenced by weight loss, caloric restriction or protein intake, but interestingly was influenced by higher intake of vitamin D during CR (409). This bone formation marker

has been recently shown to act as an important regulator of energy metabolism. Vitamin D supplementation was associated with a slight increase in osteocalcin in comparison with placebo. This increase in osteocalcin was associated with improvements in markers of glucose sensitivity in women during CR (409). Bone resorption marker NTx was not influenced by weight loss, short term CR, higher protein intake or vitamin D intake. Similar to PINP, perhaps this marker is also not sensitive to changes in body weight and/or changes in protein and vitamin D intake. Pyridinium cross links (PYD and DPD) were not influenced by short term CR or higher vitamin D intake however was influenced by higher protein intake. The increase in PYD and DPD during weight loss was attenuated by a higher protein intake compared to a normal protein intake.

7.3 BONE QUALITY AND HORMONAL MILIEU IN OBESITY

Trabecular and cortical bone in obesity

There are reports that show a higher fracture incidence in the obese (67;71;72) and poor bone quality in obese children (81;82;93), however whether or not bone quality is compromised in adult obese women is not known. Our preliminary examination in 211 women shows that although areal BMD and tibial vBMD is higher in the obese, examination of individual compartments of the tibia; reveal a compromised cortical vBMD with normal trabecular vBMD. With further adjustment of potential confounders, such as nutrient intake and lean mass, we show that excess body weight does not have an added benefit even on measures of bone strength. This suggests that bone quality may be compromised in obese adult women. This is an interesting finding because there are emerging reports of greater fracture risk in the obese population amidst the long known

concept that BMD is higher in the obese. Given a higher BMD in the obese, they should be protected against fracture risk, however they do not seem to be. Furthermore there are also reports that suggest that excess fat mass and adipokines may have a detrimental effect on bone (16). This raises the question of whether bone quality is altered in obesity and indeed using pQCT we show a compromised cortical vBMD. Although pQCT does not provide enough information on all aspects of bone quality, it provides a good estimate of trabecular and cortical compartments and bone strength in a weight bearing site such as tibia. Future studies should aim at using high resolution pQCT or other methods such as MRI to further assess other components of bone quality such as microarchitecture in obesity and assess other bone sites such as radius.

Role of higher serum parathyroid hormone in obesity

The association of PTH on bone mass in leaner hyperparathyroid population is well known (97;98). However the role of higher PTH seen in the obese is not clear and its influence on bone is not understood. Interestingly, rodent data from the Partridge (124) laboratory shows that the inflammatory cytokine MCP-1 is upregulated with PTH injections. Indeed our clinical data examining 111 women shows that serum levels of MCP-1 are dependent on higher serum levels of PTH. In normal weight women, we show that higher levels of PTH are associated with higher MCP-1 levels, but not other cytokines such as CRP and adiponectin. In the obese population, MCP-1 levels are not high in all obese, but only higher in the presence of increased PTH. So it seems that the high serum PTH levels seen in the obese is upregulating the inflammatory cytokine MCP-1. Previously it has been shown that high PTH also upregulates other inflammatory cytokines such as IL-6 and TNF- α and decreases with parathyroidectomy. It was

interesting to see whether in patients with hyperparathyroidism, parathyroidectomy will also have an influence on serum levels of MCP-1. In addition it was interesting to understand whether other measures to reduce PTH levels such as calcium or vitamin D supplementation will also have an effect on MCP-1 levels.

More recently the Partridge lab showed that the anabolic action of intermittent PTH on bone requires osteoblastic MCP-1 expression (126). Our clinical data shows that high serum PTH levels in obesity may indeed upregulate serum levels of MCP-1; however it is not known whether it will also increase osteoblastic expression of MCP-1. If indeed there is a higher osteoblastic expression of MCP-1 in obese subjects with higher PTH, this should mediate a positive effect of the osteoblast. However, extraskelatal effects of high MCP-1 are well known especially an increased risk of CVD, atherosclerosis, diabetes etc. Given this, whether or not high serum levels of MCP-1 in obesity are beneficial to the osteoblast is still not known and needs to be addressed. Also, our data do not show that higher MCP-1 is associated with higher bone turnover markers and bone variables in the obese. The effect of high PTH and MCP-1 in obesity on bone should thus be further addressed in prospective studies with data on duration of high PTH. Overall both these experiments that employ a cross sectional or case control design generate preliminary data to support the hypothesis that there is an altered bone quality and that the higher PTH in obesity upregulates certain inflammatory cytokines that is not associated with higher bone turnover in the obese.

7.4 SUMMARY

There is a very high prevalence of obesity worldwide and is even more on the rise. Reports on higher fracture incidence in the obese are surprising and challenge the notion that obese are protective against bone loss due to higher BMD. There is thus a critical need to examine bone quality and the influence of altered hormonal milieu on bone in obesity. A pilot examination in this dissertation shows altered bone quality and hormonal milieu in the obese that also leads to up-regulation of certain inflammatory cytokines. Weight loss is advocated to reduce comorbidities associated with obesity, however is associated with bone loss. Nutrient modifications such as higher protein intake and adequate calcium intake but not higher intake of vitamin D may have a positive influence on bone during weight reduction. Overall the experiments in this dissertation provide evidence on the role of nutrients on bone loss and preliminary evidence on bone quality in obesity and may help provide rational recommendations for preserving bone health in overweight- obese dieting women.

