CALORIC RESTRICTION ON BONE QUALITY IN MEN, AND THE EFFECT OF THREE DOSES OF VITAMIN D (3DD) ON BONE AND INSULIN RESISTANCE IN OLDER WOMEN

By

LILIANA CLAUDIA POP

A Dissertation submitted to the Graduate School – New Brunswick Rutgers, The State University of New Jersey in partial fulfillment of the requirements for the degree of Doctor of Philosophy Graduate program in Nutritional Sciences written under the direction of Sue A Shapses and approved by

________________________________________
________________________________________
________________________________________
________________________________________
________________________________________

New Brunswick, New Jersey
October 2016
ABSTRACT OF THE DISSERTATION

CALORIC RESTRICTION ON BONE QUALITY IN MEN, AND THE EFFECT OF THREE DOSES OF VITAMIN D (3DD) ON BONE AND INSULIN RESISTANCE IN OLDER WOMEN

By LILIANA CLAUDIA POP

Dissertation Director:
Dr. Sue A Shapses

Caloric restriction (CR) results in loss of bone mass in older populations and has specifically been shown in women, but studies examining multiple bone sites and bone quality in response to weight loss (WL) in men are missing. In our six-month WL intervention, there was no reduction in bone mineral density (BMD) ay any anatomical sites in overweight and obese men. Also, WL did not affect cortical and trabecular bone and geometry. On the contrary, despite increased BMD at some sites when maintaining excess body weight, our results showed a trend for compromised cortical bone in the weight maintenance (WM) group.

Postmenopausal women undergoing weight control to improve health outcomes, are particularly at risk for bone loss, and might benefit from supplemental vitamin D intake above the recommended allowance. A second study in this dissertation examined whether vitamin D supplementation, in healthy overweight/obese older women, affects bone mineral density (BMD) and bone structural parameters in a one year-long.
randomized, double-blind controlled trial. Results showed that higher vitamin D supplementation prevented the decline in cortical thickness, and there was a trend to attenuate trochanter BMD in older women over one year. Evidence suggest that vitamin D supplementation influences osteocalcin (OC) levels during short-term caloric restriction and this results in lower insulin resistance (IR). Hence, we addressed whether vitamin D affects markers of insulin sensitivity and OC measures in healthy overweight/obese older women over one year. We found a modest effect of vitamin D supplementation on glycemic markers and no influence on OC measures. Overall, these studies examine bone structural parameters in response to weight loss in men and the effects of vitamin D supplementation on bone quality and metabolic health in older women.
ACKNOWLEDGMENTS

I have been a very fortunate person through this journey and met so many wonderful people I have to thank for helping with my accomplishments. First, I would like to thank my wonderful advisor, Dr. Sue Shapses to whom I owe my Ph.D. Without her patience, guidance and friendship none of this could have been finalized. She is a great inspiration and her passion for science and dedication to her students have made me a better person in every aspect of my life. Thank you for teaching me kindness!

My Committee members have provided support and guidance with this work. I would like to thank Dr. Beverly Tepper, Dr. Daniel Hoffman, Dr. Stephen Schneider and Dr. Xiangbing Wang.

I would also like to express my gratitude to Dr. Yvette Schlussel for her assistance with statistical analysis, Hasina Ambia Sobhan for her support in the lab and Robert Zurfluh for his help with the clinical studies.

Special thanks to Dr. Deeptha Sukumar for her friendship and support.

My gratitude goes to all the volunteers who made this work possible.

Thank you to all the undergraduates who helped with data and laboratory work.

I would also like to thank the faculty in Nutritional Sciences Department for their passion and dedication to teaching.

Many thanks to DJ Polacik for her help and support with orders and many questions I had throughout the years.
Thanks to all my graduate fellows for their friendships: Amanda, Angela, Leslie, Marc, and Pam.

I am grateful for having wonderful parents who have helped me immensely with raising my son. I could not have done it without you. Thank you, Vlad, for your patience and for saying “I love you, mommy!” every single Skype call.

Catalin, thank you for being there, for listening and for giving me strength. Thank you for being patient and understanding. Thank you for loving me unconditionally.
Acknowledgment of previously published and collaborative work

Chapter 1 presents data originally published in Endocrine Practice Journal in August 2015. Figure 3 has been reprinted with permission from:


Chapter 2 of this dissertation was originally published in American Journal of Clinical Nutrition in March 2015. The manuscript was reprinted with permission from:

# TABLE OF CONTENTS

ABSTRACT OF THE DISSERTATION .......................................................................................... ii

ACKNOWLEDGMENTS ........................................................................................................ iv

ACKNOWLEDGMENT OF PREVIOUSLY PUBLISHED AND COLLABORATIVE WORK .............................................................................................. vi

LIST OF TABLES .................................................................................................................. x

LIST OF FIGURES ............................................................................................................... xii

CHAPTER 1: OVERVIEW .......................................................................................................... 1

Introduction .......................................................................................................................... 2

Background .......................................................................................................................... 5

Bone physiology .................................................................................................................... 5

Determinants of bone strengths and evaluation methods ...................................................... 7

Regulation of bone metabolism ........................................................................................... 10

Obesity and bone metabolism ............................................................................................... 13

Total, free and bioavailable sex steroids and bone ................................................................. 15

Weight loss and bone metabolism ......................................................................................... 16

Vitamin D physiology ......................................................................................................... 17

Vitamin D status, dietary reference intake (DRI) and sources of vitamin D ....................... 23

Body weight and vitamin D ................................................................................................. 25
Vitamin D and skeletal health ................................................................. 29  
Vitamin D and extra-skeletal health .................................................... 33  
Osteocalcin ......................................................................................... 35  
Rationale ............................................................................................ 38  
SPECIFIC AIMS OF THE DISSERTATION .............................................. 39  

CHAPTER 2: MODERATE WEIGHT LOSS IN OBESE/OVERWEIGHT MEN  
PRESERVES BONE QUALITY ................................................................. 40  
Abstract ............................................................................................. 41  
Introduction ......................................................................................... 42  
Subjects and methods ........................................................................... 43  
Results ................................................................................................ 50  
Discussion ............................................................................................ 54  
Acknowledgments and Funding Source ................................................. 59  

CHAPTER 3: THREE DOSES OF VITAMIN D, BONE MINERAL DENSITY AND  
GEOMETRY IN OLDER WOMEN DURING MODEST WEIGHT CONTROL IN A 1-  
YEAR RANDOMIZED CONTROLLED TRIAL ........................................ 68  
Abstract ............................................................................................. 69  
Introduction ........................................................................................ 70  

viii
Subjects and Methods ........................................................................................................ 71
Results ............................................................................................................................. 79
Discussion ....................................................................................................................... 84
Acknowledgments and Funding Sources ......................................................................... 90

CHAPTER 4: THREE DOSES OF VITAMIN D ON INSULIN RESISTANCE AND
OSTEOCALCIN MEASURES IN OLDER WOMEN ......................................................... 99

Abstract .......................................................................................................................... 100
Introduction ..................................................................................................................... 102
Subjects and Methods .................................................................................................. 104
Results ............................................................................................................................ 108
Discussion ....................................................................................................................... 110
Acknowledgments and Funding Sources ....................................................................... 114

CHAPTER 5: CONCLUSIONS ......................................................................................... 120

APPENDIX ..................................................................................................................... 131

Literature cited ............................................................................................................... 145
LIST OF TABLES

Table 1. Systemic and local regulators of bone metabolism ........................................... 12
Table 2. Studies with vitamin D supplementation on bone mineral density (BMD) in older women.............................................................. 32
Table 3. Studies examining the relationship between glucose and insulin metabolism and measures of osteocalcin (OC) .............................................................................. 36
Table 4. Body composition and areal bone mineral density (BMD) ....................... 61
Table 5. Trabecular and cortical volumetric bone mineral density, geometry and strength at the tibia.................................................................................................................. 62
Table 6. Sex steroids and bone regulating hormones .............................................. 63
Table 7. Multiple linear stepwise regression analysis of the explanatory variables for changes in bone mineral density (BMD) at the femoral neck, total body and cortical thickness.......................................................................................................................... 64
Table 8. Nutrient intake at baseline and average estimate over 6 months of intervention 65
Table 9. Body composition and aBMD over 12 months at three doses of vitamin D3 .... 91
Table 10. Trabecular and cortical vBMD, geometry, strength and microstructure over 12 months at three doses of vitamin D3 ........................................................................................................................................ 92
Table 11. Hormones and bone markers over 12 months at three doses of vitamin D3 .... 93
Table 12. Nutrient intake over 12 months at three doses of vitamin D3 .................... 94
Table 13. Adverse events over 12 months at three doses of vitamin D3 .............. 95
Table 14. Baseline characteristics in the three vitamin D treatment groups .......... 115
Table 15. Physical activity level scoring ................................................................................ 138
Table 16. Physical activity level in Aim 1 ........................................................................... 138
Table 17. Physical activity level in Aim 2
LIST OF FIGURES

Figure 1. Determinants of bone density, strength and fragility and bone properties assessment .......................................................................................................................... 10
Figure 2. Vitamin D metabolism in the body ................................................................................................. 20
Figure 3. Serum 25-hydroxyvitamin D (25OHD), vitamin D binding protein (DBP) and
estradiol concentrations ............................................................................................................................ 22
Figure 4. Conversion rate of 7-dehydrocholesterol to vitamin D depending on time of the
day and season in Boston (42° North). ................................................................................................... 24
Figure 5. Flowchart of study participants. ...................................................................................................... 66
Figure 6. Changes in serum 25-hydroxyvitamin D [25OHD], parathyroid hormone
(PTH), total testosterone (T) and estradiol during the intervention in men adhering to
weight loss (WL) and weight maintenance (WM) (n=38). ........................................................................... 67
Figure 7. Flowchart of study participants .................................................................................................... 96
Figure 8. The percent change in bone parameters over 12 months at three doses of
vitamin D3 ........................................................................................................................................... 97
Figure 9. Changes in 25OHD and PTH over 12 months at three doses of vitamin D3. ... 98
Figure 10. Study flowchart ............................................................................................................................. 116
Figure 11. Changes in serum 25-hydroxyvitamin D (25OHD), osteocalcin (OC),
dercarboxylated OC (ucOC) and ucOC/OC after 12 months of vitamin D treatment 117
Figure 12. Changes in markers of insulin resistance and sensitivity after 12 months of
vitamin D treatment ...................................................................................................................................... 117
Figure 13. Oral glucose tolerance test and computed area under the curve after 12 months
of vitamin D3 treatment ............................................................................................................................. 118
Figure 14. Changes in skin reflectance from winter to summer ......................... 142

Figure 15. Comparison for changes in skin reflectance from winter to summer........ 143

Figure 16. Serum 25OHD at 6 months and 12 months of vitamin D supplementation.. 144
CHAPTER 1.

OVERVIEW
Introduction

Osteoporosis affects millions of people worldwide. Progressive reduction in bone mass and structural deterioration of bone tissue cause reduced mechanical strength and increased bone fragility accompanying osteoporosis and resulting in increased fracture risk (Seeman, 2003; Felsenberg and Boonen, 2005). One in two women and one in four men over the age of 50 will have an osteoporosis-related fracture in their lifetime. By the year 2020, it is estimated that more than 61 million women and men in this age category will have osteoporosis or low bone mass (CDC, 2011). In addition to the economic burden, osteoporosis reduces the quality of life for individuals who experience fractures. One in three adults remains hospitalized in a nursing home for at least a year following hip fracture and one in five patients over the age of 50 die due to associated medical complication in the year following their hip fracture (NOF, 2011). Vertebral fractures can also have a severe health impact, resulting in chronic back pain and disability and increased mortality in older people (NOF, 2011). Although, there is a gender gap between women and men, mainly due to accelerated loss of bone mass in the years immediately after menopause in women and a delay in the hormonal changes associated with aging in men compared to women (Yialamas and Hayes, 2003), bone loss occurs at older ages independently of gender.

Obesity is another growing health burden at a global scale and a leading cause of cardiovascular disease, type 2 diabetes and certain cancers (WHO, 2015; Ogden et al., 2012). A lack of balance between caloric intake and energy expenditure contributes to this condition causing unhealthy body weight in approximately 1.9 billion adults worldwide in the year 2014 (WHO, 2015). A two-fold increase in obesity prevalence
between 1980 and 2014 (WHO, 2015) has also caused a great increase in healthcare expenditures, obesity accounting for 21% of healthcare costs (Cawley and Meyerhoefer, 2012). A healthier lifestyle with the ultimate goal of losing excess body weight by increasing energy expenditure through physical activity and reducing excess caloric intake could prevent and decrease the incidence of several comorbidities associated with obesity. Although weight loss improves health outcomes, it also results in bone loss, and this is well established in women (Shapses and Sukumar, 2012). However, differences in the aging-related pattern of hormonal changes between genders could also differentially influence bone loss due to weight loss. Nevertheless, clinical interventions in men addressing the effect of weight loss on bone mass and quality are lacking and is examined in this dissertation.

Postmenopausal women are at high risk of osteoporosis and undergoing weight control plans, designed to attenuate health due to obesity complications, could further exacerbate bone loss (Shapses and Sukumar, 2012). It has been established that vitamin D and Ca have beneficial effects on bone (IOM, 2011). However, the response to vitamin D supplementation is attenuated in the obese compared to the non-obese individuals (Gallagher et al., 2012; Drincic et al., 2013) and suggests that higher doses of vitamin D are required to reach sufficiency in obesity. However, whether additional vitamin D supplementation could further attenuate bone mass and quality is not known and will be examined in this dissertation.

The effects of the vitamin D endocrine system extend beyond calcium and bone homeostasis. However, the relationship between vitamin D and non-skeletal outcomes, such as insulin resistance, cardiovascular events, and cancer is not entirely clear (IOM,
2011; Rosen, 2012). For example, vitamin D status affects pancreatic beta-cell function and can alter insulin resistance in animal models and has also been shown in smaller clinical trials. However, studies with vitamin D supplementation have been inconsistent. This dissertation will address whether vitamin D supplementation in older postmenopausal women affects glucose homeostasis and insulin resistance. It has been shown that vitamin D increases osteocalcin gene expression, a bone formation marker that recently has emerged as a regulator of glucose and insulin metabolism. In addition, OC levels change in response to short-term vitamin D supplementation resulting in lower insulin resistance (Sukumar et al., 2015). In animal models, the undercarboxylated form of OC is considered the active hormone. The hypothesis that vitamin D effects on markers of IR are modulated by osteocalcin or its undercarboxylated form is also examined in this dissertation. Overall, it is important to determine factors influencing bone quality during caloric restriction in both older men and women, and establish nutrient recommendations to attenuate the risk of developing osteoporosis and metabolic complications.
**Background**

**Bone physiology**

Bone is a highly specialized organ that constitutes the rigid framework of the body and has a great ability to regenerate and repair. Bone fulfills the essential metabolic function within the body, including its role as a reservoir of minerals (especially calcium and phosphorus), maintaining their homeostasis. In addition, several cytokines and growth factors such as insulin-like growth factors, transforming growth factor-b and bone morphogenetic proteins can be released upon bone resorption and act both locally and systemically. Moreover, bone provides an environment for marrow (blood cells forming and fat storage) and maintains the acid – base equilibrium in the blood (Taichman, 2005).

Furthermore, bone has emerged in recent years as an endocrine organ. Bone regulates phosphate metabolism by releasing fibroblast growth factor (FGF-23), which reduces renal phosphate reabsorption (Garnero, 2014). Osteocalcin, also produced by the bone cells is involved in glucose homeostasis and energy metabolism. Osteocalcin enhances both the insulin secretion and sensitivity, in addition to boosting up the number of insulin-producing β cells and reducing stores of fat (Lee et al., 2007).

Throughout life, in response to external biomechanical forces, bones are constantly undergoing modeling and remodeling processes to adapt to changing mechanical demands and replace old, microdamaged bone with new, mechanically stronger tissue to help preserve bone strength. There are four types of bones within the body: long, short, flat, and irregular bones. The long bones (clavicles, humeri, radii, ulnae, metacarpals, femurs, tibiae, fibulae, metatarsals, and phalanges) are composed of diaphysis (hollow shaft); metaphyses (flared, cone-shaped) below the growth plates; and
epiphyses (rounded) above the growth plates. The diaphysis is composed mainly of dense (compact) cortical bone, whereas the metaphysis and epiphysis consist of trabecular meshwork (porous) bone surrounded by a relatively thin shell of dense cortical bone. Overall, 80% of the adult human skeleton is composed of cortical bone, while 20% is trabecular bone (Eriksen et al., 1994). The proportion of cortical to trabecular bone varies between skeletal sites (i.e. 75%, 50% and 25% of the bone mass in the vertebra femoral head, and radial diaphysis, respectively is trabecular).

Cortical bone is composed of osteons, cylindrical in shape with concentric lamellae surrounding the Haversian canals (Eriksen et al., 1994). Healthy cortical bone has little porosity. In aging individuals, there is increased cortical remodeling which results in thinning of the cortex and increases cortical porosity, therefore altering the mechanical strength of cortical bone (Hung et al., 2015).

Trabecular bone is composed of interconnected trabeculae, classified according to their shape as plates and rods and has a honeycomb-like microscopic structure. Bone remodeling occurs on the trabecular surface. With aging, a shift from more of a plate-like trabecular meshwork to a more rod-like microstructure occurs, and increased trabecular bone remodeling may cause a perforation in trabecular plates and breakage in trabecular rods (Eriksen et al., 1994).

Cortical bone has an outer periosteal surface and inner endosteal surface. The periosteum is a fibrous connective tissue sheath that surrounds the outer cortical surface of bone, except at joints where the bone is lined by articular cartilage. Periosteum contains blood vessels, nerve fibers, osteoblasts, and osteoclasts and has a significant role in the appositional growth and fracture repair. The endosteum is a membranous tissue
covering the inner surface of the cortical and trabecular bone and the blood vessel canals present in bone.