8. APPENDIX: PRELIMINARY METHODS

8.1 Quality control analysis for RIA's in the laboratory

8.1.1. Inter-variability among serum intact Parathyroid hormone (PTH) assays - A need
for standardization

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The measurement of parathyroid hormone (PTH) is both common and important in the fields of endocrinology for research and clinical diagnosis of hyperparathyroidism. Variations in the estimation of (PTH) have been consistently reported in the literature (411-416), but have not focused on the more commonly used intact PTH (1-84) assays. Because we recently found marked variability between two PTH immunoradiometric assays (IRMA) in the lab, we evaluated PTH by a variety of methods by using commercially available intact PTH (1-84) assay kits. This report highlights variability in PTH assay methods obtained by using various methods and assays from different companies. The goal was to define the inconsistencies between PTH assays when values range from normal to slightly high levels.

To examine serum intact PTH (1-84), we compared 42 serum samples between different assays and methods. The 3 methods used include the following: a) enzyme linked immuno sorbent assay (ELISA); b) IRMA c) chemiluminescence. We assessed 6 commercially available 2nd generation assays. The ELISA kits were provided by ALPCO (ELISA-A), MD Biosciences (ELISA-B) and Immunodiagnostic Systems Inc, (IDS; ELISA-I) and IRMA kit by Scantibodies (IRMA-A) and Diagnostic System Laboratories

(DSL; IRMA-B). Furthermore, a subset of samples at the high and low end of our range (n=4) were examined using electro-chemiluminescence (Roche, Automated random analyzer) which is used by most clinical and hospital labs. It should be noted that a very common commercial method used previously (Nichols PTH assays) is no longer available, and therefore was not tested in this analysis. Samples were performed in duplicate for each assay. We examined healthy women (aged 59 ± 4.8 years; BMI 33 ± 5.5 kg/m²) with a relatively wide range of PTH.

The mean values and ranges for the 6 commercially available PTH assay kits are shown in Table 1. There was a good and better correlation between the ELISA A and B ($R^2 = 0.97$), compared to ELISA on ice ($R^2 = 0.71$). For the IRMA, assays A and B, there was also a good correlation ($R^2 = 0.93$), although absolute values differed markedly. The range of values in our samples and confidence interval in the IRMA-B assay is larger compared to all other methods.

Table 19: Comparison of PTH assays according to methodology

METHODOLOGY	ABBREV	COMPANY & sample size required for assay (ul)	NORMAL RANGE (given by company)	RANGE (in our samples)	95% CI (in our samples)	Mean ± SD (pg/ml)
Immunoradiometric assay	IRMA-A	Scantibodies, CA (200 ul)	9 - 77	19 - 98	34-50	42 ± 23
	IRMA-B	DSL, TX (200 ul)	9 - 55	21 - 228	83-115	99 ± 54
Enzyme linked immunosorbent assay	ELISA-A	Alpco Diagnostics, NH (25ul)	9 - 77	33 - 150	63-83	73 ± 32
	ELISA-B	MD Biosciences, MN (25 ul)	9 - 77	38 - 144	63-83	73 ± 34
	ELISA-I*	IDS, AZ (200 ul)	8 - 37	17 - 79	30-38	34 ± 14
Chemiluminiscence	Chemi	Roche – 100 ul	15 - 65	22-83	—	—

N = 42 samples (except for Chemi; includes two highest and lowest samples). *ELISA-I (done at 0° C on ice (I) compared to others done at 37°C)

Lack of standardization of PTH assays is a concern and may be partially due to the synthetic 1-84 PTH molecules that are made from different species and may alter absolute values in the different assays (412). There continues to be substantial methodological variation in the intact PTH assay, and therefore a single absolute level of serum PTH should not be used to define hyperparathyroid disease. It is suggested that when examining PTH values between labs or when switching assay companies (even if it is the same methodology), that a conversion factor is determined to address the expected differences between assays. Since higher values of PTH determine treatment of hyperparathyroidism, clinicians must be aware of quantitative differences between available intact PTH assays.

8.1.2 Vitamin D external Quality Assessment Scheme (DEQAS)

There has been a consider variability in 25OHD levels based on type of assays such as RIA, ELISA, HPLC etc and has been a potential concern for interpreting vitamin D status (417-419). To assess the performance of the 25OHD assay used in the Shapses lab, we first assessed values against those obtained using mass spectrometry . We compared 25OHD values in 6 serum samples at baseline and during or after one year of intervention using the Diasorin RIA from Shapses lab and using liquid chromatography-tandem mass spectrometry in the Soldin lab- The Georgetown University Bioanalytical Center (BAC) Georgetown University and Georgetown Clinical Research Center, Washington, DC, USA. It was interesting to note that values from the RIA assay in the Shapses lab was 28-30% lower than using mass spectroscopy.

Table 20 : Comparison of 25OHD values between Mass spectrometry and Diasorin RIA

BAC		Diasorin RIA		Percent Difference	
Baseline	Wk24/52	Baseline	Wk24/52	Baseline	Wk24/52
40	39	34.2	33.1	1.16	1.18
31	47	36	34.4	0.86	1.37
33	41	30	34.2	1.11	1.2
34	29	22.2	28.2	1.55	1.04
28	39	16.5	23.1	1.7	1.69
33.20	39.00	27.78	30.60	1.28	1.30
4.44	6.48	8.25	4.89	0.34	0.25

Mean
SD

The Vitamin D assessment scheme (DEQAS) is an international standardizing agency that aims to ensure the analytical reliability of 25OHD assays. This is achieved through distribution of serum pools with standard concentrations of 25OHD at regular intervals and statistical analysis of submitted results. Standards containing an unknown

concentration of 25OHD (nmol/L) were provided by DEQAS. These samples were assessed in the Shapses Lab using the Diasorin RIA. The final values were submitted to DEQAS. DEQAS examines the data and produces an All-Laboratory Trimmed Mean (ALTM), SD and CV. The final report is sent to the Shapses lab indicating the results. The report includes the trimmed mean, SD and CV for the major method groups, together with individual histograms. $ALTM \pm 25\%$ is considered as pass.

Table 21: Comparison of 25OHD (nmolL) values between Shapses lab and ALTM values of DEQAS

	Assessment 1 (Mar 2011)		Assessment 2 (Nov 2010)		Assessment 3 (July 2010)	
Samples	DEQAS (Range , Mean)	Shapses Lab (Mean)	DEQAS (Range , Mean)	Shapses Lab (Mean)	DEQAS (Range , Mean)	Shapses Lab (Mean)
a	23.5-39.1 (31.3)	27.6	65.6- 109.4 (87.5)	75.5	35.3-58.8 (47.0)	71.3
b	44.7-74.5 (59.6)	56.8	30.0-50.0 (40.0)	36.8	14.4-24.0 (19.2)	26.0
c	57.5-95.9 (76.7)	69.0	56.9-94.8 (75.8)	64.0	55.4-92.3 (73.8)	75.5
d	36.3-60.5 (48.4)	45.6	22.4-37.3 (29.8)	23.5	33.0-55.0 (44.0)	54.3
e	44.2-73.6 (58.9)	50.2	44.0-73.4 (58.7)	47.5	45.2-75.4 (60.3)	79.5

	Assessment 4 (June 2010)		Assessment 5 (Mar 2010)		Assessment 6 (Jan 2010)	
Samples	DEQAS (Range , Mean)	Shapses Lab (Mean)	DEQAS (Range , Mean)	Shapses Lab (Mean)	DEQAS (Range , Mean)	Shapses Lab (Mean)
a	36.1-60.1 (48.1)	47.9	36.4-60.6 (48.5)	47.9	16.6-27.6 (22.1)	17.5
b	50.0-83.4 (66.7)	69.2	49.7-82.9 (66.3)	69.2	30.5-50.8 (40.6)	31.0
c	13.1-21.9 (17.5)	17.2	13.2-22.0 (17.6)	17.2	48.8-81.3 (65.0)	47.3
d	33.8-56.4 (45.1)	52.0	34.0-56.6 (45.3)	52.0	65.4-109.0 (87.2)	59.0
e	18.5-30.9 (24.7)	25.5	18.5-30.9 (24.7)	25.5	37.8-63.0 (50.4)	38.8

8.2 Precision data for DXA

Background: The ISCD position statement 2007 (333) suggests the following

- *Each DXA facility should determine its precision error and calculate the LSC.*
- *The precision error supplied by the manufacturer should not be used.*
- *If a DXA facility has more than one technologist, an average precision error combining data from all technologists should be used to establish precision error and LSC for the facility, provided the precision error for each technologist is within a pre-established range of acceptable performance.*
- *Every technologist should perform an in vivo precision assessment using patients representative of the clinic's patient population.*
- *Each technologist should do one complete precision assessment after basic scanning skills have been learned (e.g., manufacturer training) and after having performed approximately 100 patient-scans.*
- *A repeat precision assessment should be done if a new DXA system is installed.*
- *A repeat precision assessment should be done if a technologist's skill level has changed.*

Methods: 33 Subjects were measured 2 times, repositioning the patient after each scan twice at the femoral neck, hip, spine, radius and total body. Least significant changes were calculated using average and standard deviation of the two values.

CV= SD/average; LSC= CV *2.77

Table 22: Comparison of Least significant changes at bone sites between Shapses Lab and ISCD.

Site	ISCD (LSC %)	Shapses Lab(LSC %)
Lumbar Spine	5.3	3.5
Femoral Neck	6.9	4.2
Total Hip	5.0	2.3
Total Body	NA	2.6
Radius	NA	6.1

Conclusions: Our least significant change values at all sites are below the maximum recommended values (2.2-6.1%).