**Determinants of bone strengths and evaluation methods**

Bone strength is not only determined by bone mass, but also by bone geometry, cortical and trabecular microstructure, and the bone tissue properties (Felsenberg and Boonen, 2005; Compston, 2006). Bone tissue comprises 65% mineral, 35% organic matrix, cells, and water. Bone mineral is in the form of hydroxyapatite, located within and between collagen fibers, predominantly type-I collagen which dominates the organic matrix. The mineral provides mechanical rigidity and load-bearing strength of bone, whereas the organic matrix provides flexibility and elasticity. When new bone is formed, the osteoblasts (bone forming cells), produce and deposit the collagen matrix first, which then undergoes mineralization. The mineral content increases up to 70% of the final levels within the first few days (primary mineralization), followed by a gradual maturation of the mineral content (secondary mineralization). Bone packets are not deposited at the same time in a remodeling cycle. Therefore, the mineral content of bone matrix is not uniform and depends on the time since its deposition. Hence, both average bone mineral density and heterogeneity of mineralization contribute to mechanical properties of bone tissue and affect bone strength.

Bone geometry and composition are also important contributors to bone strength. At a similar bone mineral density, larger bones are stronger compared to smaller bones. As bone size increases from its axis, the strength of bone is proportional to the radius of the bone raised to the fourth power (Bilezikian, 2008). Furthermore, the amount and the ratio of cortical to the trabecular bone at a given skeletal site, also affect bone strength.
In addition, genetic determinants compromising bone material properties (e.g., collagen defects decrease bone strength in osteogenesis imperfecta, impaired γ-carboxylation of Gla proteins, resulting in bone weakness) could influence bone strength (Clarke, 2008). Disease states such as osteomalacia, fluoride therapy, or hypermineralization states can also affect bone strength. Bone microstructure affects bone strength also. Low bone turnover results in microfractures (Clarke, 2008). On the other hand, high bone turnover, with bone resorption exceeding formation, also causes microarchitectural deterioration (van der Meulen et al., 2001). Overall, bone mass, geometry, and bone tissue properties maintain bone structural integrity. Bone strength results from both the amount of bone (quantity) and the geometric and tissue properties (quality) that resist fracture.

Although many factors affect bone strength, the most widely used method to measure bone mass, diagnose osteoporosis and evaluate fracture risk is dual X-ray energy absorptiometry (DXA) (Cummings et al., 2002). However, DXA method is limited by the inherent axial nature of the measurement (Genant et al., 1996). Therefore, an accurate geometric evaluation of the bone is not possible, and bone strength is only estimated, providing limited indications of relative fracture risk. Furthermore, DXA has limited use in individuals with spinal abnormalities, in patients undergoing spinal surgery or with vertebral compression fractures or osteoarthritis. In addition, in the obese subjects with excess fat tissue surrounding the bone or in those experiencing weight reduction and changes in the bone surrounding soft tissue the accuracy of the projected based axial DXA measurements could suffer (Bolotin, 1998). Hence, a growing interest among clinicians and researchers has led to the development of complementary measures of bone quality that could improve measurement accuracy and fracture risk prediction.
Peripheral quantitative computed tomography (pQCT) measures peripheral sites 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 volumetric BMD (total, cortical and trabecular), bone geometry (area, circumference), bone mineral content (BMC) and bone strength indices. Therefore, by measuring the metabolically active bone compartments, pQCT is a sensitive method for detecting actual treatment effects or the degree of bone loss (i.e. osteoporosis). In addition, pQCT evaluates the cross-sectional geometry and bone composition (trabecular to cortical bone ratio) at a variety of skeletal sites at a tridimensional bone structure.

A more recent in vivo method for assessing bone microarchitecture and volumetric BMD in high-quality 3D has emerged: high resolution peripheral quantitative computed tomography (HR-pQCT). The method uses computerized processing of X-ray attenuation to obtain sectional images. From the slices, it is possible to produce a three-dimensional high-quality model. The software creates the different compartments based on image processing and mathematical algorithms. The most valuable parameters are bone volume, bone volume density, structure model index, trabecular thickness, trabecular separation, trabecular number and connectivity density, cortical thickness, and the degree of cortical porosity. In Aim 2 of this dissertation, a custom software package, combining threshold-based and region-growing algorithms was used to measure apparent trabecular microstructure, providing novel information about bone changes in response to nutrient supplementation.
Figure 1. Determinants of bone density, strength and fragility and bone properties assessment

Abbreviations: BMD, bone mineral density; BMC, bone mineral content; DXA, dual energy X-ray absorptiometry

Regulation of bone metabolism

Bone metabolism is controlled by several hormones acting at a systemic level and factors that affect bone locally (Table 1). Primary systemic regulators include parathyroid hormone (PTH), calcitonin and vitamin D metabolites, hormones that maintain calcium and phosphate homeostasis (Raisz, 1998). Parathyroid hormone promotes bone resorption by enhancing osteoclasts activity and induces the release of calcium and phosphates from the bone (Boyle et al., 2003). Calcitonin antagonizes PTH action and inhibits osteoclast activity, therefore limiting bone resorption and enhancing bone deposition (MacDonald, 1986). Calcitriol (1,25-dihydroxycholecalciferol, vitamin D derivative) stimulates calcium and phosphate absorption in the small intestine. Thus, these minerals become
readily available for bone formation. In addition, calcitriol promotes collagen production by the osteoblasts (Clarke, 2008).

Many other systemic hormones are involved in bone metabolism. Estrogens inhibit osteoclast activity through local factors and inhibit bone formation (Slemenda et al., 1987; Jilka et al., 1992). Growth hormone mediates the formation of local growth factors such as insulin-like growth factors (IGF-1 and IGF-2) and stimulates bone formation (Giustina, 2008). Insulin acts as an anabolic agent in bone and increases osteoblasts activity (Clarke, 2008). Glucocorticoids increase bone resorption and reduce bone formation, inducing rapid bone loss (Seibel, 2016). Thyroid hormone may act either directly on osteoclast affecting calcium metabolism or by regulating the osteoblasts, which in turn mediate osteoclastic bone resorption (Britto et al., 1994).

Local contributors regulating bone metabolism act by stimulating bone cell differentiation. Several factors promote osteoblast differentiation, including the bone morphogenic factor, insulin-like growth factor 1, platelet-derived growth factor, and fibroblast growth factor, whereas other factors such as M-Colony stimulating factor, interferons and interleukins stimulate osteoclast differentiation (Clarke, 2008). Various biochemical markers are indicative of bone metabolism. In addition to calcium and phosphates, serum levels of several bone-regulating hormones (PTH, vitamin D/calcitriol, estrogens, glucocorticoids, etc.) are used as markers of bone metabolism (Goltzman, 2010). Furthermore, enzymes and proteins released during bone formation and degradation products produced during bone resorption are useful tools to monitor bone turnover and detect metabolic imbalance (Ross and Knowlton, 1998). Bone formation markers include: serum bone–specific alkaline phosphatase (BSAP), serum
osteocalcin (OC) or serum type 1 procollagen (C-terminal/N-terminal; C1NP or P1NP). Markers of bone resorption are: bone isoenzyme of acid phosphatase (tartrate-resistant), carboxy-terminal telopeptides of collagen (U-ICTP), urinary total pyridinoline (PYD), urinary free deoxypyridinoline (DPD), urinary collagen type 1 cross-linked N-telopeptide (NTX), urinary or serum collagen type 1 cross-linked C-telopeptide (CTX), and bone sialoprotein (BSP) (Seibel, 2005).

Table 1. Systemic and local regulators of bone metabolism

<table>
<thead>
<tr>
<th>Hormones</th>
<th>Resorption</th>
<th>Formation</th>
</tr>
</thead>
<tbody>
<tr>
<td>PTH</td>
<td>↑</td>
<td>↑</td>
</tr>
<tr>
<td>Calcitriol</td>
<td>↑</td>
<td>↑</td>
</tr>
<tr>
<td>Calcitonin</td>
<td>↓</td>
<td>-</td>
</tr>
<tr>
<td>Cortisol</td>
<td>↑</td>
<td>↓</td>
</tr>
<tr>
<td>Growth hormone</td>
<td>-</td>
<td>↑</td>
</tr>
<tr>
<td>T3, T4</td>
<td>↑</td>
<td>↑</td>
</tr>
<tr>
<td>Insulin</td>
<td>-</td>
<td>↑</td>
</tr>
<tr>
<td>Estrogen and testosterone</td>
<td>↓</td>
<td>↑</td>
</tr>
</tbody>
</table>

**Local factors**

<table>
<thead>
<tr>
<th>Growth factors (i.e. IGFs)</th>
<th>↑</th>
<th>↑</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bone morphogenetic proteins</td>
<td>↓</td>
<td>↑</td>
</tr>
<tr>
<td>Inflammatory cytokines</td>
<td>↑</td>
<td>↓</td>
</tr>
</tbody>
</table>

Abbreviations: PTH, parathyroid hormone; T3, triiodothyronine; T4, thyroxine; IGF, insulin-like growth factor.
**Obesity and bone metabolism**

**Hormonal environment in obesity**

Obesity has been traditionally associated with higher bone mass and lower risk of osteoporosis (Reid et al., 1992; Kopelman, 2000; Villareal et al., 2005). One of the proposed mechanisms for the higher BMD in obese individuals is greater mechanical loading conferred by higher body weight, which stimulates bone formation by stimulating osteoblasts and osteocytes differentiation and decreases cell apoptosis through the Wnt/β-catenin signaling pathway (Robling et al., 2006; Bonewald and Johnson, 2008). In addition, hormonal dysregulations accompanying obesity and altered endocrine function of excess adipose tissue (adipokines secretion) influence bone. Obese individuals have higher circulating estrogen and PTH, and lower 25OHD, 1,25(OH)\(_2\)D\(_3\) and sex hormone binding globulin (SHBG) (Shapses and Sukumar, 2012; Slemenda, 1987; Cao, 2011). Moreover, pancreatic hormones such as insulin, amylin and preptin, known for their anabolic effects on bone are increased in obesity (Clowes et al., 2005; Cornish et al., 2007). Several adipokines including leptin, adiponectin, resistin, visfatin, vaspin and apelin have altered levels in obesity (Lenchik et al., 2003; Jurimae et al., 2005). These factors have distinct anabolic or catabolic actions on the osteoblast (Pistili et al., 2007; Zhang et al., 2010; Ducy et al., 2000; Karsenty, 2001; Karsenty and Elefteriou, 2008; Takeda and Karsenty, 2001; Takeda et al., 2002). Obese individuals have also higher levels of inflammatory cytokines, such as interleukin-6 (IL-6), monocyte chemoattractant protein-1, and C-reactive protein (CRP) and tumor necrosis factor α (TNF-α) (Calabro et al., 2008). Proinflammatory cytokines appear to modulate osteoclast differentiation and bone resorption. However, their role in regulating bone in obesity is not entirely clear (Jilka et al., 1992; Koh et al., 2005; Bertolini et al., 1986). Lastly,
altered hormonal milieu in obesity and dietary factors (high-fat intake) may interfere with calcium absorption and affect bone (Shapses et al., 2012).

**Bone quality in obesity**

Despite the positive association between body weight and bone mineral density (BMD), recent data show that obese population is not protected against fracture (Nielson et al., 2012; Premaor et al., 2013). In fact, when correcting for body weight, BMD is lower in obesity compared to normal weight individuals (De Laet et al., 2005) and there is higher bone resorption compared to formation markers in obese vs. lean individuals (Cifuentes et al., 2003; Papakitsou et al., 2004). As previously mentioned, although BMD is most often used to diagnose osteoporosis, a decrease in BMD can estimate the relative fracture risk. However, a high BMD does not eliminate the risk of fracture.

In addition to BMD, other factors predict fracture risk, including bone quality and its components: bone structure and microarchitecture responsible for bone strength (Compston, 2006). Indeed, in recent years studies have found a negative relationship between excess adiposity and bone quality in diverse populations (Nielson et al., 2012; Premaor et al., 2013; Janicka et al., 2007; Sukumar et al., 2011). Cortical bone, evaluated by peripheral QCT is altered in obesity and this is detrimental to bone microarchitecture and in the long term could induce bone fragility (Nielson et al., 2012; Premaor et al., 2013; Sukumar et al., 2011). A higher serum PTH in obesity has enhanced catabolic effects on cortical bone (Shapses and Sukumar, 2012), while serum 25OHD which is positively associated with cortical and trabecular bone in studies (Shapses and Sukumar, 2012), is low in obese individuals. In addition, other factors besides an altered hormonal
milieu in obesity, such as genetics, nutritional factors or physical activity could influence bone quality in obesity.

**Total, free and bioavailable sex steroids and bone**

It is well established that altered levels of steroid hormones are detrimental to bone health in both genders (Sanyal et al., 2008; Leder et al., 2003). The free hormone hypothesis, based on a physiological and mathematical model, postulates that unbound (free) fractions of vitamins or steroid hormones, rather than the protein-bound forms, are the biologically active forms of the compounds (Mendel, 1989). Both estradiol and testosterone can be found in circulation nonspecifically bound to albumin (bioavailable), specifically bound to SHBG, and, in a small proportion, unbound (free). The free and bioavailable fractions are considered more reliable clinical biomarkers compared to total levels which can undergo further metabolization and inactivation in the tissues (Vermeulen et al., 1999). Excessive fat mass is associated with an increased aromatization of androgens to estrogens in adipose tissue. Also, the obese have low serum SHBG levels with increased levels of free sex steroids (Gonnelli et al., 2014). Higher estrogens in obesity could be responsible for higher BMD due to excess body weight (Rosen and Bouxsein, 2006). Higher estrogens in obesity could be responsible for higher BMD due to excess body weight (Rosen and Bouxsein, 2006). In addition, increased aromatase and hydroxyl steroid dehydrogenase, adipose derived enzymes, have anabolic effects on osteoblasts (Folkestadt et al., 2009). On the other hand, in obese men, there are lower levels of total and free testosterone and lower SHBG (Cohen, 1999; Stanworth and Jones, 2009). In both older men and women, bioavailable estradiol and testosterone, have been positively associated with trabecular microstructure (Khosla
et al., 2006) while sex hormone deficiency is responsible for the age-induced loss in
cortical bone (Riggs et al., 2008). In young men, free estradiol is negatively associated
with cortical parameters (cross-sectional area, periosteal and endosteal circumference),
but has a positive relationship with cortical BMD at both the tibia and radius (Lorentzon
2005). On the other hand, free testosterone is independently associated with the cortical
cross-sectional area, periosteal and endosteal circumference, but not with BMD
(Lorentzon et al., 2005). In addition, SHBG is associated with cortical cross-sectional
area and periosteal and endosteal circumference (Lorentzon et al., 2005). However, free
testosterone is an independent predictor of BMD in elderly men (Mellström et al., 2006).
Weight loss increases testosterone levels and decreases circulating estrogen. Whether
changes in testosterone, estradiol and their free and bioavailable fractions influence
cortical and trabecular BMD in middle-aged and older men has not been specifically
examined and is addressed in this dissertation.

**Weight loss and bone metabolism**

Weight loss has a positive effect on health outcomes in overweight and obese individuals,
but, a 10% body weight reduction will also cause 1-2% bone loss at most sites, especially
in older women and men (Shapses and Sukumar, 2012). Furthermore, only about 5%
weight loss also results in bone loss and increased fracture risk in older populations
(Shapses and Sukumar, 2012). Factors associated with weight loss that contributes to
bone loss, include decreased nutrient intake (calcium and other nutrients), decreased
calcium absorption (Cifuentes et al., 2004), reduced weight bearing and/or hormonal
changes associated with weight loss (Shapses and Sukumar, 2012). Other factors such as
initial body weight/composition, the amount of weight loss, age, and ethnicity can also
the amount of bone that is lost following weight reduction (Shapses and Sukumar, 2012). In addition, hormonal changes associated with aging occur at an older age in men compared to women (Yialamas and Hayes, 2003) and bone loss occurs at older ages in men. Therefore, weight loss effects on bone could differ between men and women. Middle-aged overweight men examined who lost 6.4% body weight in a 1-y trial, experienced 1.5% total body BMD loss, but no other bone sites were measured (Pritchard et al., 1996). In another 1-y trial that with older men and women (~1:3) showed that a 9.7% weight loss resulted in a 2.6% hip BMD loss and negatively affected bone geometry (Villareal et al., 2011). In other smaller weight loss studies with bone loss conducted in adults, there was no control group or the majority of the subjects were women. Nevertheless, in a large observational prospective study in elderly men who lost 5% body weight, there was a bone loss at the hip, and this was greater in men who decreased serum testosterone concentrations (Ensrud et al., 2006). However, weight loss interventions in men examining multiple bone sites, compartments, and geometry have not been conducted and is addressed in this dissertation.

**Vitamin D physiology**

The role of vitamin D and its biologically active metabolite 1,25-dihydroxyvitamin D3 [1,25(OH)2D3] in mineral homeostasis and bone health is well established. In recent years, a role for vitamin D in extraskeletal health has been suggested, including metabolic syndrome, cancer, cardiovascular diseases, autoimmune diseases and neurological disorders (Holick, 2007).
Metabolism

Vitamin D or calciferol is a fat-soluble vitamin that occurs in two major forms: vitamin D2 and vitamin D3. Vitamin D2 (ergocalciferol) is mainly photosynthesized or fortified in foods, while vitamin D3 (cholecalciferol) is synthesized in the skin, exposed to solar UVB radiation, from 7-dehydrocholesterol and can also be found in animal-based products. The difference in their side chain structure does not affect their bioactivation. Vitamin D, produced in the skin or from dietary/supplemental intake, is transported to the fat cells where it can be deposited or it is metabolized in either the D2 or D3 forms, which are considered inactive prohormones before undergoing enzymatic hydroxylation. The first hydroxylation in the liver, mediated by 25-hydroxylase yields the major circulating form of vitamin D which is 25-hydroxyvitamin D (25OHD). The second enzymatic reaction in the kidney, mediated by 1α-hydroxylase, converts 25OHD to the biologically active hormone, 1,25-dihydroxyvitamin D [1,25(OH)2D3] or calcitriol. The majority of 25OHD and 1,25(OH)2D3 is bounded in the circulation by vitamin D binding protein (DBP), about 10–15% is bound to albumin, while free 25OHD and 1,25(OH)2D3 account for less than 1% (Bikle, 1986). Since 25OHD or 1,25(OH)2D3 are weakly bound to albumin compared to DBP, the loosely albumin-bound fraction and the free fraction is considered bioavailable 25OHD. Only the free form of 1,25(OH)2D3 can activate vitamin D receptor (VDR) and regulates transcription and expression of vitamin D target genes that will control cell proliferation, differentiation, invasion and angiogenesis (Nagpal et al., 2005; Pike et al., 2012). The renal production of 1,25(OH)2D3 is stimulated by parathyroid hormone (PTH), and inhibited by calcium, phosphate, and fibroblast growth factor 23 (FGF23) (Shimada et al., 2004). The final products of
25OHD and 1,25(OH)2D3 are catabolized by 24-25 hydroxylase into water-soluble calcitroic acid, which is biologically inactive and is excreted in the bile (Raval-Pandya et al., 1998) (See Figure 2).