8.3: Comparison of 8 vs 24 hour pooled urine samples for estimation of calcium absorption

Aim: We have previously analyzed whether a 10 hour pooled urine sample has a similar identical calcium isotope excretion compared to a 24 pooled collection. The goal was to understand whether a 8 hour pooled sample has identical calcium isotope excretion compared to a 24 pooled collection.

Subjects and Methods: Ca^{42} and Ca^{43} ratio was measured in 14 women at 8 hours and 24 hours after isotope administration using ICMS.

Results: The $\text{Ca}^{43/42}$ ratio was 19% lower in the 8 hour sample as compared to the 24 hours sample. The correlation between the 24 hour and 8 hours sample was strong ($r=0.8$, $p < 0.05$), however absolute values were lower.

Conclusions: A 8 hour pooled urine sample is not an appropriate method to estimate calcium absorption.

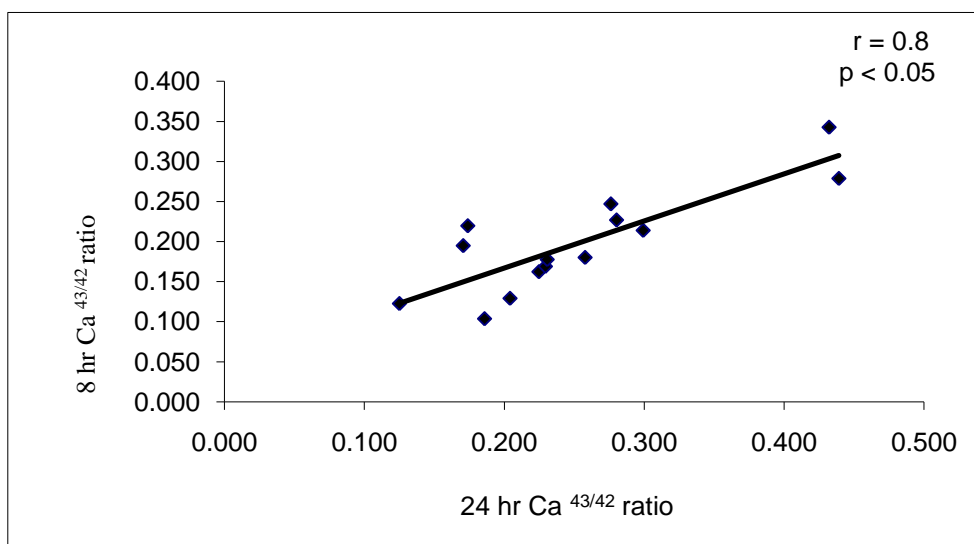


Figure 19: Comparison of calcium isotope excretion at 8 vs 24 hour urine collection

8.4 MCP-1 does not reduce after weight reduction if PTH remains elevated

Aim: Previous studies have shown that weight reduction reduced serum levels of MCP-

1. Our previous findings in women showed that women with higher PTH have higher MCP-1 levels. We thus hypothesized that MCP-1 levels will only decline with weight loss if there is a decrease in PTH.

Subjects and Methods: In a group of 26 women who underwent gastric bypass surgery we analyzed PTH and cytokines before and after 6 months of weight reduction..

Results:

Table 23: MCP-1 and PTH levels before and after massive weight loss in 26 women

	Baseline	6 months	p
Weight (lbs)	309 ± 66	225 ± 79	<0.0001
MCP-1 (pg/ml)	491 ± 526	525 ± 726	0.212
Adiponectin (ng/mL)	5230 ± 2780	8339 ± 7940	0.006
CRP (ng/mL)	8345 ± 4400	4435 ± 3493	<0.0001
PTH	78 ± 30	77 ± 31	0.859

Conclusions: Weight loss is not associated with a reduction in MCP-1 if PTH remains elevated.

8.5 Standard operating procedures

8.5.1 Vertebral Exclusion

Measurement errors in a lumbar spine measurement are due to a local structural change or artifact. Furthermore osteoarthritis seen in the obese can also influence lumbar spine measurements. A vertebral exclusion assessment improves the precision of a L1-L4 measurement. The vertebral body assessment for exclusion followed the criteria specified by the ISCD 2007 Official Positions (333). This analysis was employed in aim 1 of this dissertation.

- *Use L1-L4 for spine BMD measurement*
- *Use all evaluable vertebrae and only exclude vertebrae that are affected by local structural change or artifact. Use three vertebrae if four cannot be used and two if three cannot be used*
- *Anatomically abnormal vertebrae may be excluded from analysis if:*
 - *They are clearly abnormal and non-assessable within the resolution of the system; or*
 - *There is more than a 1.0 T-score difference between the vertebra in question and adjacent vertebrae*
- *When vertebrae are excluded, the BMD of the remaining vertebrae is used to derive the T-score*

8.5.2 High Resolution Scan to examine trabecular microarchitecture.

A High Resolution scan is performed to reveal the microarchitecture of the trabecular and the following steps explain how to export a scan from XCT 3000.

1. Obtain scan at 0.2mm thickness at the 4% slice.
2. Note the CT# of the HiRes file that you want to export (ie 2000**).
3. Main menu > OPTIONS
4. EXPORT
5. Select the drive location (best to select your local drive C)
6. Select CT number
7. Give the CT# to export (ie 2000**)
8. What this will do is create a AV002 directory in C.
9. Exit the program.
10. Just copy everything in this AV002 (onto a memory stick) and send for analysis.

Output variables: Trabecular number, thickness, separation, maximum hole size, hole avg, BV/TV (Bone volume/Total Volume).

8.5.3 Bone marrow fat analysis

The bone marrow fat (BMF) is a useful indicator of extent of adiposity in the bone marrow that has been shown to be inversely correlated with BMD (420) . Bone marrow fat has been previously assessed using the gold standard whole-body magnetic resonance imaging (MRI), however it is also a possible output variable assessed using the pQCT. Whether or not the tibial BMF assessed by the pQCT is comparable to that obtained by the MRI is not known and needs to be addressed in future studies.

To compute tibial bone marrow fat from the 4% slice of tibia.

1. Delete ROI for slice 4 muscle bone area
2. Select calcbd, Set contor mode=2, peel mode=2

4. Inner threshold= 100
5. Run and rename as marrow fat
6. The trab density value reflects bone marrow fat value

8.5.4 Protein score

In aim 1 of this protocol, a protein score was used to assess compliance to a high protein intake. The protein score is based on 4 factors; increase from baseline to intervention, avg of protein intake during intervention, % protein intake during intervention and an increase from baseline in BUN. Percent protein doesn't provide consistent information about protein intake due to variable caloric intake, so this alone cannot be used to assess total protein intake. But this is important to include since this helps to define the proportion of the diet that contains protein, and therefore should contribute to physiological outcomes. Total protein is good estimate, but can't be the only way to define protein intake because a large person who only has 83 g/d, is actually consuming slightly lower amount for their body size. Delta protein intake is important, so we know how they've modified their intake due to the intervention, and ensure it differs between the 2 groups. BUN is an important biological marker and also would help define delta protein intake (but has the limitation of being assessed at only 3 time points (4, 24 and 52 wk) vs. diet information collected monthly (11 time points), so this is not used alone to estimate the response of bone to protein intake. All participants can receive a maximum score of 8 based on their protein intake and BUN values. The protein score is based on 4 parameters:

Delta increase from baseline to avg of protein intake from 1-12 mo :Maximum

score given =1. (Each subject is scored from -1 to +1 (HP = < 5 g increase from baseline=0; +25-49% =0.3; +50-90% = 0.6; +100% =1; same for negative (NP))

Average protein intake: Maximum score give = 3 (We calculated median protein intake (76.1)

and calculated difference from median for each person and then gave a score of +3 to -3 (+/- 5 = .5; +/- 5-10 =1; +/- 11-15 = 1.5; +/- 16-20 = 2; +/- 21-25 = 2.5; +/- 26+ = 3))

% protein intake: Maximum score give = 3 (We calculated median % protein intake (19.7) and

calculated difference from median for each person and then gave a score of +3 to -3 (0-2 = 1; 2-4 = 1.5; 4-6 = 2; 8-10=3))

Delta increase in BUN: Total possible BUN score = 1 (We averaged BUN values (4, 24, 52)

or (4, 24) and calculated delta increase from baseline and a score of + 3 to -3 was given).

On an average we had at least 34 days of food diaries. We take 3 day avg for FD which means we had FD at least in 11 time points for each person. But, we have BUN data only at 3 time points (apart from wk-0), hence we decided that BUN is weighed less while diet is weighed more to calculate total protein score.

8.6 Adverse events reporting for aims

11 adverse symptoms were recorded for Aim 1 and Aim2 for all participants who completed the study and was reported to the External data safety monitoring board (DSMB)- Shapses DSMB report 2011

8.6.1 Frequency of symptoms- Aim 1 (NP and HP treatment)

To assess if the frequency of adverse symptoms (AS) assessed between high protein and normal protein differs by treatment groups.

11 AS were assessed once every month during the 12 month study and the frequency of occurrence of each event at baseline, 6 months and 12 months in the HP and NP group is presented. Results show no statistically significant differences for the change between groups for individual symptoms except dizziness that decreased more in HP than NP group ($p < 0.05$).