The classic function of vitamin D is to regulate intestinal calcium absorption by stimulating the expression of the epithelial calcium channel and the calbindin 9K (calcium binding protein; CaBP) (Akhter et al., 2007) and maintains its homeostasis. It also promotes mineralization of newly formed osteoid tissue in bone and plays a role in preosteoclast differentiation to mature osteoclast. It is well known that vitamin D deficiency is detrimental to the skeleton, causing rickets in children and osteomalacia in adults (Thacher et al., 2011). Less severe vitamin D deficiency may be associated with secondary hyperparathyroidism, bone loss, muscle weakness and falls and fragility fractures in older people (Holick, 2007). The biologically active hormone, 1,25(OH)2D3, plays a role in bone formation by increasing osteocalcin expression and osteoblasts mineralization and in bone resorption by increasing the number and activity of osteoclasts and osteopontin expression. 1,25(OH)2D3 is recognized by its receptor in osteoblasts causing an increase in the expression of receptor activator of NFkB ligand (RANKL) while RANK receptor on the preosteoclast binds RANKL and induces the preosteoclast differentiation (Khosla, 2001).
Figure 2. Vitamin D metabolism in the body

Vitamin D Binding Protein (DBP)

Vitamin D binding protein (calbindin-D28 K), encoded by the Gc (group-specific component) gene, functions as a specific transporter of circulating vitamin D metabolites and protects vitamin D metabolites from hydroxylase-mediated catabolism, modulates their cellular uptake and biological activity. It is assumed that serum DBP concentrations (much higher than its ligands) and binding properties would affect serum free metabolite concentrations (Bouillon et al., 1981; Bouillon et al., 2008; Bouillon et al., 2016). Several factors such as pregnancy, use of exogenous estrogen and obesity affect serum DBP concentrations and therefore, may influence bioavailable 25OHD. In addition, aging affects DBP concentrations (Yousefzadeh et al., 2014). However, data from our lab suggest that when there is no change in endogenous estrogen levels, serum DBP
concentrations might remain stable with aging. Therefore, low estrogen levels in postmenopausal women could result in low DBP concentrations and low free and bioavailable serum 25OHD (Pop et al., 2015) (See Figure 3). Whether the relationship between serum total 25OHD, free and bioavailable 25OHD with regard to DBP with vitamin D supplementation changes is not known and will be addressed in this dissertation.

*Vitamin D receptor*
Following the activation by 1,25(OH)2D3, VDR binds to specific vitamin D response elements (VDRE) in the promoter regions of numerous genes and regulates both, directly and indirectly, their expression and modulates biological responses (Haussler et al., 1998). Furthermore, the widespread presence of VDR in tissues such as pancreas, skin, brain, immune and bone as well, raised the possibility that its ligand, 1,25(OH)2D3, is involved in many physiological functions besides maintaining calcium homeostasis (Bhalla et al., 1983; DeLuca, 2008).
Figure 3. Serum 25-hydroxyvitamin D (25OHD), vitamin D binding protein (DBP) and estradiol concentrations

Groups include premenopausal (<27 ng/mL, n=18; >27 ng/mL, n= 31); and postmenopausal (<27 ng/mL, n=65; >27 ng/mL, n=51) women. Different superscripts indicate means differ significantly, p < 0.05. Abbreviations: 25OHD, 25-hydroxyvitamin-D; DBP, vitamin D binding protein; pre, pre-menopausal women; post, post-menopausal women.

Vitamin D status, dietary reference intake (DRI) and sources of vitamin D

Serum level of 25OHD is the gold standard indicator of vitamin D status. It represents both vitamin D intake and vitamin D that is produced from sun exposure. Vitamin D sufficiency is defined as a serum levels of 25OHD > 20ng/mL (50 nmol/L) and insufficiency as serum levels < 16ng/mL (40 nmol/L) (IOM, 2011). 25OHD has a longer half-life of ~ 15 days compared to its biologically active form 1,25(OH)2D3 that has a half-life of ~ 15 hours (Jones, 2008). In addition, circulating levels of 25OHD are far greater compared to 1,25(OH)2D3. Moreover, PTH maintains calcium levels by increasing calcium reabsorption in the kidney, mobilization of calcium from the bones and by increasing the renal production of 1,25(OH)2D3 (Norman, 2009). Therefore, the increase in PTH levels with vitamin D deficiency or insufficiency is accompanied by normal or increased levels of 1,25(OH)2D3, making 1,25(OH)2D3 an inaccurate marker of vitamin D status.

Development of the DRI for vitamin D, by the Institute of Medicine in 2011, was based on bone health, as well as calcium absorption and biomarkers such as serum 25OHD and PTH (IOM, 2011). In the USA, DRI for vitamin D intake is 600 IU/d for adults and 800 IU/d for elderly (IOM, 2011) with an upper limit (UL) set at 4000 IU/d. The major source of vitamin D is from sun exposure (Holick, 2007). However, vitamin D production in the skin varies with latitude, season and time of the day, therefore during winter at ~33 degrees latitudes North and South, respectively, vitamin D3 in the skin is not being synthesized (Wacker and Holick, 2013). For example, farther North in Boston, at 42° latitude, vitamin D3 cannot be produced in the skin from November through February (See Figure 4). In addition, skin pigmentation affects vitamin D production and
darker skin is associated with lower 25OHD levels. Also, vitamin D synthesis in the skin is diminished in individuals older than 65 years of age and with the use of sunscreen topical agents (Matsuoka et al., 1987).

Food sources rich in vitamin D are fatty fish (such as salmon, tuna, and mackerel) and fish liver oils (IOM, 2011). Smaller amounts are in beef liver, cheese, and egg yolks. Various amounts of vitamin D2 can be found in some mushrooms (Calvo et al., 2004). Fortified foods, such as milk (100 IU/cup), breakfast cereals, some brands of orange juice and yogurt and less common cheese and ice cream also provide vitamin D in the American diet (IOM, 2011). A large variety of dietary supplements is also available on the market. Although, controversy regarding the effectiveness of vitamin D form present

Figure 4. Conversion rate of 7-dehydrocholesterol to vitamin D depending on time of the day and season in Boston (42° North).

in fortified foods and supplements (D\textsubscript{2} or D\textsubscript{3}) in maintaining serum 25OHD levels, studies have not found any difference between the two forms (Cranney et al., 2007; Holick et al., 2008).

**Body weight and vitamin D**

The majority of fat-soluble vitamin D is stored in the adipose tissue (Holick, 2007; Blum et al., 2008). Studies have reported lower serum 25OHD concentrations in the obese compared to lean individuals and the negative relationship between vitamin D status and anthropometric measurements of obesity has been observed regardless of age and ethnic group (Saneei et al., 2013; Zitterman et al., 2014).

Lower serum 25OHD concentrations in the obese compared to lean subjects is attributed to differences in volume dilution between normal and higher body weight individuals (Drincic et al., 2012; Gallagher et al., 2012; Zitterman et al., 2014). Alternatively, fat-soluble vitamin D compounds stored in the adipose tissue, lead to a reduced serum concentration, but through lipolysis they can be released into the circulation (Drincic et al., 2012). Moreover, in larger non-obese individuals, height and body surface area are positively associated with 25OHD, further providing evidence that only the obese have lower 25OHD (Pazaitou-Panayiotou et al., 2012). This relationship may be attributed to an increased synthesis of vitamin D in the skin as a result of the increasing body surface area without excess adiposity (Pazaitou-Panayiotou et al., 2012). In addition, recent associations between obesity–related SNPs and low concentrations of 25OHD could explain 25OHD levels in obesity (Vimaleswaran et al., 2013).
Several studies have explored possible mechanisms underlying the association between obesity and vitamin D deficiency other than volume dilution into adipose tissue. Lower 25OHD concentrations in obesity may also occur due to reduced synthesis of vitamin D3 in the skin since the response to a given ultraviolet (UV) B radiation dose is attenuated in obese subjects compared to lean individuals (Wortsman et al., 2000). Moreover, the ability of the skin to release vitamin D into the circulation may be altered in obesity (Wortsman et al., 2000) and is not necessarily due to insufficient sunlight exposure (Wortsman et al., 2000; MacDonald et al., 2011). In general, the obese have reduced skin area exposure to sunlight due to wearing excess clothing and a lack of outdoor activity (Kühn et al., 2013). In addition, hepatic biosynthesis of the vitamin D pathway might be altered in the obese, in the presence of nonalcoholic fatty liver disease, which is often found together with vitamin D deficiency in obese individuals (Barchetta et al., 2013; Kühn et al., 2013).

Other possible causes for lower serum 25OHD have been attributed to a greater vitamin D catabolism in obesity (elevated 24-hydroxylase) found in the obese compared to lean subjects (Wamberg et al., 2013). Low serum 25OHD in obesity has also been attributed to the negative feedback control of hepatic 25OHD synthesis exerted by their elevated 1,25-dihydroxyvitamin D and parathyroid hormone concentrations (Drincic et al., 2012). In addition, because there is a higher prevalence of obesity in minority groups, it is possible that the ethnic/racial differences in DBP and VDR genetic polymorphisms are contributing to the lower circulating 25OHD in many obese individuals (Ahn et al., 2010).
The response to vitamin D supplementation in the obese

Studies addressing serum 25OHD response to vitamin D supplementation in normal to overweight populations (BMI < 30 kg/m²) estimated the rise in serum 25OHD to ~0.28 ng/mL per μg of vitamin D intake/d or greater, ranging from 0.40 to 0.79 ng/mL/μg (Heaney et al., 2003; Holick et al., 2008; Gallagher et al., 2012; Autier et al., 2012). These differences were attributed to differences in the type of vitamin D used, or varying characteristics of the subjects (age, BMI, etc.), compliance issues, or assay variability (Autier et al., 2012). Since the response to vitamin D supplementation is greatly influenced by body weight, more recent studies have addressed the dose-response relationship between serum 25OHD and vitamin D intake in obese populations.

The influence of body weight on serum 25OHD response to vitamin D intake was also examined in a systematic review, including 94 independent studies using placebo vs vitamin D doses ranging from 200 IU to 10,000 IU (Zitterman et al., 2014). The results confirmed that body weight was an important predictor for the rise in 25OHD for a given dose of vitamin D. Besides body weight, other important predictors included the type of supplement (vitamin D₂ or D₃), age, use of calcium supplements and baseline 25OHD levels. Based on these findings, the authors suggested a formula using body weight, age and baseline 25OHD levels to calculate the increase in circulating 25OHD in response to vitamin D dose [Incremental change in nmol/l = 49.4 + 16.03 × Ln vitamin D dose (μg per kg body weight per day) + 0.22 × age (years) − 0.13 × baseline 25OHD (nmol/l)] (Zitterman et al., 2014). However, there remains variability in vitamin D response even when correcting for body weight, and this might be partially attributed to genetic factors, such as variance in the genotypes of VDR, DBP and hydroxylation enzymes (Wang et al., 2010).
**Weight loss and vitamin D**

Studies with moderate weight loss in overweight/obese individuals are accompanied by a rise in vitamin D status, and it could be hypothesized that this may contribute to the beneficial health consequences of reducing body weight. Studies in obese women reported a 1.9, 2.7 and 5.0 ng/mL increase in serum 25OHD for those who did not lose weight, those who lost 5%–10% and those who lost >10% body weight, respectively (Rock et al., 2012). In addition, 83% of those who achieved a normal BMI after 24 months met the recommended serum concentrations (>20 ng/ml). Greater increases in serum 25OHD with greater weight loss, independent of baseline vitamin D status or changes in dietary vitamin D intake was also found in overweight and obese postmenopausal women randomly assigned to diet, exercise, diet + exercise intervention, or control (Mason et al., 2011). In weight loss studies conducted in our lab, we have shown a rise in 25OHD levels with weight loss that is greater than individuals who were weight stable (Riedt et al., 2005).

**Vitamin D Supplementation during Weight Loss**

Weight loss has beneficial health consequences; however, it may also decrease calcium absorption and negatively affect bone (Shapses and Sukumar, 2012). Therefore, supplemental intake during weight loss interventions may attenuate bone loss. In studies where vitamin D supplementation is given during weight loss in a randomized controlled trial, those individuals who lose weight will increase serum levels of 25OHD to a greater extent than those who lose less weight (Mason et al., 2014; Zitterman et al., 2009). For example, a study in our laboratory in women given 2500 IU/d vitamin D3 intake who lost even a modest amount of weight (~3%) compared to those who didn’t lose weight
showed a 20% and 9% rise in serum 25OHD, respectively (Shapses et al., 2013. In addition, poor vitamin D status has been linked to metabolic disorders and elevated chronic inflammation in both obese and normal weight individuals (Hewison et al., 2010; Sung et al., 2012). Hence, additional vitamin D is expected to improve metabolic health independent of weight loss. Several randomized controlled trials showed a beneficial effect of vitamin D on lipid profile and inflammation as well as insulin sensitivity and insulin resistance (Zitterman et al., 2009; Nagpal et al., 2009; von Hurst et al., 2010). The effect of vitamin D supplementation on inflammation was addressed in a randomized, placebo controlled weight loss trial comparing a daily dose of 2000 IU vitamin D₃ vs placebo in 218 overweight/obese postmenopausal women with serum 25OHD levels between 10-32 ng/mL. The vitamin D supplement was effective at reducing IL-6 levels, but not other cytokines (TNFα, IL1β, IL8, and IL10) or adipokines (adiponectin, leptin) (Duggan et al., 2015). Although the question whether vitamin D supplementation can promote weight loss has also been raised, studies found no effect of vitamin D vs placebo at improving weight loss.

**Vitamin D and skeletal health**

Healthy vitamin D status is essential for normal skeletal development in children and for achieving and maintaining bone health in adults (IOM, 2011). Vitamin D deficiency leads to secondary hyperparathyroidism, increased bone turnover, bone loss, and fracture risk as well as muscle weakness (Lips et al., 2011). Large epidemiological studies have found a positive relationship between serum 25OHD levels and bone mineral density in both men and women. However, the optimal vitamin D dose and serum levels that could attenuate bone loss and reduce the risk of fracture in older populations have been
controversial. This controversy has arisen from the lack of association between vitamin D supplementation and fracture prevention as well as the inconsistent relationship between vitamin D and BMD in randomized controlled trials (Reid et al., 2014). Inadequate vitamin D doses or high baseline serum 25OHD levels could explain the lack of effect with vitamin D supplementation on BMD in some studies (Reid et al., 2014). Furthermore, it has been suggested that the bioavailable 25OHD, rather than total 25OHD is a more accurate marker of vitamin D status (Bikle et al., 2013) and therefore should be a better predictor of BMD. This relationship will be addressed in this dissertation.

In particular, postmenopausal women are at high risk of bone loss and microstructural deterioration, causing bone fragility and increased fracture risk. However, studies with vitamin D supplementation alone did not result in reduced risk of fractures or increased BMD. Nevertheless, some trials found a positive effect on bone with daily supplementation (but not with intermittent treatment regimens) (Table 2).

In weight-stable women, it has been previously shown that 400 IU/d compared to placebo does not prevent bone loss that occurs with aging over 1 year (Ooms et al., 1995). In other trials, some found that BMD loss is attenuated with 700-800 IU/d (Chapuy et al., 1992; Dawson-Hughes et al., 1995) and calcium supplements (500-1,200 mg) or, at similar doses there was no effect (Meier et al., 2004; Aloia et al., 2005). Higher doses of vitamin D on BMD also yielded inconsistent results. In some studies with higher vitamin D$_2$ doses (1428 IU/d) and calcium (1000 mg) compared to calcium alone, there was no effect on bone (Cooper et al., 2003), while others using 5000 IU D$_3$/d and 320 mg calcium/d compared to placebo found higher BMD at the lumbar spine and hip (Mocanu et al., 2009). Nevertheless, all of these previous studies were conducted in weight stable
individuals and a few of them assessed bone quality. Postmenopausal women often overweight and obese undergo weight control plans to improve health outcomes. Although beneficial, weight loss decreases calcium absorption and promotes bone loss. Even a modest 3-4% body weight reduction can reduce BMD in women (Salamone et al., 1999). A positive relationship between 25OHD levels and bone quality at the radius and tibia was found in older Caucasian men (Barbour et al., 2011). Some studies reported a catabolic effect of PTH on the cortical bone in both obese and non-obese individuals and increased endocortical resorption, cortical thinning and cortical porosity at high PTH levels (Dempster et al., 2007. On the other hand, excess PTH has a beneficial effect on trabecular bone (Duan et al., 1999). However, studies in older women examining the relationship between 25OHD and bone compartments at the tibia are missing. One study found a positive relationship between 25OHD and cortical vBMD (negative with PTH), but neither 25OHD nor PTH was associated with trabecular vBMD (Lauretani et al., 2008). The effect of vitamin D supplementation during modest weight loss on bone quality will be examined in this dissertation.
### Table 2. Studies with vitamin D supplementation on bone mineral density (BMD) in older women

<table>
<thead>
<tr>
<th>Author &amp; year</th>
<th>Sample N, Age, Body weight</th>
<th>Baseline 25OHD (ng/mL)</th>
<th>Vitamin D dose</th>
<th>Spine BMD</th>
<th>Total Hip BMD</th>
<th>Femoral Neck BMD</th>
<th>Total Body BMD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dawson-Hughes et al. 1991</td>
<td>N=276 F, 62 yrs, 68 kg</td>
<td>28.6</td>
<td>400 IU/d vs placebo</td>
<td>+</td>
<td></td>
<td></td>
<td>NS</td>
</tr>
<tr>
<td>Dawson-Hughes et al. 1995</td>
<td>N=261 F, 64 yrs, 68 kg</td>
<td>26.6</td>
<td>100 IU/d vs 700 IU/d</td>
<td>NS</td>
<td></td>
<td>+</td>
<td>NS</td>
</tr>
<tr>
<td>Ooms et al. 1995</td>
<td>N=348 F, 80 yr, 71 kg</td>
<td>10.4</td>
<td>400 IU/d vs placebo</td>
<td></td>
<td>NS</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>Komulainen et al. 1999</td>
<td>N=227 F, 53 yrs, 70 kg</td>
<td>10.8</td>
<td>300 IU/d vs placebo</td>
<td>NS</td>
<td></td>
<td>NS</td>
<td></td>
</tr>
<tr>
<td>Cooper et al. 2003</td>
<td>N=187 F, 56 yrs, 67 kg</td>
<td>32.8</td>
<td>10 000 IU/week vs placebo</td>
<td>NS</td>
<td>NS</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>Aloia et al. 2005</td>
<td>N=208 F, 61 yrs, 79 kg</td>
<td>18.4</td>
<td>800 IU/d for 2 years then 2000 IU/d vs placebo</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>Zhu et al. 2008</td>
<td>N=302 F, 77 yrs, 73 kg</td>
<td>17.6</td>
<td>1000 IU/d vs placebo</td>
<td>NS</td>
<td></td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>Nieves et al. 2012</td>
<td>N=127 F, 62 yrs, 82 kg</td>
<td>11.6</td>
<td>1000 IU/d vs placebo</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>Grimnes et al. 2012</td>
<td>N=297 F, 63 yrs, 25 kg/m²</td>
<td>28.4</td>
<td>800 IU/d vs 6500 IU/d</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>McDonald et al. 2013</td>
<td>N=305 F, 65 yrs, 25 kg/m²</td>
<td>13.5</td>
<td>1000 IU/d vs 400 IU/d</td>
<td>NS</td>
<td></td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>Hansen et al. 2015</td>
<td>N=230 F, 75 yrs, 30 kg/m²</td>
<td>20.5</td>
<td>50000 IU 2x/mo vs 800 IU/d vs placebo</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
</tr>
</tbody>
</table>

NS, nonsignificant findings. Overall, there is a lack effect on BMD in one-year vitamin D supplementation studies in older women. However, these studies did not impose the stress of caloric restriction on bone during the study period and did not measure bone microarchitecture and geometry.
Vitamin D and extra-skeletal health

In addition to the well-known effects on calcium metabolism and bone health, lately, vitamin D has been highly investigated for its extra-skeletal effects, including diabetes and insulin resistance, cardiovascular disease, and cancers. Mice and cell studies support a role for calcitriol in the synthesis and secretion of insulin in beta islet cells (Cade and Norman, 1987; Pittas et al., 2007) suggesting that vitamin D could improve insulin sensitivity in diabetes. However, the mechanism is not entirely clear and a paracrine effect of vitamin D to upregulate 1,25(OH)2D3 synthesis in the beta cell and to decrease tissue inflammation or fat infiltration in skeletal muscle have been proposed (Muscogiuri et al., 2014).

Observational studies have shown an inverse relationship between serum 25OHD and the prevalence of diabetes, although the associations remain inconclusive (Mitri et al., 2011; Seida et al., 2014). Some vitamin D supplementation studies suggest that vitamin D could influence insulin secretion and improve glucose homeostasis (Dutta et al., 2014; Naharci et al., 2012; Harinarayan et al., 2013). Vitamin D may also have a role in delaying the onset of type 2 diabetes in adults at a high risk of developing the disease (Harinarayan et al., 2013; Baz-Hecht et al., 2010). However, most studies show that vitamin D supplementation in patients with clinical diabetes has no effect on glucose homeostasis, insulin resistance or diabetes prevention (Kampmann et al., 2014; Seida et al., 2014). In many of these studies, combined supplementation of vitamin D with calcium makes it difficult to separate the effects of those two nutrients. Moreover, vitamin D doses (suboptimal) and duration of supplementation (short duration interventions) vary between studies and could explain the lack of beneficial effects of
vitamin D supplementation on diabetes-related outcomes (Seida et al., 2014). In this dissertation the effects of three vitamin D3 doses on markers of insulin resistance over 12 months of intervention will be examined.

**Insulin sensitivity assessment: HOMA-IR and QUICKI**

The Homeostasis model assessment, HOMA-IR, is a mathematical model assessing insulin resistance using plasma insulin and glucose concentrations at multiple time points representing degrees of β-cell deficiency and insulin resistance (Matthews et al., 1985). The model assumes that impaired β-cell function results in an attenuated response of the β-cell to glucose-stimulated insulin secretion, and insulin resistance represent a less efficiently suppressed glucose production in the liver by insulin. The most simple and widely used method is HOMA-IR= (fasting plasma insulin x fasting plasma glucose)/22.5. The denominator (22.5) is the product of normal fasting plasma insulin of 5 µU/ml and a normal fasting plasma glucose of 4.5 mmol/l= 22.5 so that an individual with “normal” insulin sensitivity has an HOMA-IR=1. HOMA-IR has been used in large cohort studies, cross-sectional epidemiology studies and intervention studies in healthy populations (Wallace et al., 2004).

The Quantitative Insulin Sensitivity Check Index, QUICKI, was developed to replace the clamp and Frequently Sampled Intravenous Glucose Tolerance Test in larger studies and in clinical practice (Katz et al., 2000). It is a mathematical transformation of fasting glucose and insulin concentrations generating an index, which has a high positive correlation with the clamp: 1/[log (fasting plasma insulin) + log (fasting plasma glucose)]. Similar to HOMA-IR, QUICKI has the advantage of being a less expensive and invasive tool and it is a highly reproducible index (Sarafidis et al., 2007).
**Osteocalcin**

Osteocalcin is the most abundant noncollagenous protein (46-50 amino acids) of bone matrix, produced by osteoblasts and traditionally considered a bone formation marker (Hauschka et al., 1989). Osteocalcin gene has a VDR binding site approximately 485 base pairs upstream of the OC gene’s transcriptional start site (Ozono et al., 1990). Osteocalcin transcription is directly stimulated by vitamin D while vitamin K regulates carboxylation processes. Carboxylated Gla residues promote calcium and hydroxyapatite binding, favoring osteocalcin deposition in the mineralized bone matrix (Razzaque, 2011). On the contrary, noncarboxylated osteocalcin with low affinity for hydroxyapatite is being released into the circulation. However, both the carboxylated and the undercarboxylated forms, as well as the total osteocalcin, are detectable in the circulation. However, while circulating undercarboxylated osteocalcin levels are influenced by vitamin K daily intakes and status, total OC levels are dependent on bone cell activity and are not by vitamin K status (Booth et al., 2013).

Mice studies show a role for OC on energy metabolism and glucose homeostasis (Lee et al., 2007). In particular, in mice, undercarboxylated form is considered the active hormone which increases islet cell growth and insulin synthesis and maintains glucose homeostasis in mice. However, there are differences in the molecular size, and some amino acids of osteocalcin between mice and humans, and while mice contain three osteocalcin gene, there is only one osteocalcin gene in humans (Desbois et al., 1994; Pi et al., 2005). Therefore, the exact role of OC and ucOC in glucose metabolism is unclear in humans and will be examined in this dissertation.
Table 3. Studies examining the relationship between glucose and insulin metabolism and measures of osteocalcin (OC)

<table>
<thead>
<tr>
<th>Study &amp; year</th>
<th>Sample (N, age, BMI)</th>
<th>Results</th>
</tr>
</thead>
<tbody>
<tr>
<td>Zhou et al. 2009</td>
<td>N= 254 M &amp; 180 F, 40-60 yrs, 25 kg/m² (T2DM vs control)</td>
<td>Serum OC was lower in adults with T2DM vs controls.</td>
</tr>
<tr>
<td>Kindblom et al. 2009</td>
<td>N=1010 M, 75 yrs, 26 kg/m² (T2DM vs control)</td>
<td>Serum OC was lower in T2DM and inversely correlated with BMI, fat mass and fasting glucose.</td>
</tr>
<tr>
<td>Kanazawa et al. 2009</td>
<td>N=179 M &amp; 149 F, 52 yrs * (T2DM)</td>
<td>OC correlated negatively with fasting glucose and HbA1c.</td>
</tr>
<tr>
<td>Pittas et al. 2009</td>
<td>N=380 M&amp;F, 71 yrs, 26 kg/m² (T2DM and healthy)</td>
<td>Serum OC inversely correlated with fasting glucose, insulin and IR.</td>
</tr>
<tr>
<td>Saleem et al. 2010</td>
<td>N=2493 M&amp;F, 59 yrs, 31 kg/m² (healthy)</td>
<td>Serum OC inversely correlated with BMI, fasting glucose, and IR.</td>
</tr>
<tr>
<td>Kanazawa et al. 2009</td>
<td>N= 50 M&amp;F, 50 yrs * (T2DM)</td>
<td>Serum OC level increased and the ratio of ucOC/OC decreased after one month of improved glycemic control.</td>
</tr>
<tr>
<td>Kanazawa et al. 2011</td>
<td>N=180 M &amp; 109 F, 58 yrs, 23 kg/m² (T2DM)</td>
<td>Serum ucOC was inversely correlated with fasting glucose and HbA1c in men but not in women.</td>
</tr>
<tr>
<td>Iki et al. 2012</td>
<td>N= 2174 M, &gt;65 yrs * (T2DM and healthy)</td>
<td>Serum ucOC was inversely associated with glycemic index and IR after adjusting for serum OC levels.</td>
</tr>
<tr>
<td>Caglar et al. 2014</td>
<td>N=87 F, 54 yrs, 28% obese (healthy)</td>
<td>No relationship between OC and fasting glucose, insulin and IR.</td>
</tr>
<tr>
<td>Lee et al. 2015</td>
<td>N=194 F, 55 yrs, 24 kg/m² * (Metabolic syndrome and healthy)</td>
<td>Serum ucOC negatively correlated with fasting insulin and HOMA-IR.</td>
</tr>
</tbody>
</table>

Cross-sectional studies. *BMI, age-NA. T2DM, type 2 diabetes mellitus
Low levels of OC have been found in subjects with glucose intolerance or in those at increased risk of type 2 diabetes (Im et al., 2008; Pittas et al., 2009; Díaz-López et al., 2013) while improving glycemic control normalizes OC levels (Rosato et al., 1998; Sayinalp et al., 1995). Similarly, increased serum ucOC levels were associated with greater insulin sensitivity in middle-aged men (Hwang et al., 2009). In several other studies, serum ucOC osteocalcin level was inversely correlated with plasma glucose levels and insulin sensitivity in non-diabetic subjects (Kindblom et al., 2009). However, in some studies ucOC was inversely correlated with fasting plasma glucose and HOMA-IR after adjusting for total OC, while total osteocalcin was not associated with these parameters after adjusting for ucOC (Iki et al., 2012). Although results are inconsistent and studies are sparse, OC may play a role in the prevention and treatment of obesity and associated comorbidities such as type 2 diabetes and metabolic syndrome, therefore, requires clarifications.

On the other hand, lower 25OHD levels are associated with lower OC concentrations (Fonseca et al., 1988). However, some reported that vitamin D supplementation alone had no effect on total OC in adults (von Hurst et al., 2010; Je et al., 2011) or ucOC in children (O’Connor et al., 2010) and no relationship was found between total OC and VDR polymorphism in adolescents (Ozaydin et al., 2010). Nevertheless, a previous study conducted in our lab showed that the interaction between short-term vitamin D supplementation and weight loss in overweight/obese individuals has significant effects on OC measures and insulin resistance but ucOC was not measured (Sukumar et al., 2015). Whether long-term vitamin D supplementation on serum OC and ucOC will attenuate markers of IR is not known and will be addressed in this dissertation.
Rationale

Obesity and osteoporosis affect an increasing number of individuals. Although a higher body weight is associated with higher bone mass, evidence shows that obesity-induced hormonal imbalance and chronic inflammation alter bone quality and increase fracture risk. On the other hand, despite attenuating health outcomes, weight loss results in endocrine changes and nutrient deficiency and induces bone loss, especially in the context of age-related hormonal decline. Although in men this decline occurs later in life, they are not protected against osteoporosis but, studies in men addressing weight loss effects at specific bone sites are currently lacking. In contrast, in women, it is well established that even a modest weight loss results in bone loss. The beneficial effects of vitamin D on bone health are known, but there is controversy regarding vitamin D dose and the optimal serum 25OHD levels to maximize bone health. In addition, the response to vitamin D treatment is attenuated in the obese suggesting that higher than recommended doses are needed to raise serum 25OHD levels to sufficient ranges (> 75 nmol/L) in these individuals. Higher vitamin D intake is expected to lower PTH and attenuate bone quality. However, vitamin D supplementation effects on bone compartments have been less examined. In addition, vitamin D supplementation has been shown to improve beta-cell function and insulin sensitivity. Whether, by increasing the expression of OC, a novel regulator of energy metabolism and glucose homeostasis, long-term doses of vitamin D attenuate insulin resistance is not known. Furthermore, the relationship between the undercarboxylated form of OC (the active form of OC in mouse models) and IR in humans is not clear. Elucidating these questions is important to help understand the mechanisms regulating bone quality with weight loss and optimize strategies to attenuate bone loss and IR with weight control.
SPECIFIC AIMS OF THE DISSERTATION

1. To examine the effect of caloric restriction on bone mineral density (BMD), geometry and strength in middle-aged and older overweight/obese men.

*Endocrine changes due to weight reduction affect bone and this is clear in women.*

*Because the steroid decline in aging men occurs in older age compare to women, bone due to weight loss may differ in men.*

2. Three doses of vitamin D3 supplementation in postmenopausal overweight/obese women over 1 year during modest caloric restriction will be examined:

a. To determine the effect on bone turnover markers, BMD, geometry, strength, and microstructure.

*We hypothesize that vitamin D3 supplementation during moderate caloric restriction will raise serum 25OHD and will suppress parathyroid hormone (PTH) in a dose-dependent manner and in turn, will influence geometry and microstructure of both cortical and trabecular bone compartments.*

b. To determine whether serum 25OHD influences osteocalcin (a bone turnover marker) and insulin resistance.

*It is currently unclear whether 25OHD affects total serum OC, and if there is a differential effect on total and ucOC. Due to the evidence for a greater effect of ucOC on IR in murine studies, total, carboxylated and ucOC will be examined. It is hypothesized that vitamin D will increase total OC (with no effect on other bone turnover markers) and both the undercarboxylated and total OC will predict IR.*
Chapter 2.

Moderate weight loss in obese/overweight men preserves bone quality

Abstract

Weight loss negatively affects bone mineral density (BMD) in older populations and has specifically been shown in women. This prospective controlled trial examines parameters of bone quality and endocrine changes following intentional weight loss in men. Thirty-eight overweight and obese (body mass index of 31.9 ± 4.4 kg/m²; age 58 ± 6 years) men were recruited to either weight loss (WL) through caloric restriction or weight maintenance (WM) for six months. There was a -7.9 ± 4.4% and +0.2 ± 1.6% change in body weight in the WL and WM groups, respectively. There was a greater increase in femoral neck and total body BMD and bone mineral content (BMC) in the WM group when compared to the WL group (interaction effect; p < 0.05). In contrast, there was a trend for tibia cortical thickness and area to decrease more in the WM than WL group (p < 0.08). There was a decrease in periosteal circumference in both groups over time (p < 0.01) and no significant changes in trabecular bone. The circulating total, free and bioavailable estradiol decreased in the WL compared to the WM group and the changes were different between groups (p < 0.05). Serum total and bioavailable testosterone increased in both groups (p < 0.01). Serum 25-hydroxyvitamin D increased to a similar extent in both groups (p < 0.05). Moderate weight loss in overweight/obese men did not decrease BMD at any anatomical site or alter cortical/trabecular bone and geometry. Also, despite an increased BMD at some sites when maintaining excess body weight, cortical bone shows a trend in the opposite direction.
Introduction

Promoting weight loss in a rising number of overweight and obese individuals seems the rational way to improve health outcomes. However, despite numerous health benefits in older adults (Darmon, 2013) weight loss can also promote bone and muscle loss with older age (Shapses and Sukumar, 2012; Villareal et al., 2011). In addition, changes in hormonal status with aging in both genders have direct effects on skeletal health (Khosla, 2013). Postmenopausal women undergoing voluntary weight loss, experience a 1-2.5% bone loss when compared to a weight stable group (Nguyen et al., 1998; Riedt et al., 2005). Similarly, epidemiological studies of older men show that weight loss (both voluntary and involuntary) is associated with bone loss and increased fracture risk (Knoke et al., 2003, Mussolino et al., 1998). A delay in age-related sex steroid decline (Yialamas and Hayes, 2003) in men when compared to women might explain the later onset of bone loss in this population. As a result, weight loss-induced bone loss in aging men may differ compared to that observed in older women.

Weight reduction studies in men examining multiple bone sites, compartments and geometry are currently lacking. In a one year intervention with middle-aged overweight men, whole body composition was performed in dieters who lost 6.4% body weight and 1.5% total body-BMD; specific bone sites were not measured in this study (Pritchard et al., 1996). Another one year trial that examined hip BMD and included elderly men and women (~1:3), showed that a 9.7% weight loss resulted in a 2.6% BMD loss and negatively affected bone geometry (Villareal et al., 2011; Villareal et al., 2012). Other smaller intervention studies of weight loss conducted in older adults suggesting bone loss either do not have a control group or examine primarily a female population (63-98%)
(Jensen et al., 1994; Villareal et al., 2006). Nevertheless, a large observational prospective study found hip bone loss in elderly men (age 74 y) who experience 5% weight loss, and this was even greater in men who decreased serum testosterone concentrations (Ensrud et al., 2006).