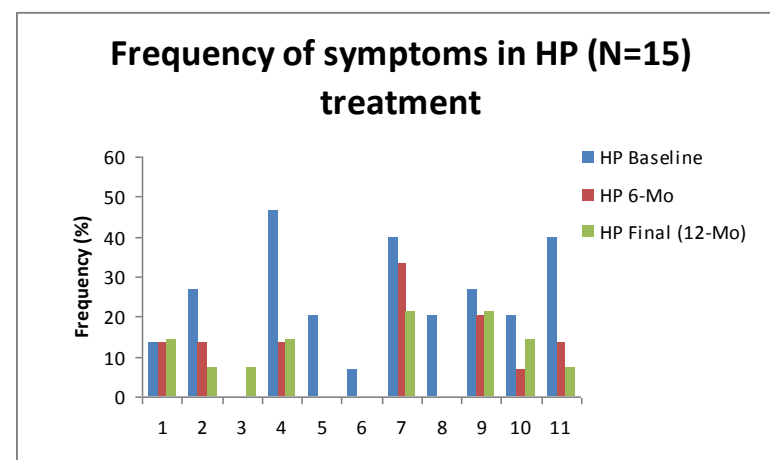
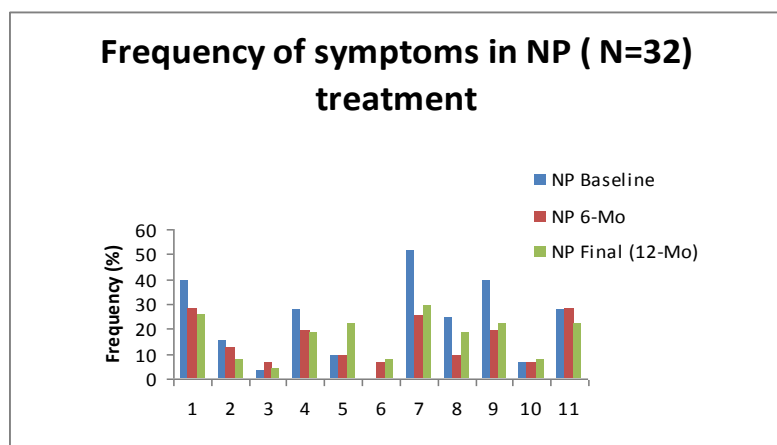


Figure 20: Frequency of adverse symptoms in protein study- Aim 1

1 = Pain or heaviness in legs

2 = Swelling in the legs

3 = Pain or heaviness in the chest

4 = Headaches

5 = Dizziness

6 = Nausea

7 = Fatigue

8 = Muscle Weakness

9 = Urinary Frequency

10 = Abdominal Pain

11 = Muscle Aches

8.6.2 Frequency of symptoms- Aim 2 (Vitamin D and placebo)

To assess if the frequency of adverse symptoms assessed between high protein and normal protein differs by treatment groups. 11 AS were assessed once every month during the 6 week study and the frequency of occurrence of each event at baseline and after 6 weeks in the vit D and placebo group is presented. Results show greater frequency ($p = 0.036$) for headaches at 6 weeks in treatment A as compared to treatment B. (Note: A subset of these women were enrolled in a follow up study to continue same treatment for a 1 year study and there was no effect of treatment on headaches in at 6 months).

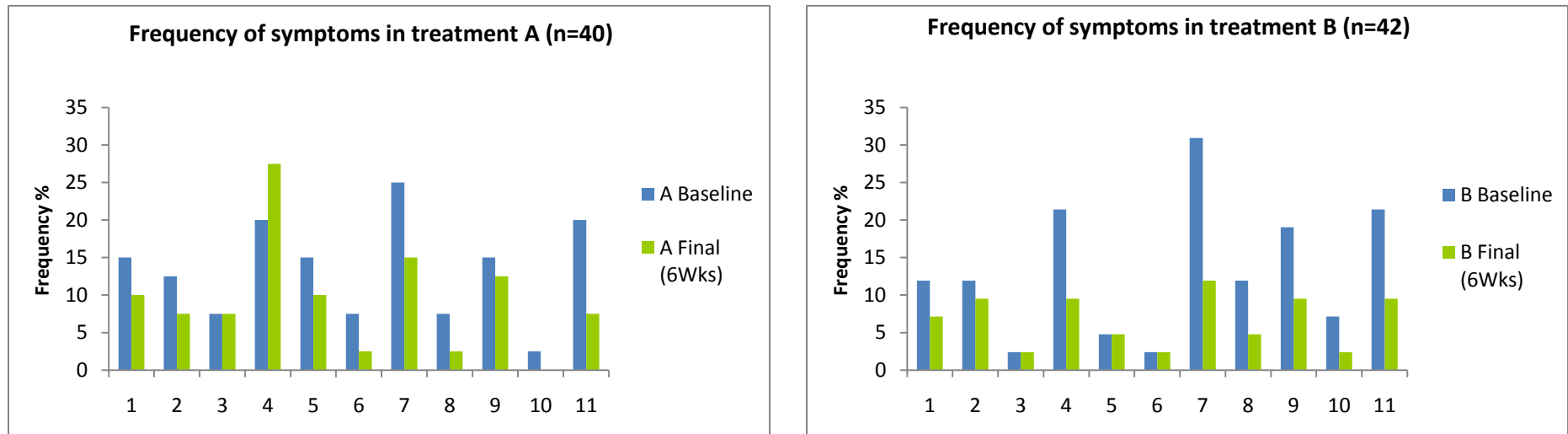


Figure 21: Frequency of adverse symptoms in vitamin D study- Aim 2

1 = Pain or heaviness in legs	2 = Swelling in the legs	3 = Pain or heaviness in the chest	4 = Headaches	5 = Dizziness	6 = Nausea
7 = Fatigue	8 = Muscle Weakness	9 = Urinary Frequency	10 = Abdominal Pain	11 = Muscle Aches	

8.7 Calculation of dietary measures of acid production due to high protein intake.

Aim: The negative effect of dietary protein on bone is primarily attributed to the increase in endogenous acid production that ultimately leads to bone resorption to neutralize pH. The Potential Renal Acid Load (PRAL), Net Endogenous Acid Production (NEAP), and Sulfur (mEq) levels are dietary measures of acidity and was assessed in Aim 1.