While a higher body weight is associated with higher bone mass, there is evidence that bone quality is compromised in the obese (Nielson 2012, Premaor 2013, Sukumar 2011). It is possible that these heavy individuals undergo more dieting or weight cycling which may be detrimental to bone (Shapses 2012, Fogelholm 2001). Hence, understanding whether there are changes in bone quality due to weight loss is important to better predict fracture risk, especially in this population. In this controlled trial, the effect of caloric restriction on BMD, geometry and strength is examined to determine the risk-benefit ratio of weight loss in middle aged and older obese/overweight men. Because weight loss alters sex steroids, 25-hydroxyvitamin D (25OHD) and parathyroid hormone (PTH), these hormones were investigated to determine if they could explain the association between bone changes due to weight loss.

SUBJECTS AND METHODS

Subjects

Overweight and obese men (BMI range 25-39 kg/m2, age range 50-72 years) were recruited in the nutrition and weight loss lab at Rutgers University following local newspaper, electronic and radio station advertisements. Subjects were recruited during winter months to either maintain or lose weight. Telephone interviews were conducted by trained staff to determine whether subjects met the experimental criteria. Men under
treatment known to influence Ca or bone metabolism, or diagnosed with diseases known
to influence Ca metabolism (i.e. metabolic bone disease, hyperparathyroidism, untreated
thyroid disease, significant immune, hepatic, or renal disease, kidney stone in the last 5
years, diabetes, significant cardiac disease, active malignancy or cancer therapy within
the past year) were excluded. Men experiencing weight loss 3 months prior to recruitment
were also excluded. All eligible volunteers underwent biochemical and physical
screening including: comprehensive chemistry panel, complete blood count and physical
examination to ensure they were healthy and had no evidence of undiagnosed diseases
(i.e., diabetes, anemia). Subjects signed an informed consent approved by the Rutgers
University Institutional Review Board for the protection of human subjects in research
prior to initiation of the study protocol. This trial was also monitored by an external
advisory review board and was registered at clinicaltrials.gov as NCT00472745. The
protocol met the ethical standards in accordance with the Helsinki Declaration.

Study design

Subjects were recruited to either lose or maintain weight over a six months study period
using the same criteria. Subjects who volunteered for the weight loss treatment were
counseled and offered weekly classes for the first 2 months of the study followed by
biweekly classes until 6 months. They followed a standard behavior modification
nutrition education weight loss program that has been shown to be effective to increase
compliance in our previous studies conducted at our weight loss unit and at the NY
Obesity and Nutrition Research Center, as described previously (Riedt 2005). Caloric
intake was individualized, consisting of 500-600 kcal deficit/day and designed to achieve
a moderate weight loss.
Participants were instructed to provide detailed information on portion size and methods of food preparation to fill out food diaries. Adherence to the diets was assessed through at least three 24-hour recalls twice during the intervention. The dietitian reviewed these diaries with the subjects to increase compliance and ensure accuracy. In this prospective adaptive study design, individuals originally in the WL group who were not compliant and did not lose weight within the first 7 weeks were eligible for the WM group. If they agreed to continue in the WM group and still met eligibility criteria, this was considered their final group. Subjects recruited for weight maintenance group were asked to maintain weight during the intervention. In addition, these individuals were offered free nutrition education classes with the dietitian at the end of the study. Weight maintenance was defined as less than 2.5% change in body weight from baseline to the end of the 6 month study. Similar to the WL group, the WM subjects were weighed and measured at the same intervals (baseline, and months 1, 3 and 6). At these time points, we collected food diaries, and assessed for calcium intake and adverse events.

Supplements and nutrient analysis

Calcium intake was assessed through food frequency questionnaires during initial screening with a goal to achieve a daily intake of 1.2 g of calcium for subjects in both groups beginning one month prior to and throughout the intervention. Our goal was to examine how caloric restriction under controlled intake of calcium and vitamin D affected bone parameters. A multivitamin/mineral tablet with 400 IU of vitamin D3 and 200 mg Ca (Nature Made Multi 50 +, Mission Hills, CA) was given to all the volunteers. As needed, if dietary Ca plus the supplement in the multivitamin did not reach 1.2 g/day, then Ca intake was adjusted accordingly, using 200 mg Ca tablets (Ca citrate, Bayer Inc,
NJ). All subjects were asked to refrain from taking any other supplements during this study. Dietary and supplemental calcium intake was assessed for the WL and WM groups at baseline and months 1, 3 and 6 using calcium intake questionnaires. To assess nutrient intake, food diaries were analyzed in all the study participants from 2 non-consecutive weekdays and one weekend day at baseline and twice during the intervention using FoodWorks software (Version 11, FoodWorks, Long Valley, NJ).

Physical activity

Subjects were encouraged to follow their usual exercise regimen throughout the study. Men were given a physical activity questionnaire to assess their physical activity level (PAL) (Ainsworth et al., 2011). A numerical score was used to calculate PAL with a range from 0 to 3 (0-inactivity, 1-low activity, 2-moderate activity and 3-high activity) to reflect an estimation of energy expenditure in metabolic equivalent-minutes per week (Ainsworth et al., 2011). In addition, subjects were asked to record the amount and type of physical activity on a specified area of their food diary forms.

Body weight and height

A balance beam scale and stadiometer (Detecto, Webb City, MO, USA) were used for anthropometric measures. Body weight was obtained at the regular morning visits while wearing light clothing. Body mass index (BMI) was calculated as weight / height (kg/m²).

Soft tissue and BMD

Dual energy x-ray absorptiometry measurements were performed with a total body scanner (Lunar Prodigy Advanced; GE-Lunar, Madison, WI; CV: ≤ 1% for all sites) at
baseline and six months. Total hip, lumbar spine, femoral neck, trochanter and total body BMD and BMC were measured. In addition, soft tissues were evaluated including total tissue, fat (android/gynoid) and fat free soft tissue compartments. Vertebral exclusion criteria and corrections to the DXA lumbar spine measurements (specified by the International Society of Clinical Densitometry) (Lewiecki 2007) were performed by a trained physician. Vertebrae showing signs of local structural change and/or anatomic abnormality or artifact were excluded if the T-score difference between the abnormal vertebra and the adjacent one was more than 1.0.

*Peripheral quantitative computed tomography*

Peripheral quantitative computed tomography (pQCT, Stratec XCT 3000, Orthometrix, NY) was used for bone measurements performed at specific sites (4% and 38%) at the distal nondominant tibia and included volumetric (v) total BMD, trabecular, cortical BMD and BMC, geometry and strength indices at the tibia. The scans were analyzed using Stratec XCT software, version 5.4. A scout view allowed the positioning of the cross-sectional measurements along the tibia. The voxel size for all the scans was 0.5-mm and the slice thickness was 2.4 mm. The precision error (coefficient of variation) was less than 1.7% for all the measurements.

*Laboratory Methods*

After a 12 hour overnight fast, venous blood and a spot urine sample was taken from each participant in the study at baseline, and at 1, 3 and 6 months. Samples were analyzed in batch analysis for the following hormones: 25OHD (radioimmunoassay, RIA; DiaSorin, Stillwater, MN, CV <12.5%), intact PTH (immunoradioassay, IRMA; Scantibodies,
Santee, CA, CV <6.8%), and sex hormones: ultra-sensitive estradiol (E2) (RIA; DSL, Webster, TX, CV<8.9%), total testosterone and free testosterone (enzyme-linked immunosorbent assay, ELISA; Alpco Diagnostics, Salem, NH, CV<9.6% and <12.4 respectively). Sex hormone binding globulin (SHBG) (ELISA; Alpco Diagnostics, Salem, NH, CV <12.1%) and osteocalcin (RIA; Biomedical Technologies, Stoughton, MA, CV <9%) were measured at baseline and after 6 months.

Calculations

Concentrations of total testosterone, total E2, SHBG and measured albumin were used to calculate bioavailable testosterone, and free and bioavailable E2 according to the algorithm described previously by Vermeulen et al (Vermeulen et al., 1999). The algorithm for free sex hormone calculation assumes that the concentration of the free sex steroids in blood is the result of the interaction between SHBG/albumin, and total hormone concentration (testosterone or E2) through different affinity constants of the peptides for these sex hormones, without any interaction with other hormones in the blood that could influence the equilibrium described below:

\[
[FT] = \frac{([T] - (N \times [FT]))/(K_{T} \{SHBG - [T] + N[FT]\})}{(K_{T} \{SHBG - [T] + N[FT]\})}, \text{ respectively}
\]

\[
[FE2] = \frac{([E2] - (N \times [FE2]))/(K_{E2} \{SHBG - [E2] + N[FE2]\})}{(K_{E2} \{SHBG - [E2] + N[FE2]\})},
\]

where \([T]\) and \([E2]\) are total testosterone and total E2 concentrations, respectively; \([fT]\) and \([fE2]\) are \(fT\) and \(fE2\) concentrations; \(K_{T}\) and \(K_{E2}\) are the affinity constants of SHBG for T and E2; \(N1 = KaT \, Ca + 1\), and \(N2 = KaE2 \, Ca + 1\), where \(Ca\) is the albumin concentration (BSA, Fisher Scientific, CV <2.9%), and \(KaT\) and \(KaE2\) are the affinity constants of albumin for T and E2.
Safety

Serious adverse events and adverse symptoms were recorded throughout the study duration. Adverse symptoms included the following: headaches, pain in legs, swelling in leg, pain or heaviness in chest, nausea, dizziness, fatigue, muscle weakness, urinary frequency, abdominal pain or muscle aches. The presence of a symptom included categories “sometimes”, “often” or “always” whereas the absence of a symptom required one of the 2 following responses “never” or “rarely”. Volunteers were asked to fill out an adverse symptoms form during baseline measurements and during months 1, 3 and 6 at the study meetings.

Statistical analysis

Analyses were conducted using the SAS statistical package (SAS Institute, Cary, NC, USA; v 9.2). The groups were compared for baseline characteristics by using one-way ANOVA. Variables considered clinically important, even if not significantly different, were used as covariates in the analyses (i.e., body weight). Additional covariates included age, season, PAL, sex hormones (total testosterone, free testosterone, estradiol), SHBG and 25OHD. Mixed-model ANCOVA was used to analyze the two main effect determinants: group and time (6 months intervention period) on bone parameters, fat, fat free soft tissue, hormones and nutrient intake. When the interaction group-by-time was significant, post-hoc analysis was conducted with the Bonferroni correction for multiple comparisons. For hormones with more than 2 time point measurements (0, 1, 3, 6 months), repeated measures ANCOVA using mixed-models with age, season, PAL and baseline weight as covariates was also conducted followed by pairwise comparisons with Bonferroni correction. Pearson correlation coefficients were used to assess the
relationship between changes in the outcomes and changes in the measured variables. Stepwise multiple linear regression analysis (forward selection technique) was performed to select the explanatory variables that would be considered the most important predictors for bone changes. We controlled for micronutrients including calcium and vitamin D during these studies so this was not included in the model.

In a previous study conducted in overweight/obese men, a 6.4 kg body weight loss resulted in a total body bone mineral content loss of $42 \pm 35$ grams (Pritchard et al., 1996). With a power of 90% and $\alpha$ set at 0.05, a sample size of 13 per group was required to detect significant differences for total body BMC between groups. Secondary outcomes were bone regulating hormones and sex steroids in response to weight loss. To allow for one covariate and due to anticipated dropouts and noncompliance, we recruited additional subjects in each group. Evaluating the success or failure to lose weight (Furlow and Anderson, 2009) was not the goal in this study, but rather to examine whether successful weight loss affects bone, and hence we report data for subjects who met final eligibility criteria and completed the study. In addition, in a separate analysis of all the subjects ($n=44$), the success or failure of weight loss in the original group was used as a covariate to analyze for changes in bone parameters. Values are reported as mean $\pm$ SD, and graphs with SEM. Categorical values are expressed in percentages to represent a portion of the sample. Significance was considered at a p value $< 0.05$.

**RESULTS**

*Participants*

Out of the 67 men who were screened, 44 met the inclusion/exclusion criteria and were in either WM ($n=22$) or WL ($n=22$) groups. Three men dropped out within the first month
of the study in the WM group due to personal reasons. One recruited volunteer, who underestimated his weight in the telephone screen, was excluded because after weight measurement he no longer met the BMI inclusion criteria. Two subjects dropped due to non-compliance. After 7 weeks of dietary counseling, five subjects were unsuccessful at losing weight and therefore, were excluded from the WL group. These men were asked to maintain weight and be part of the WM group. Thirty-eight men (31.9 ± 4.4 kg/m²; 58 ± 6 years) that included 36 Caucasians, 1 African American and 1 Asian completed the study (Figure 5).

**Weight, body composition and bone mineral density**

Weight, body composition and bone results at baseline and after 6 months of diet intervention are presented in Table 1. There were no statistically significant differences at baseline (Table 4). Subjects in the WL group lost 7.9 ± 4.4% weight, 16.1 ± 19.5% total body fat, 2.2 ± 3.9% fat-free soft tissue, and 4.1 ± 6.6% loss of total body BMC that differed significantly than the WM group (p < 0.02). There was an interaction between group and time for femoral neck and total body BMD (p < 0.05) (Table 4). In addition, the change in total body BMD in the WL group (-1.0 ± 2.5%) differed compared to the WM group (1.5 ± 2.7%) (p < 0.05). The interaction between group and time was not significant for radius, lumbar spine and total hip BMD (Table 4). Hip BMD indicated a significant time effect (p < 0.02) in both groups. In the analysis using the original groups, which included men who did not lose weight in the WL group, there were no significant differences at baseline or changes between groups over time.
Trabecular and cortical bone at the tibia

There were not significant changes in trabecular parameters (Table 5). There was an interaction between group and time that approached significance for cortical thickness (p ≤ 0.06) (Table 5), showing a change of -0.8 ± 2.9% and 0.7 ± 2.3% in the WM and WL groups, respectively. A trend was also observed for the interaction between group and time for cortical area (p < 0.08). In addition, there were trends for endosteal circumference and polar moment of inertia to decrease over time in both groups (p < 0.08). There were no other changes in cortical parameters between groups or over time (Table 5). In the analysis using the original groups, the change over 6 months in cortical vBMD differed between the WL (-0.1 ± 0.5%) and WM (0.6 ± 1.2%) groups (p<0.05 for the interaction effect). However, cortical area and polar moment of inertia decreased more in the WM (-0.6 ± 4.0% and -2.4 ± 4.2%, respectively) vs WL group (-0.1 ± 2.1% and -1.6 ± 2.7%, respectively) (p < 0.05 for the interaction effect).

Sex steroids and bone regulating hormones

Serum sex steroids and bone regulating hormones are shown in Table 6. Forty-two percent of the men had serum 25OHD concentrations below 50 nmol/L, and 21% were above 75 nmol/L. The interaction between group and time was significant for serum total, free and bioavailable E2 (p < 0.05; Table 6). In addition, compared to baseline, there was an increase in serum free and bioavailable E2 (p < 0.001). Serum 25OHD and total testosterone concentrations increased over time in both groups (p < 0.05) (Figure 6). The increase in total serum estradiol concentration in the WM differed compared to the small decrease in the WL group (p < 0.05 for the interaction) (Figure 6). In addition, there was no change in serum PTH (Figure 6). Serum osteocalcin was 1.4 ± 0.6 nmol/L and 1.2 ±
0.4 nmol/L in the WL and WM groups, respectively, and did not differ significantly between groups or change over time (data not shown).

*Nutrient intake and physical activity level*

As expected, during the intervention, there was a lower intake of total calories in the WL group (1595 ± 182 kcal/d) compared to the WM group (2097 ± 248 kcal/d) (p < 0.05) (Table 8). Additionally, the WL group had lower protein (81 ± 8 g/d) and fat intake (61 ± 18 g/d) compared to the WM group (87 ± 11 g protein/d and 89 ± 11 g fat/d) (p < 0.05). The daily Ca intake from diet and supplement was 1179 ± 251 mg/d and 1176 ± 89 mg/d in the WL and WM groups, respectively (Table 8). Compliance with supplemental multivitamin and calcium intake was 90% ± 22% (WL: 92 ± 22% and WM: 89 ± 22%). Diaries indicated that most of the men were sedentary, and there was no difference in the PAL change between groups, and the score rose slightly over time in both groups (1.1 ± 1.0 to 1.2 ± 1.1; p < 0.05 time effect).

*Pearson correlations and stepwise regression.*

Body composition - Changes in total body BMD correlated positively to changes in body weight (r=0.43, p<0.01), fat free soft tissue (r=0.44, p=0.005) and total body fat (r=0.31, p < 0.05). In contrast, total body fat tended to inversely correlate with stress:strain index (r= -0.30; p = 0.06).

Hormones - Total serum testosterone and total body BMD changes were inversely correlated (r= -0.34, p < 0.05). Changes in serum 25OHD concentrations were positively correlated with changes in femoral neck BMD (r = 0.39, p < 0.01) and tibia cortical thickness (r=0.39, p < 0.01). Changes in serum free E2 inversely correlated with changes
in cortical thickness (r = -0.49; p < 0.002), polar moment of inertia (0.33; p < 0.05) and stress:strain index (r = -0.32; p < 0.05) and tended to positively correlate with endosteal circumference (r = 0.30; p = 0.06). A stepwise regression was performed with bone outcomes as the dependent variables and hormonal and body composition changes as independent variables. Only those independent variables that reached significance were included in the final model (Table 7). Changes in body weight and 25OHD serum concentrations explained 25% (model R2) of the variance in femoral neck BMD. Changes in fat free soft tissue explained 19% of the changes in total body BMD while the changes in free E2 explained 14% of the changes in cortical thickness at the tibia, controlling for prior variables. Models constructed to explain changes in femoral neck, total body BMD and cortical thickness for each group individually did not reach significance.

Adverse events and symptoms

There were no serious adverse events recorded during this study. The frequency of non-serious adverse symptoms did not change from baseline or differ between groups.

DISCUSSION

Osteoporosis and bone loss in older men is an increasing public health burden in an aging society (Watts 2012) and a history of weight loss in older men or women is known to increase fracture risk (Mussolino 1998, Langlois 2001, Ensrud 2003). The use of calorie restriction to prevent obesity complications has been shown to result in bone loss in postmenopausal women (Shapes 2012). The current study was designed to examine how voluntary weight reduction in moderately older men affects BMD, geometry and strength and this has not been examined previously in a controlled trial. In
addition, a secondary goal was to examine if the endocrine response to caloric restriction could explain bone changes with weight reduction. In this weight loss study, even though the change in femoral neck and total body BMD differed from weight stable men, the decrease was not significant and there were no changes in cortical or trabecular bone parameters.

Men who are 50-72 years of age and lose 8% of their body weight show little or no change in BMD, geometry or strength. This differs from the significant decrease in BMD found in older women using a similar protocol in our lab (Riedt 2005) and in other weight loss studies (Shapses 2012). One early study examined weight loss in an overweight middle-aged male population (43 years of age) and only measured total body BMD (Pritchard 1996) and found 1.5% loss with 6 kg weight loss. Bone loss due to weight loss might be greater if there is a lower initial weight or greater loss of lean body mass, such as when there is lower protein intake (Sukumar 2011) or less physical activity (Pritchard 1996, Villareal 2006, Ryan 1998). In this study, the absence of femoral neck BMD loss with weight loss differs from previous weight loss findings in women and mixed gender populations (Shapses). There were no changes in trabecular or cortical bone compartments with weight loss in men in this study; yet there was a trend to decrease cortical thickness and area only in the overweight/obese men who maintained their body weight. This finding is consistent with evidence that obesity is associated with altered cortical bone that is detrimental to bone microarchitecture (Nielson et al., 2012; Premaor et al., 2013; Sukumar et al., 2011). In previous studies of weight loss in women, there was also no reported change in cortical and trabecular vBMD, strength or geometry (Sukumar et al., 2011; Uusi-Rasi et al., 2010). In the study by Uusi-Rasi et al. (Uusi-Rasi
et al., 2010), the amount of weight loss (range of 2-19%) did not predict the loss of bone after 3 months of caloric restriction in premenopausal women nor did it predict bone loss after an additional 9 months of weight maintenance measured by pQCT. The absence of bone loss in premenopausal women (Uusi-Rasi et al., 2010) might be explained by their younger age and higher estrogen status and is consistent with no areal BMD loss found in another study of premenopausal women (Riedt et al., 2007). However, in a more recent study, we also found that postmenopausal women (mean of 58 years) showed no change in trabecular and cortical bone parameters with 7% weight loss after 1 year, despite a decrease in areal BMD at a few central bone sites (Fogelholm et al., 2001). In another study of elderly men and women (72 years of age), it was found that hip geometric properties including cortical thickness, calculated from areal BMD, decreased with moderate weight loss after 12 months (Villareal et al., 2012). It may be that central bone sites respond differently than peripheral sites or that bone in the elderly is more vulnerable to a decline in cortical bone properties.

In the current study with moderate weight loss, there was a significant difference in total estradiol change between groups, although the decline from baseline was not as great as reported in elderly men (73 years) who decrease weight (Ensrud et al., 2006). It is notable that serum estradiol concentrations reported here (102 pmol/L) were slightly higher than those found in the MrOs study of older men (66-92 pmol/L) (Cauley et al., 2010). A younger age or higher adiposity may explain the higher estradiol concentrations in the current study. In addition, the cause for a slight rise in serum estradiol in weight stable obese men is not entirely clear. Possible explanations include aging during the study, an increase in aromatase enzyme concentrations (Bjornerem et al., 2004), or a small rise or
redistribution of adipose tissue (Wake et al., 2007). Studies show a positive effect of estradiol on areal BMD in men (Cauley et al., 2010; Khosla and Riggs, 2005; Lorentzon et al., 2005), yet a negative association between free estradiol and cortical bone size (Lorentzon et al., 2005), similar to findings in this study. Also, other factors associated with excess adiposity such as altered cytokines, growth factors and calcitropic hormones attenuate the protective effect of estrogen on cortical bone in obesity (Shapses and Sukumar, 2012; Donath et al., 2011).

Circulating testosterone is a positive determinant of BMD and lower concentrations are associated with faster bone loss (Cauley et al., 2010; van den Beld et al., 2000). In this study, the absolute concentration of testosterone would be negatively influenced by their advanced age and excess adiposity (van den Beld et al., 2000; Pasquali et al., 2006). The levels of testosterone in these obese men averaged 11.4 ± 5.0 nmol/L and this is at the lower end of normal range (10.4-34.6 nmol/L) (Swerdloff et al., 2011). The men who lost weight in this study showed a 35% increase in testosterone that could be considered physiologically significant (41-43). A rise in serum testosterone possibly combined with the rise in 25OHD (Riedt et al., 2005), might have attenuated bone changes in this study.

There are some limitations and strengths of this study. The precision of dual energy x-ray absorptiometry measurements is influenced by abnormally high amounts of soft tissue surrounding the bone or changes in total body fat (Knapp et al., 2012; Yu et al., 2012). For example, it was found that 6 kg fat (but not less), overlying bone will affect the precision of areal BMD measurements, but not volumetric BMD measurements by pQCT (Yu et al., 2012). It should be noted that, while men in the treatment group lost 6.7 kg of fat in the current study, the amount of loss at a single anatomical site would be
significantly less. Hence, the precision errors to estimate BMD using DXA are less likely to occur during the moderate weight loss that was achieved in this study. In addition, while previous studies have shown that a 6 month intervention induces bone loss, a longer duration may have resulted in greater bone loss (Shapses and Sukumar, 2012). However, the goal here was to examine bone while subjects were still close to their weight nadir rather than after regain or maintenance. Nevertheless, a future intervention trial to examine the long-term effect of weight change on BMD and bone quality in men is indicated. The separate recruitments for each group may have led to the higher, although statistically insignificant, body weight in the weight loss than weight stable group of men. A larger sample size would have limited the influence of weight variability between groups. Other limitations of this study are lack of randomization and masking and the multiple comparisons that increase the risk of Type I error and reduce the reliability of p-values greater than 0.005. Also, the adaptive study design was used to retain the eligibility criteria and cases that were recruited for the sake of adequate power and thereby reduce problems arising from a lack of efficacy (Chow et al., 2008; Chang et al., 2005). In addition, our goal was to examine bone changes in response to weight loss rather than the success or failure of the weight loss diet whereas other studies employ motivational interviewing to achieve the goal of weight loss (West et al., 2007). A strength in this study is the additional information about bone microarchitecture by pQCT that was not captured by areal BMD measurements alone, and these measurements are especially important in studies of the obese who have evidence of compromised bone quality (Nielson et al., 2012; Premaor et al., 2013; Sukumar et al., 2011). Another strong point of this study is that by excluding vertebrae with structural abnormalities such as
osteophytes or compression fracture for lumbar spine BMD which is more common in
the obese (Samartzis et al., 2012), the risk of overestimating values and reporting changes
due to artifacts was reduced. Also, participants in this study were recruited in the same
season (winter) so that the seasonal influence on 25OHD concentrations did not differ
between individuals or groups.

In conclusion, 8% weight loss in overweight/obese men did not decrease areal or
volumetric BMD or show evidence of altered bone geometric properties. Also, our
findings suggest that maintaining an obese/overweight status may lead to detrimental
changes in cortical bone compared to weight loss. These results indicate the need for a
larger longitudinal study that specifically examines obese men and factors contributing to
changes in bone quality over time.

Acknowledgments and Funding Source

We thank the technical staff for the laboratory assistance, and R Zurfluh, RD and the
other clinical staff for their invaluable clinical assistance, as well as the commitment of
the volunteers. The effort of the external data safety monitoring board was appreciated
(Lester Katzel, MD, Robert Recker, MD and Bruce Barton, PhD). We would also like to
thank our colleagues, Drs. M Watford, D Hoffman and G Henderson for reviewing our
findings. SAS: Study design and conception, data analysis and interpretation, and
primary responsibility for final content. LCP: record keeping, data collection,
management and interpretation, laboratory and statistical analysis. DS: coordination of
the study, laboratory and statistical analysis. KT: data collection, management and
interpretation, laboratory and statistical analysis. YS: Study design and statistical analysis
and interpretation. SHS: Study design, study physician, interpretation of results and
contributed to safety report. CLG: imaging analysis and data interpretation. XW: calculations of data, interpretation of results. All authors contributed to the interpretation of the results, the data analysis and reviewed and contributed to writing the manuscript. LCP, DS, KT, YS, SHS, CLG, XW and SAS had no conflicts of interest. This study was supported by NIH-AG12161.
**Table 4. Body composition and areal bone mineral density (BMD)**

<table>
<thead>
<tr>
<th></th>
<th>Weight Loss (n=19)</th>
<th>Weight Maintenance (n=19)</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Baseline</td>
<td>Final</td>
<td></td>
</tr>
<tr>
<td>Age (years)</td>
<td>57.7 ± 6.6</td>
<td>59.7 ± 4.9</td>
<td></td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>32.4 ± 4.7</td>
<td>30.0 ± 4.8</td>
<td></td>
</tr>
<tr>
<td>Weight (kg)</td>
<td>103.1 ± 18.5</td>
<td>95.1 ± 18.5</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Total body fat (kg)</td>
<td>34.2 ± 10.9</td>
<td>28.7 ± 12.4</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Fat-free soft tissue (kg)</td>
<td>64.0 ± 8.7</td>
<td>62.5 ± 8.6</td>
<td>0.01</td>
</tr>
<tr>
<td>Bone mineral content (g)</td>
<td>3184.8 ± 416.9</td>
<td>3069.7 ± 451.4</td>
<td>0.94</td>
</tr>
<tr>
<td>BMD (g/cm²)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>UD radius</td>
<td>0.439 ± 0.044</td>
<td>0.431 ± 0.048</td>
<td>0.19</td>
</tr>
<tr>
<td>1/3 radius</td>
<td>0.804 ± 0.075</td>
<td>0.798 ± 0.075</td>
<td>0.81</td>
</tr>
<tr>
<td>Lumbar spine</td>
<td>1.238 ± 0.148</td>
<td>1.258 ± 0.151</td>
<td>0.21</td>
</tr>
<tr>
<td>Trochanter</td>
<td>0.891 ± 0.159</td>
<td>0.888 ± 0.155</td>
<td>0.91</td>
</tr>
<tr>
<td>Femoral neck</td>
<td>0.965 ± 0.137</td>
<td>0.958 ± 0.143</td>
<td>0.04</td>
</tr>
<tr>
<td>Total hip</td>
<td>1.041 ± 0.152</td>
<td>1.033 ± 0.155</td>
<td>0.11</td>
</tr>
<tr>
<td>Total body</td>
<td>1.285 ± 0.094</td>
<td>1.272 ± 0.084</td>
<td>0.004</td>
</tr>
</tbody>
</table>

1Observed mean ± SD (all such values). A mixed-model ANCOVA analysis was performed with time (0, 6 months) and group (WL or WM) as independent variables with age, season, physical activity level, body weight, sex hormones (total testosterone, free testosterone, and estradiol), sex hormone binding globulin, 25-hydroxyvitamin D as covariates. aBaseline differs from final WM; p < 0.05. Abbreviations: BMI, body mass index; BMD, bone mineral density; UD radius, ultra-distal radius; 1/3 radius, radial BMD at 1/3 of the distance from the distal end.
Table 5. Trabecular and cortical volumetric bone mineral density, geometry and strength at the tibia

<table>
<thead>
<tr>
<th></th>
<th>Weight Loss (n=19)</th>
<th>Weight Maintenance (n=19)</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Baseline</td>
<td>Final</td>
<td>Group</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Time</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Group x x Time</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>vBMD (mg/cm(^3))</td>
<td>331.0 ± 45.2</td>
<td>333.2 ± 44.5</td>
<td>0.95</td>
</tr>
<tr>
<td>BMC (mg)</td>
<td>407.6 ± 49.9</td>
<td>406.7 ± 51.43</td>
<td>0.06</td>
</tr>
<tr>
<td>Area (mm(^2))</td>
<td>1266.4 ± 128.1</td>
<td>1258.4 ± 118.9</td>
<td>0.53</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>0.25</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>0.93</td>
</tr>
<tr>
<td><strong>Trabecular</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>vBMD (mg/cm(^3))</td>
<td>241.8 ± 28.4</td>
<td>242.5 ± 29.3</td>
<td>0.11</td>
</tr>
<tr>
<td>BMC (mg)</td>
<td>135.5 ± 23.6</td>
<td>141.0 ± 20.1</td>
<td>0.06</td>
</tr>
<tr>
<td>Area (mm(^2))</td>
<td>360.0 ± 38.2</td>
<td>360.9 ± 40.4</td>
<td>0.20</td>
</tr>
<tr>
<td></td>
<td></td>
<td>328.9 ± 60.8</td>
<td>0.17</td>
</tr>
<tr>
<td></td>
<td></td>
<td>324.2 ± 60.2</td>
<td>0.08</td>
</tr>
<tr>
<td>Thickness (mm)</td>
<td>5.8 ± 0.6</td>
<td>5.9 ± 0.7</td>
<td>0.06</td>
</tr>
<tr>
<td>Periosteal (mm)</td>
<td>80.2 ± 4.2</td>
<td>80.0 ± 4.2</td>
<td>0.06</td>
</tr>
<tr>
<td>Endosteal (mm)</td>
<td>43.5 ± 5.6</td>
<td>43.0 ± 5.4</td>
<td>0.06</td>
</tr>
<tr>
<td>Polar moment of inertia (mm(^4))</td>
<td>40180 ± 8283</td>
<td>40082 ± 8718</td>
<td>0.57</td>
</tr>
<tr>
<td>SSI (mm(^3))</td>
<td>2214 ± 369</td>
<td>2209 ± 377</td>
<td>0.93</td>
</tr>
</tbody>
</table>

\(^1\)Observed mean ± SD (all such values). A mixed-model ANCOVA analysis was performed with time (0, 6 months) and group (WL or WM) as independent variables with age, season, physical activity level, body weight, sex hormones (total testosterone, free testosterone, and estradiol), sex hormone binding globulin, 25-hydroxyvitamin D as covariates. The covariates tested in the model had no significant influence on bone parameters at any site.
Abbreviations: BMC, bone mineral content, SSI; stress-strain index, vBMD, volumetric bone mineral density.

**Table 6. Sex steroids and bone regulating hormones**

<table>
<thead>
<tr>
<th></th>
<th>Weight Loss (n=19)</th>
<th>Weight Maintenance (n=19)</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Baseline</td>
<td>Final</td>
<td></td>
</tr>
<tr>
<td>Total-E(_2) (pmol/L)</td>
<td>101.6 ± 30.4</td>
<td>99.0 ± 33.0</td>
<td>0.20</td>
</tr>
<tr>
<td>Free-E(_2) (pmol/L)</td>
<td>2.9 ± 1.7</td>
<td>2.2 ± 1.2</td>
<td>0.08</td>
</tr>
<tr>
<td>Bioavailable-E(_2) (pmol/L)</td>
<td>58.4 ± 40.8</td>
<td>42.2 ± 49.4</td>
<td>0.14</td>
</tr>
<tr>
<td>Total-T (nmol/L)</td>
<td>11.2 ± 3.8</td>
<td>15.3 ± 5.5</td>
<td>0.31</td>
</tr>
<tr>
<td>Free-T (pmol/L)</td>
<td>33.0 ± 10.7</td>
<td>33.8 ± 11.6</td>
<td>0.001</td>
</tr>
<tr>
<td>Bioavailable-T (nmol/L)</td>
<td>5.0 ± 2.7</td>
<td>7.1 ± 6.2</td>
<td>0.65</td>
</tr>
<tr>
<td>SHBG (nmol/L)</td>
<td>60.1 ± 24.7</td>
<td>70.4 ± 39.1</td>
<td>0.03</td>
</tr>
<tr>
<td>25OHD (nmol/L)</td>
<td>57.5 ± 19.7</td>
<td>68.0 ± 24.2</td>
<td>0.94</td>
</tr>
<tr>
<td>Intact PTH (ng/L)</td>
<td>36.4 ± 19.1</td>
<td>36.1 ± 19.2</td>
<td>0.66</td>
</tr>
</tbody>
</table>

\(^{a}\)Observed mean ± SD (all such values). A mixed-model ANCOVA analysis was performed with time (0, 6 months) and group (WL or WM) as independent variables with age, season, physical activity level and body weight as covariates. \(^{b}\)Baseline differs from final WM; p < 0.05.