Methods: Using the food diaries, PRAL, NEAP and sulfur levels were analyzed in aim 1 of this dissertation in 26 women consuming the HP diet and 21 women consuming the NP diet (421;422) and was calculated using the following equations

$$\text{PRAL} = 0.49 \text{ Protein (g)} + 0.37 \text{ Phosphorus (mg)} - 0.021 \text{ Potassium (mg)} - 0.02 \text{ Magnesium (mg)} - 0.13 \text{ Calcium (mg)}$$

$$\text{NEAP} = 54.5 [\text{Protein (g)}/\text{Potassium(mEq)}] - 10.2$$

$$\text{Sulfur (mEq)} = 2 [\text{Methionine (mg)}/149.2] + 2 [\text{Cystine (mg)}/240.3]$$

Results: At baseline, the levels of PRAL, NEAP and Sulfur were similar between groups. However during the intervention the PRAL, NEAP and sulfur were higher in the HP group ($p < 0.05$) and sulfur was lower in the NP group.

Table 24: Dietary measures of acid production at baseline and intervention in HP and NP diets

Baseline

Variable	HP diet	NP diet	P value
PRAL(mEq/d)	14.8 ± 16.6	13.8 ± 17.6	0.85
NEAP	57.9 ± 17.3	58.4 ± 19.9	0.93
Sulfur (mEq)	8.8 ± 9.2	6.6 ± 6.6	0.38

Intervention

Variable	HP diet	NP diet	P value
PRAL (mEq/d)	18.2 ± 9.5	10.2 ± 8.4	0.005
NEAP	61.6 ± 10.6	54.4 ± 13.1	0.05
Sulfur (mEq)	9.0 ± 6.0	6.0 ± 3.4	0.03

Conclusion: A high protein intake of 24% of total calories is associated with significantly higher dietary acid measures.

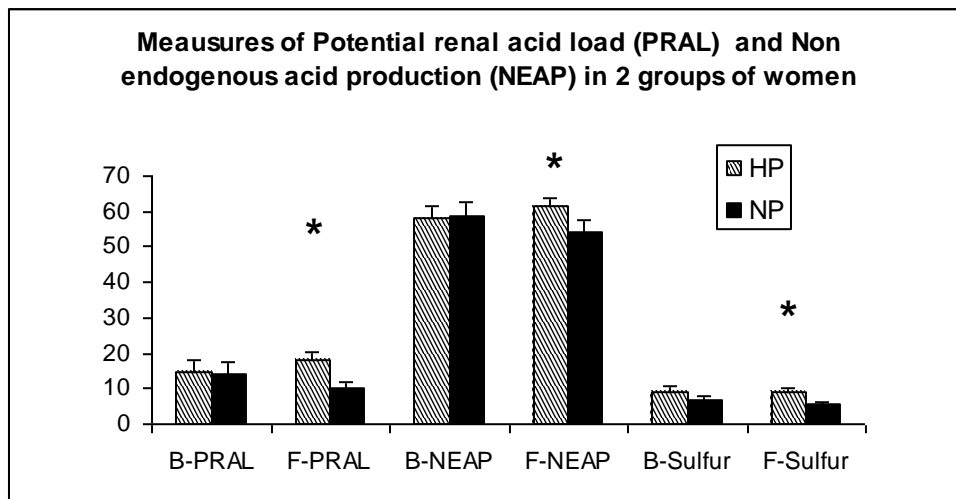


Figure 22: Differences in PRAL and NEAP in women with 2 levels of protein intake during the intervention

*p<0.05

8.8 Sources of protein in diets

Aim: To identify sources of protein in diets in aim 1 of this dissertation in women consuming a HP or NP diet.

Methods: Protein sources were identified using food diaries and food frequency questionnaire and the following was used to estimate 1 serving size