Abbreviations: E\(_2\), estradiol; PTH, parathyroid hormone, SHBG; sex hormone binding globulin; T, testosterone; 25OHD, 25-hydroxyvitamin D.
Table 7. Multiple linear stepwise regression analysis of the explanatory variables for changes in bone mineral density (BMD) at the femoral neck, total body and cortical thickness

<table>
<thead>
<tr>
<th>Variable</th>
<th>R²</th>
<th>β</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Femoral neck BMD</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Body weight</td>
<td>0.15</td>
<td>0.32</td>
<td>0.01</td>
</tr>
<tr>
<td>25OHD</td>
<td>0.10</td>
<td>0.42</td>
<td>0.03</td>
</tr>
<tr>
<td>Total body BMD</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fat free soft tissue</td>
<td>0.19</td>
<td>0.44</td>
<td>0.05</td>
</tr>
<tr>
<td>Cortical thickness</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Free E₂</td>
<td>0.14</td>
<td>-0.36</td>
<td>0.02</td>
</tr>
</tbody>
</table>

1Analysis was done for change in variables over six months (n=38).
2The final model does not include variables that did not reach significance (physical activity level, total body fat, parathyroid hormone, total and free testosterone). BMD, bone mineral density; E₂, estradiol; 25OHD, 25-hydroxyvitamin D.
3A separate analysis was conducted for each individual group (not in table). In the WL group, there was a trend for the change in fat free soft tissue to explain 18% of the change in total body BMD (β = 0.42, p=0.06) and there were no other significant explanatory variables for the femoral neck BMD or cortical thickness. There were no explanatory variables for the WM group.
Table 8. Nutrient intake at baseline and average estimate over 6 months of intervention

<table>
<thead>
<tr>
<th></th>
<th>WL (N=19)</th>
<th>WM (N=19)</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Baseline</td>
<td>Intervention</td>
<td>Baseline</td>
</tr>
<tr>
<td>Energy (kcal)</td>
<td>2034 ± 376</td>
<td>1595 ± 182</td>
<td>2178 ± 264</td>
</tr>
<tr>
<td>Protein (g)</td>
<td>94.2 ± 23.8</td>
<td>81.1 ± 8.4</td>
<td>87.4 ± 6.8</td>
</tr>
<tr>
<td>Carbohydrate(g)</td>
<td>235.1 ± 8.4</td>
<td>185.8 ± 8.4</td>
<td>259.8 ± 55.9</td>
</tr>
<tr>
<td>Total Fat (g)</td>
<td>82.4 ± 26.2</td>
<td>60.7 ± 17.6</td>
<td>90.3 ± 10.1</td>
</tr>
<tr>
<td>Protein (% of energy)</td>
<td>18.3 ± 1.8</td>
<td>20.5 ± 2.8</td>
<td>16.3 ± 3.0</td>
</tr>
<tr>
<td>Carbohydrate (% of energy)</td>
<td>46.6 ± 7.7</td>
<td>46.9 ± 5.4</td>
<td>47.3 ± 4.5</td>
</tr>
<tr>
<td>Fat (% of energy)</td>
<td>36.2 ± 8.4</td>
<td>33.6 ± 6.7</td>
<td>37.4 ± 3.0</td>
</tr>
<tr>
<td>Calcium (mg)²</td>
<td>1198 ± 176</td>
<td>1179 ± 251</td>
<td>1102 ± 94</td>
</tr>
<tr>
<td>Magnesium(mg)</td>
<td>421 ± 63</td>
<td>374 ± 51</td>
<td>481 ± 65</td>
</tr>
<tr>
<td>Phosphorus(mg)</td>
<td>1520 ± 445</td>
<td>1225 ± 169</td>
<td>1466 ± 128</td>
</tr>
<tr>
<td>Sodium(mg)</td>
<td>3500 ± 773</td>
<td>2715 ± 439</td>
<td>2974 ± 563</td>
</tr>
<tr>
<td>Vitamin D(mcg)</td>
<td>10.6 ± 1.2</td>
<td>10.9 ± 1.3</td>
<td>10.9 ± 0.8</td>
</tr>
<tr>
<td>Vitamin K(mcg)</td>
<td>85.5 ± 31.4</td>
<td>81.7 ± 45.8</td>
<td>92.5 ± 60.5</td>
</tr>
</tbody>
</table>

¹Observed mean ± SD (all such values). A mixed-model ANCOVA analysis was performed with time (0, 6 months) and group (WL or WM) as independent variables with age, body weight and physical activity level as covariates. Nutrient intake is an average over 6 months (3 day food diaries from each month).

²Intake includes multivitamins (210 mg calcium, 10ug of vitamin D, 48 ug of phosphorous, 120 mg magnesium, 20ug vitamin K), individualized calcium supplement (200 mg Ca citrate).

³Pre-stabilization intake of calcium was 804 ±333 and 728 ± 129 mg in WL and WM groups respectively.

⁴Throughout the intervention, total calorie, protein, carbohydrate and total fat intakes were lower in the WL group than the WM group.
Figure 5. Flowchart of study participants.

1Non-compliance was defined as weight loss of <2.5% of initial body weight; WL, weight loss; WM, weight maintenance. 2Personal reasons: distance, time commitment.
Figure 6. Changes in serum 25-hydroxyvitamin D [25OHD], parathyroid hormone (PTH), total testosterone (T) and estradiol during the intervention in men adhering to weight loss (WL) and weight maintenance (WM) (n=38).

Repeated measures ANCOVA analysis using mixed models was performed with time (0, 1, 3 and 6 months) and group (WL or WM) as independent variables with age, season, physical activity level and body weight as covariates. *time effect in WL and WM groups (p < 0.05) with no difference between groups; †WM differs from WL group (interaction effect over time, and at 6 mo post-hoc test), p < 0.05. Diamond dotted line represents WL and square solid line represents WM.
Chapter 3.

Three doses of vitamin D, bone mineral density and geometry in older women during modest weight control in a 1-year randomized controlled trial
Abstract

Studies examining how bone responds to a standard dose of vitamin D supplementation have yielded inconsistent results. In addition, the effects of higher doses on bone mineral density (BMD) and quality are not known. Postmenopausal women undergoing weight control to improve health, are particularly at risk for bone loss, and might benefit from supplemental vitamin D intake above the recommended allowance. This one year-long, randomized, double blind controlled study addresses whether vitamin D supplementation, in healthy overweight-obese older women, affects bone mineral density (BMD) and bone structural parameters. In addition, bone turnover, and serum total, free and bioavailable 25-hydroxyvitamin D (25OHD) response to one of three daily levels vitamin D$_3$ (600 IU; 2000 IU; 4000 IU) with 1.2 Ca g/d while during weight control were examined. Fifty-eight women (age, 58 ± 6 years; body mass index, 30.2 ± 3.8 kg/m$^2$, serum 25OHD, 27.3 ± 4.4 ng/ml) were randomized to treatment. After one year, serum 25OHD concentrations increased to 26.5 ± 4.4, 35.9 ± 4.5 and 41.5 ± 6.9 ng/ml, in groups 600, 2000 and 4000 IU, respectively, and differed between groups (p<0.01). Weight change was similar between groups (-3.0 ± 4.1%). There was a trend for trochanter BMD to decrease more in the 600 IU compared to higher intakes of vitamin D (p < 0.06). Cortical (Ct) thickness of the tibia changed by -1.5 ± 5.1%, +0.6 ± 3.2% and +2.0 ± 4.5% in the 600 IU, 2000 IU and 4000 IU, respectively and each group was significantly different from each other (p < 0.05). The decline in Ct thickness was prevented with higher vitamin D$_3$ supplementation, and there was a trend to attenuate trochanter BMD in older women over one year. Whether these findings translate to changes in biomechanical properties leading to reduced fracture risk should be addressed in future studies.
Introduction

Vitamin D maintains calcium and phosphate homeostasis and is responsible for bone health throughout life (DeLuca, 2008; Reichel and Norman, 1989). However, the optimum vitamin D dose and serum 25-hydroxyvitamin D (25OHD) concentration for best skeletal outcomes is less clear. The controversy has been fueled by an inconsistent relationship between vitamin D and bone mineral density (BMD) in cross-sectional studies, as well as a lack of association between vitamin D supplementation and fracture prevention (Reid et al., 2014). Insufficient vitamin D dosing necessary to increase serum 25OHD concentrations to healthy ranges or high baseline serum 25OHD have been suggested as reasons for the non-significant effects of vitamin D supplementation on BMD in some studies (Lappe et al., 2012; Jorde et al., 2015). In addition, it has been argued that the bioavailable 25OHD, rather than total 25OHD, concentrations are a better assessment of vitamin D status and therefore, should be more strongly associated with BMD (Powe et al., 2011; Chun et al., 2014).

A negative relationship between body weight and serum 25OHD has been reported previously (Liel et al., 1988; Parikh et al., 2004; Jungert et al., 2012) and it has been attributed to factors such as adipose tissue acting as a depot for the steroid and impaired vitamin D bioavailability in the obese, inadequate sunlight exposure, true vitamin D deficiency, genetic polymorphisms, or other unknown factors (Chun et al., 2014; Jungert et al., 2012; Drincic et al., 2012; Wortsman et al., 2000). Moreover, excess adiposity attenuates serum 25OHD response to vitamin D supplementation in the obese compared to lean individuals (Wortsman et al., 2000; Drincic et al., 2012; Gallagher et al., 2013). In addition, serum 25OHD response to vitamin D treatment could be further
influenced by age, ethnicity, baseline serum 25OHD, type and level of vitamin D intake (Zittermann et al., 2014).

Older individuals and in particular postmenopausal women are at high risk of bone mineral loss and changes in structural elements and this leads to loss of bone strength and increased fracture risk (Andreopoulou and Bockman, 2015). In addition, because postmenopausal women often experience weight gain, many undergo weight control plans designed to reduce obesity associated comorbidities. Although beneficial, weight loss decreases calcium absorption and promotes bone loss (Shapses and Sukumar, 2012). It has been shown that even a modest loss of 3-4% body weight can reduce BMD in women (Salamone et al., 1999; Uusi-Rasi et al., 2001). The beneficial effects of a standard vitamin D supplementation in combination with calcium on BMD and bone quality measures have been shown previously, but studies examining higher doses of vitamin D have not been conducted (IOM, 2011).

Whether bone health further improves with higher doses of vitamin D above the recommended levels has been questioned (Reid et al., 2014). Moreover, vitamin D interventions on bone quality are not available. The goal in this study is to determine whether vitamin D supplementation, in healthy overweight/obese postmenopausal women (≤70 y of age), attenuates bone changes accompanying weight control over 1 year. We addressed whether there is a dose-dependent effect of vitamin D$_3$ supplementation on BMD, geometry, strength and bone microstructure over 12 months.

**Subjects and Methods**

**Subjects**
The 3DD study (3 doses of vitamin D) is a randomized controlled double blind trial to determine the effects of vitamin D₃ supplementation on BMD and bone microarchitecture in older women. We recruited healthy, postmenopausal women (age 50-70 years old; BMI 25-40 kg/m²) through advertising in local newspapers and email list servers from January through February, over a 3-year period. Subject’s enrollment and randomization were conducted during winter months to minimize seasonal fluctuations in serum 25OHD concentration.

Participants passing an initial telephone screening questionnaire, followed by laboratory and physical screening were considered eligible. Subjects were not eligible if they were less than 2 years postmenopausal, or experiencing more than 5% body weight loss or gain within 3 months prior to recruitment. Subjects diagnosed with diseases known to influence Ca metabolism (i.e. metabolic bone disease, hyperparathyroidism, untreated thyroid disease, significant immune, hepatic, or renal disease, kidney stone in the last 5 yrs., significant cardiac disease, active malignancy or cancer therapy within the past year) or under treatment known to influence Ca or bone metabolism were excluded. Only volunteers with serum 25OHD concentrations <30 ng/ml were allowed to participate.

During the first visit to the Nutrition and Bone laboratory subjects signed an informed consent approved by the Rutgers University Institutional Review Board and an external data safety-monitoring advisory board. This trial was registered at clinicaltrials.gov as NCT01631292. The protocol met the ethical standards in accordance with the Helsinki Declaration.

*Study design*
This was a 1-year, randomized, double-blind, controlled trial conducted at Rutgers University, New Brunswick, NJ. One month prior to treatment assignments, subjects underwent a stabilization period during which they were asked to maintain body weight and were given a multivitamin/mineral and calcium supplement to total 600 IU/d vitamin D and 1.2 g Ca/d (diet and supplement) that continued throughout the study duration. When daily calcium intake was <1.2 g from the diet and the daily multivitamin/mineral supplement combined, an extra calcium supplement without added vitamin D was provided to those subjects (200 mg Ca/tablet; Citracal; Bayer Health care LLC). The multivitamin/mineral supplement (NatureMade Multi 50+) given was started 1 month prior to baseline measurements (during stabilization) and continued throughout the study to meet dietary requirements. Each serving size contained 10 μg (400 IU) vitamin D$_3$. At the end of the stabilization period, participants were randomly assigned using SAS, Version 9.3 (SAS Institute, Cary, NC, USA) in a double-blind manner to one of three vitamin D treatment groups (habitual dietary intake + supplements) estimated at 600 IU, 2000 IU and 4000 IU per day.

Vitamin D$_3$ capsules and identical-looking placebos were purchased from Bio Tech Pharmacal and manufacturer’s stated D$_3$ content was 5000 IU vitamin D$_3$/capsule. Vitamin D$_3$ capsules were analyzed by reversed-phase high performance liquid chromatography (C18 column) and UV detection in our lab after the study ended, and indicated a vitamin D$_3$ content/capsule of 4898 IU. Participants were asked to consume vitamin D$_3$ capsules or placebo during 5 consecutive weekdays with their largest meal of the day. The 600 IU group received 5 placebo capsules/week; the 2000 IU group received 2 vitamin D$_3$ capsules + 3 placebo/week and the 4000 IU group received 5 vitamin D$_3$
capsules/week. All subjects also received 400 IU from the multivitamin/mineral supplement. Total daily vitamin D intake (diet + supplements) is reported in Table 12.

Behavior-modification/nutrition-education - Weekly nutrition-education classes were offered during the stabilization period and at monthly intervals thereafter. Subjects followed a standard behavior modification nutrition education/weight control program and adhered to individualized moderate energy-restricted diets with modest weight-loss goals. Participants recorded their caloric intake with food diaries on a weekly basis to enhance compliance with the nutrition-education plan. Food diaries were analyzed from 2 non-consecutive weekdays and one weekend day at baseline and at regular intervals three times during the intervention using nutrient analysis software (Version 17, FoodWorks, Long Valley, NJ). A registered dietitian reviewed these diaries and discussed them at weekly visits in the first two months and monthly thereafter during group and individualized counseling sessions.

The volunteers were asked to follow their physical activity routine throughout the study, with no specific instructions. Physical activity level was recorded on their food diaries. A numerical score was calculated using a range from 0 to 3 (0-inactivity, 1-low activity, 2-moderate activity and 3-high activity) to reflect an estimation of energy expenditure in metabolic equivalent-minutes per week (Ainsworth et al., 1998).

Measurements

Assessment of sun exposure - Quantification of skin color changes with season was performed by using a reflectance spectrophotometer (model CS-100, Konica Minolta Optics Inc., Osaka, Japan) at two different skin areas: mid outer forearm (exposed) and
waist (unexposed). Measurements are reported using Yxy color-indexing system where Y measures reflectance ranging from 0 (black) to 100 (pure white) (Takiwaki, 1998).

*Anthropometric measurements* - Anthropometric measurements were performed during monthly visits. A balance beam scale and stadiometer (Detecto, Webb City, MO) were used for this purpose. Body weight and height were measured to the nearest 0.25 kg and cm, respectively. Body mass index (BMI) was computed as weight in kilograms divided by the square of height in meters (kg/m²).

*Bone and body composition measurements*

Baseline and 12 months measurements of BMD and BMC at total hip, lumbar spine, femoral neck, trochanter and total body were performed using a dual energy x-ray absorptiometry total body scanner (Lunar Prodigy Advanced; GE-Lunar, Madison, WI; CV ≤ 1% for all sites). In addition, soft tissues were evaluated including total tissue, fat (android/gynoid) and lean tissue compartments.

Volumetric (v) total BMD, trabecular, cortical BMD and BMC, geometry (cortical thickness, Ct.Th) and strength indices (IP, polar moment of inertia; SSI, stress-strain index) at the distal tibia were measured at specific sites using peripheral quantitative computed tomography (pQCT) (Stratec XCT 3000, Orthometrix, NY). The scans were analyzed using STRATEC XCT-3000 software, Version 5.4. A scout view allowed the positioning of the cross-sectional measurements along the tibia. The voxel size for all the scans was 0.5-mm and the slice thickness was 2.4 mm. The precision error (coefficient of variation) was less than 1.7 % for all the measurements.
A custom software package, pQCT OsteoQ (Inglis Software Solutions Inc., Hamilton, ON) combining threshold-based and region-growing algorithms was used to measure apparent trabecular microstructure (trabecular separation (Tb.Sp), bone volume fraction (BV/TV), trabecular number (Tb.N) and trabecular thickness (Tb.Th). Short-term validity of bone microstructure measurements indicated a precision error of less than 5% (Wong et al., 2014).

**Laboratory methods**

Fasting serum and urine samples were collected at baseline and month 6 and 12 and were analyzed in batch analysis. Serum was analyzed for: 25OHD (radioimmunoassay; DiaSorin) (CV <12.5%), intact PTH (immunoradioassay; Scantibodies) (CV < 6.8%) and ultrasensitive estradiol (radioimmunoassay; DSL) (CV <8.9%). The performance of our 25OHD assay has been monitored and issued a proficiency certificate by the vitamin D External Quality Assessment Scheme.

Calcium and albumin concentrations were measured using colorimetric assays on an automated analyzer. Concentrations of serum vitamin D binding protein (DBP) were determined using a commercial enzyme-linked immunosorbent assay kit (ALPCO) (CV<12.7%) that uses two monoclonal antibodies in a sandwich format. Serum concentrations of free and bioavailable 25OHD were calculated by using a validated algorithm (Vermeulen et al., 1999).

Bone turnover markers were measured at baseline and after 1 year. Propeptide of type 1 collagen (P1NP) and C-telopeptide of type 1 collagen (CTX) were measured by ELISA
Urinary Ca was measured with a colorimetric reagent kit (Pointe Scientific, Canton, MI, USA, CV <4.6%).

**Compliance**

Adherence to treatment protocol was assessed by pill count at each counseling session. Dietary Ca intake was estimated from food records. In addition, compliance with vitamin D intervention was assessed by measuring serum concentrations of 25OHD at fixed intervals throughout the study.

**Safety**

Study outcomes and adverse events were reviewed periodically by an internal Data Safety Monitoring Committee and Rutgers University IRB. A serious adverse event was defined as resulting in death; it is life-threatening; requires inpatient hospitalization or results in persistent or significant disability/incapacity. Adverse events such as: pain in legs, swelling in legs, pain or heaviness in chest, headaches, dizziness, nausea, fatigue, muscle weakness, muscle aches, abnormal urinary frequency, and abdominal pain were categorized as non-serious adverse events and were recorded during the study to assess the safety of vitamin D supplementation. Adverse events forms were filled in at baseline and during monthly counselling sessions by all subjects. Serum and urinary calcium were measured at baseline and after 12 month of treatment.

**Sample size**

In a previous study conducted in overweight postmenopausal women, mean age of 61 years, it was found that a moderate weight loss resulted in significantly more BMD loss at the trochanter (-0.031 ± 0.029 g/cm²) in the group supplemented with normal
compared to high Ca intake (-0.010 ± 0.034 g/cm²) (Riedt et al., 2005). To be able to
detect a similar difference at the trochanter BMD between three levels of vitamin D₃
intake and to allow for two covariates, a sample size of 18 per group (power of 80% and
α set at 0.05) was calculated. Based on previous clinical trials conducted in our lab, an
additional 4 subjects per group were recruited to account for dropouts. We used single-
block randomization for vitamin D group assignments generated by the statistical
consultant of the study and the principal investigator (PI) assigned the participants in
randomly mixed block sizes until the required sample size was met. The PI remained
blinded to the treatment assignments throughout the study. In addition, all clinical
personnel and subjects were blinded to the randomization status.