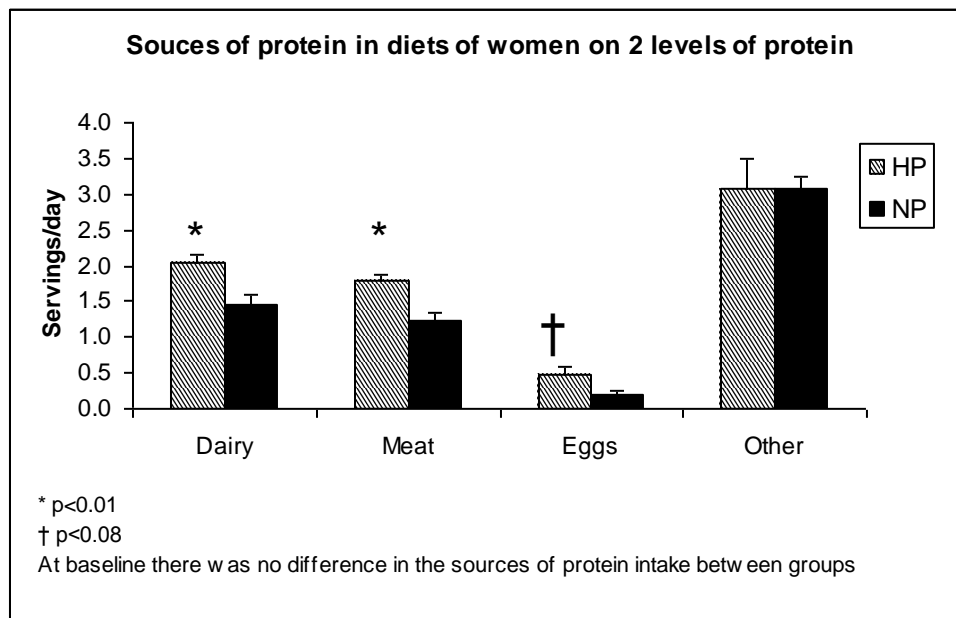


Figure 23: Sources of protein at baseline and after 12 months in women on 2 levels of protein intake

Servings = Dairy (8 oz. Milk, 6 oz. Yogurt, 1 Slice of Cheese), **1 Large Egg**), **Meats, Fish, Poultry** (3-4 oz. serving cooked), **Other High Protein Foods**= Beans, Tofu, Lentils, Legumes (1 cup), Grains (including Bread – 1 slice / Pasta ½ cup), Peanut Butter (2 Tablespoons), Nuts (roasted, raw, ½ cup / 3 oz.) Soy Milk (8oz. / 1 cup)

Conclusions: During the 1year intervention the intake of dairy, meat and eggs was higher in the HP compared to NP diet.

8.9 Physical activity assessment in Aim 1

Aim : To assess physical activity levels of participants in Aim 1, during the year long intervention. This is important to assess to ensure that a higher physical activity that has shown to preserve bone during weight loss is not a confounding factor that influenced results in this study.

Methods: To assess the physical activity levels 2 data collection methods were used

- a) Subjects were given pedometers and were asked to record steps per day (Scoring of 0-3 was based on (423)

(Score): Description:

- (1) None: < 5000 steps/d
- (2) Low: 5000-7499 steps/d
- (3) Moderate: 7,500-9,999 steps/d
- (4) High: >10,000 steps/d

- b) Subjects were asked to list type, duration and frequency of activity. Scoring of 0-3 was based on (424;425)

(Score): Description

- (1) None: <180 METs*min/wk = < 20mins/d moderate activity 3 days/week
- (2) Low: 180-449 METs*min/wk
- (3) Mod: 450-750 METs*min/wk = >30 mins/d moderate activity 5 days/week or > 20 mins/d vigorous activity 3 days/week

- (4) High: $>750 \text{ METs} \cdot \text{min}/\text{wk} = >30 \text{ mins}/\text{d}$ moderate activity 5 days/week
 AND $> 20 \text{ mins}/\text{d}$ vigorous activity 3 days/week, OR $> 60 \text{ mins}/\text{d}$
 moderate activity 5 days/week OR $> 40 \text{ mins}/\text{d}$ vigorous activity 3
 days/week

Results:

Based on 0-3 point assessment from both these methods, a cumulative score was given at baseline and an average during the intervention.

Table 25: Cumulative score for physical activity at baseline and during intervention in both groups

	High protein		Normal protein		P value for delta
	Baseline	Intervention	Baseline	Intervention	
Cumulative score	1.56 ± 1.26	1.95 ± 0.96	1.50 ± 0.94	1.84 ± 0.76	0.88

Conclusions:

There was no significant difference in physical activity levels in both groups at baseline or during the intervention.

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10. CURRICULUM VITAE

DEEPTHA SUKUMAR

EDUCATION

2011	PhD, Nutritional Sciences, Rutgers, The State University of New Jersey, New Brunswick, NJ
2004	M.Sc. Foods and Nutrition, University of Madras, Chennai, India
2002	B.Sc. Nutrition, Food service management and Dietetics University of Madras, Chennai, India

PROFESSIONAL EXPERIENCE

2007- 2011	Graduate Research Assistant , Rutgers, The State University of New Jersey, New Brunswick, NJ
2006- 2007	Teaching Assistant, Rutgers, The State University of New Jersey, New Brunswick, NJ
2004- 2005	Chief Dietitian and teaching instructor, Malar Hospitals, Chennai India

PUBLICATIONS

Sukumar D, Partridge N, Xiangbing X, Shapses SA “The high serum monocyte chemoattractant protein-1 in obesity is influenced by high parathyroid hormone and not adiposity. Journal of Clinical Endocrinology and Metabolism. 2011 Jun;96(6):1852-8

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