Statistical analysis

Statistical analyses were conducted with SAS, version 9.3 (SAS Institute, Cary, NC,
USA). An intention-to-treat analysis (ITT), including all randomly assigned participants,
was used to examine the effect of vitamin D₃ supplementation on bone outcomes. The
last observation carried forward was used for the subjects with missing data. The groups
were compared for baseline characteristics by using one-way ANOVA. Variables
considered clinically important, even if they did not reach statistical significance (i.e.
body weight) and other factors such as age and years since menopause, were used as
covariates in the analyses. Mixed-model ANCOVA was used to compare differences in
bone parameters, fat, fat free soft tissue, hormones and bone turnover markers over time
between treatment groups. Two fixed factors were examined: group (with doses of
intervention) and time (baseline and 12 month). When the interaction (group-by-time)
was significant, post-hoc analysis was conducted with Bonferroni correction for multiple
comparisons. Mixed-model ANCOVA has the advantage of adjusting for dependencies between repeated responses and allows for missing data without introducing bias. Pearson correlation coefficients were used to assess the relationship between changes in independent and outcome variables. Stepwise multiple linear regression analysis (forward selection technique) was performed to select the explanatory variables that would be considered the most important predictors for bone changes. Calcium intake was controlled and adjusted to meet the requirements in all the participants throughout the study period; therefore we did not need to include this as a covariate in the analysis. The recruitment and study start-up procedures were conducted during winter months to minimize the effect of sunlight on serum 25OHD. In addition, seasonal skin color changes were quantified and all subjects began the intervention in the winter months. Hence we did not include season as a covariate in the analysis.

In addition, an as-treated analysis for subjects who completed the study and who were at least 80% compliant with vitamin D supplementation was conducted for all the outcomes. Values are reported as mean ± SD, and graphs with SEM. Categorical values are expressed in percentages to represent a portion of the sample. Values ≤0.05 were considered significant.

**Results**

**Participants**

The study participant’s flowchart is shown in Figure 7. Of the 210 women assessed for eligibility, 102 met the inclusion criteria and were recruited for the study. Twenty-one women withdrew before treatment initiation due to time commitment (n = 10), lack of interest in the study (n=7) and lack of adherence to the stabilization period intervention (n
Eighty-one women were randomized to one of the three vitamin D₃ groups: 600 IU/d (n = 24), 2000 IU/d (n = 28), or 4000 IU/d (n = 29). During the intervention, 23 women in all groups, discontinued the study due to time commitment (n = 10), moving away (n = 5) or were lost to follow-up (n = 7). One volunteer, randomized to 600 IU group, lost a dramatic amount of weight (>30% body weight) and therefore did not meet the criteria for as-treated analysis, and was dropped from the study. Final analysis includes fifty-eight women (age, 57.6 ± 5.6 years; BMI, 30.2 ± 3.7 kg/m²), predominantly Caucasian (85%). At screening, 68% of the subjects were taking multivitamins and 18% of them were taking additional vitamin D supplements. Calcium supplementation was used by 63% of the subjects. Reported habitual intake of vitamin D and Ca (diet and supplements) in the volunteers prior to stabilization month ranged from 64 to 1085 IU/d and from 510 to 1600 mg, respectively. During the stabilization month, vitamin D and Ca intake from diet and supplements averaged 565 ± 14 IU and 1186 ± 71 mg/d, respectively. At screening, serum 25OHD concentration was 22.8 ± 6.4 ng/mL and increased to 27.2 ± 4.3 ng/mL after one month of stabilization with a multivitamin/mineral pill.

Skin reflectance (Y) values at the beginning of the study (winter) averaged 47 ± 16 cd/m² at the exposed area and 67 ± 14 cd/m² at the unexposed area (p<0.01) and there were no significant differences between groups. Similarly, after the summer months, skin reflectance was lower (42 ± 11 cd/m²) at the exposed compared to the unexposed area (58 ± 21 cd/m², p<0.01) and the groups did not differ significantly. The difference between skin reflectance at the unexposed areas compared to exposed areas remained significant in all subjects throughout the study (p<0.01).
**Nutrient intake and physical activity**

Nutrient intake at baseline was similar between groups (Table 12). Across all groups, there was a decrease in energy, fat, phosphorus, magnesium, sodium and vitamin K intake associated with caloric restriction. Vitamin D intake was significantly different between treatment groups (p<0.001) (Table 12). Supplement compliance, as calculated by monthly pill counts was similar between groups and averaged 83 ± 10% and 89 ± 11% for the vitamin D and multivitamin capsules, respectively. Diaries showed that most of the women had a low to moderate level of physical activity at baseline (2.1 ± 0.8), and over the 12 month study, there was no significant change in habitual level of activity over time or between groups (data not shown).

**Weight, body composition, and BMD**

Baseline and 12 month measurements for weight, body composition and BMD at multiple sites are presented in Table 9. There were no significant differences for baseline characteristics between groups. After 12 months, subjects lost 3.0 ± 4.1 % body weight and 3.1 ± 8.5% fat mass with no significant difference between groups and the findings were similar for both ITT and as-treated analyses (p value for time< 0.02). ITT analysis showed no difference over time between groups for fat-free soft tissue, total body BMC and BMD with intervention. When using the as-treated analysis of the completers, there was a trend for trochanter BMD to differ between groups over 12 month of vitamin D treatment (p=0.056) (Table 9). No differences between groups with vitamin D₃ treatment were found for femoral neck BMD, radius, lumbar spine, total hip and total body BMD with the as-treated analysis (Table 9, Figure 8A).
Trabecular and cortical bone at the tibia

The ITT analysis yielded a statistically significant interaction between treatment and time for cortical thickness (p<0.03). There was also a loss of total and trabecular BMC over time in all the groups (p<0.05). Similarly, the completer’s analysis showed that cortical thickness changed differently between groups over time: -1.5 ± 5.1%, +0.6 ± 3.2% and +2.0 ± 4.5% in the 600 IU, 2000 IU and 4000 IU, respectively (p < 0.05) and each group differed significantly from all others (Bonferroni-corrected p value < 0.05) (Table 10, Figure 8B). In addition, in the as-treated analysis there was a weak trend for cortical vBMD to differ over time between groups (p<0.1). No other changes in cortical variables between groups or over time were observed (Table 10). Both ITT and as-treated analyses showed a decrease over time in trabecular volumetric BMD and BMC in all women (p<0.05) and the change was not significantly different between groups. As-treated analysis indicated an increase in trabecular separation (+3.1 ± 5.2%) and a decrease in trabecular number (-1.6 ± 3.0%) in the 600 IU/d group compared to baseline measurements (p < 0.05). In addition, in the 600 IU/d group, there was a trend for bone volume/tissue volume to decrease from baseline (-1.3 ± 2.5%) with no change for trabecular thickness. However, the interaction effect between treatment and time was not significant for any of this trabecular parameters. No other parameters were found to differ significantly between groups.

Bone regulating hormones and bone turnover markers

Bone regulating hormones and bone turnover markers are shown in Table 11. The increase in serum 25OHD in response to 600, 2000 or 4000 IU vitamin D$_3$/d in this overweight/obese population was 3.8 ± 4.1, 7.4 ± 6.5, and 14.1 ± 8.1 ng/ml, respectively.
(p value for interaction between time and treatment <0.01) (Table 11, Figure 9). No subject had a serum 25OHD level below 20 ng/mL after any of the interventions. In the placebo group (600 IU) 58% of subjects had 25OHD concentrations < 30 ng/ml, whereas in the higher dose vitamin D groups, only 1-2 subjects in each group had values < 30 ng/mL. In the 2000 IU and 4000 IU groups, 20% and 42% of the women had 25OHD concentration above 40 ng/ml. The change in free 25OHD and bioavailable 25OHD differed with treatment dose (p value for interaction <0.02) (Table 11). Serum DBP concentration decreased over time in all groups (p< 0.05), but there was no significant difference due to vitamin D₃ dose. After 12 month of treatment, PTH decreased by 10.6 ± 16.6 % and the change did not differ significantly between groups (Table 11, Figure 9). The bone turnover markers, CTX and P1NP, increased by 10.0 ± 15.9% and 17.6 ± 20.2%, respectively from baseline to 12 months (p<0.05), and the rise did not differ significantly between groups (Table 3). A trend for serum estradiol to decrease by 4.5 ± 22.6 % in all the groups was observed (p<0.07).

*Pearson correlation and stepwise regression*

The relationship between changes in body weight, body composition and bone parameters, hormones and bone turnover markers was analyzed within each treatment group. In the 600 IU group, trochanter BMD loss correlated with weight loss (r=0.475, p< 0.03) and total body fat loss (r=0.531, p<0.02). Furthermore, in the 600 IU group there was a trend for bone loss at the femoral neck to correlate with weight loss (r=0.435, p<0.07). There was no significant relationships between any variables at higher levels of vitamin D₃ intake (2000 and 4000 IU). An inverse relationship between changes in serum
CTX, but not P1NP, and body weight (r=-0.640, p<0.01) and fat mass (r=-0.659, p<0.01) was found in the 600 IU group, but not in the other treatment groups.

**Stepwise regression.** Using bone outcomes as dependent variables and changes in body-composition and hormone levels as independent variables, a stepwise regression was performed. Only the independent variables that reached significance were included in the final model. Analysis indicated that during weight loss, at 600 IU vitamin D intake, changes in trochanter BMD were explained by changes in body weight (R²=23.5%, p<0.05). At higher levels of vitamin D intake, changes in body weight were not significantly associated with changes in trochanter BMD. In addition, weight loss was the major predictor for the increase in CTX (R²=46%, p=0.002) in the 600 IU group, but it did not reach significance at higher levels of intake.

**Safety**

There were no significant differences for serum calcium concentrations between groups at baseline or after the intervention, as measured. No subject presented with hypercalcemia (>10.5 mg/dL) at 12 month. The urinary calcium excretion (corrected for creatinine) and creatinine clearance were similar between groups over 12 month of intervention (Table 11). No serious adverse events were reported during the study (Table 13). The cumulative frequency of non-serious events was not significantly different between groups at any time point during the study.

**Discussion**

Vitamin D is essential for bone health and prevents rickets and osteomalacia by promoting calcium absorption required for bone mineralization. However, to what extent
vitamin D may prevent bone loss during moderate caloric restriction and attenuates bone quality is largely unknown. In this one year long trial, we examined the effect of three vitamin D doses on aBMD and bone quality during modest weight loss. In this overweight/obese population of postmenopausal women, the increase in serum 25OHD in response to 600, 2000 or 4000 IU vitamin D₃/d occurred in a dose response manner. After 12 month of intervention and 3% weight reduction, there was a decrease in cortical thickness and a trend to decrease trochanter BMD in the 600 IU group but not in the higher vitamin D groups, whereas no other parameters (bone geometry, strength and microstructure) differed due to the dose of vitamin D₃ intake.

It is well established that weight loss (3-10%) decreases BMD at various bone sites as compared to weight stable women especially at older ages (Riedt et al., 2005; Villareal et al., 2006; Uusi-Rasi et al., 2009). This decrease is attributed to at least a few factors including a decrease in nutrient intake (Jensen et al., 2001; Ricci et al., 2001) or a decrease in calcium absorption (Cifuentes et al., 2004; Shapses et al., 2013) reduced mechanical loading (Villareal et al., 2006) and reduced estradiol or insulin-like-growth factor levels accompanying weight reduction (Shapses and Sukumar, 2012). In this one year trial, there was no bone loss over time which may be attributed to the small amount of weight loss while consuming the recommended intake of Ca and vitamin D (or higher intakes) in all groups of women. However, there was an increase in bone turnover possibly due to aging or the slight energy restriction, but this did not differ between groups.

In weight stable women, there are many studies examining the effect of supplemental vitamin D intake on bone (Chapuy et al., 1992; Dawson-Hughes et al., 1995; Ooms et al.,
1995; Cooper et al., 2003; Meier et al., 2004; Aloia et al., 2005; Jorde et al., 2010; Grimnes et al., 2012). Ooms et al showed that 400 IU/d compared to placebo did not prevent bone loss that occurs with aging over 1 year (Ooms et al., 1995). In some trials BMD loss was attenuated with 700-800 IU/d (Chapuy et al., 1992; Dawson-Hughes et al., 1995) and calcium supplements (500-1,200 mg) while others, using similar doses, found no effect of vitamin D on BMD (Meier et al., 2004; Aloia et al., 2005). Higher doses of vitamin D on BMD also yielded inconsistent results. While daily doses of 1428 IU D$_2$ and 1000mg calcium compared to calcium alone did not influence bone (Cooper et al., 2003), others reported higher BMD at the lumbar spine and hip with 5000 IU D$_3$/d and 320mg calcium/d compared to placebo (Mocanu et al., 2009). Nevertheless, none of these studies included bone quality measures or used a model of energy restriction. In addition, it is possible that the inclusion of supplemental micronutrients in the current study may have influenced the effect on bone in our study compared to other trials.

To date, most interventions with vitamin D supplementation have used areal BMD derived from dual energy x-ray absorptiometry as a surrogate measure of fracture risk (Kanis et al., 2008). However, the 2-dimensional measurement cannot adequately assess bone geometry and consequently bone strength that are independent of changes in areal bone mineral density. Peripheral quantitative computerized tomography captures geometrical changes at the cortical and trabecular bone compartments, which respond differently to physiological and pathological factors (Griffith et al., 2008). In this study, supplemental vitamin D$_3$ did not affect aBMD, but the higher intakes influenced bone geometry, specifically an increase in cortical thickness, that may translate to improved biomechanical strength.
A positive association between 25OHD levels and bone quality at the radius and tibia was found in older Caucasian men (Barbour et al., 2011) and some reported a catabolic effect of PTH on the cortical bone in both obese and non-obese individuals (Charopoulos et al., 2006). On the other hand, excess PTH has been shown to maintain or even have anabolic effects on trabecular bone (Duan et al., 1999) while high levels of PTH can result in an increased endocortical resorption, cortical thinning and cortical porosity (Dempster et al., 2007). Few studies have examined the relationship between 25OHD and the cortical and trabecular bone of the tibia in older women, but one reported a positive association between 25OHD and cortical vBMD (that was negative with PTH), but no relationship of either 25OHD or PTH with trabecular vBMD (Lauretani et al., 2008). It is thus hypothesized that suppression of PTH that can be achieved by vitamin D supplementation, may improve cortical vBMD in obese women, independently of modest body weight changes. Weight loss, on the other hand, has been shown to either decrease total vBMD with trend to decrease cortical vBMD in postmenopausal women (Sukumar 2011) but not in premenopausal women (Uusi-Rasi et al., 2009). Reports of energy restriction in rodent models with more dramatic weight loss show a decrease in cortical bone parameters, such as vBMD, BMC and CtTh (Hamrick et al., 2008) and another study indicated that trabecular parameters were also affected (BV/TV and TbTh) (Hamrick et al., 2008; Mardon et al., 2008). The current study suggests that weight loss (time effect) causes a decrease in trabecular vBMD, BMC and BV/TV and increase in separation with no significant effect on cortical bone. The addition of vitamin D had no effect in preventing the trabecular decline with modest weight loss over 1 year. Also,
even at high intakes of vitamin D₃, cortical vBMD was unaffected, but there was a positive effect on cortical thickness.

Determining an optimum level of vitamin D supplementation and serum 25OHD concentration may be important in the older population to minimize bone loss or changes in quality during energy controlled diets. In addition, the overweight or obese population typically has lower serum 25OHD than normal weight persons, and is at risk of bone loss due to energy restriction (Shapses and Sukumar, 2012). The rise in serum 25OHD was 0.19 and 0.27 ng/mL per μg intake when consuming 2000 or 4000 IU/d, respectively. A slightly lower rise in 25OHD (0.16 ng/mL/μg/day) was found in a previous study when given similar vitamin D₃ doses and may be due to their higher BMI (>35 kg/m²) (Dhaliwal et al., 2014) compared to 30 kg/m² in the current study. Free and bioavailable 25OHD also increased with increasing doses of vitamin D intake and remained at a steady 0.02% and 7% of total 25OHD concentration, respectively, throughout the study and in all groups.

Furthermore, although there are seasonal influences on 25OHD levels, the current study was 1 year, and measurements of skin reflectance, as a marker of sun exposure, indicated no significant differences between groups or from baseline to final measurements.

After one year of intervention, bone turnover markers increased in all subjects with no significant differences between groups. Studies with vitamin D supplementation on bone turnover in postmenopausal women are scarce and have yielded mixed results. In a previous study in women (50-80 yr) with similar baseline serum 25OHD concentrations, after 1 yr (no weight loss) vitamin D supplementation reduced bone turnover, but the reduction was greater in their standard dose (800 IU/d) compared to the high dose
treatment (6500 IU) (Grimnes et al., 2012). In another study, there was no effect of vitamin D treatment at any dose (400, 800 or 1000 IU) on bone turnover (MacDonald et al., 2013). In the current study, the rise in bone turnover was likely due to the combined effect of aging (Ensrud et al., 1995) and weight loss (Riedt et al., 2005; Villareal et al., 2006; Uusi-Rasi et al., 2009).

There are several limitations in this study including sample population consisting of healthy postmenopausal women. The results may not be applicable to other populations with low serum 25OHD concentrations or with chronic conditions. However, the intention of this study was to examine whether higher than recommended vitamin D doses in individuals has additional beneficial effects on bone. In addition, a transient increase in BMD remodeling with vitamin D and calcium supplementation is possible in a one year study, and bone changes would ideally be examined in a longer intervention consisting of more remodeling cycles (Aloia et al., 2008). Another limitation in this study is the analysis of multiple endpoints raising the possibility of chance results, but we used Bonferroni correction for multiple comparison to correct for this. Although errors associated with DXA measurements are possible, especially in overweight/obese subjects with excess body fat surrounding the bone or due to changes in total body fat in these individuals (Knapp et al., 2015; Yu et al., 2012) the modest weight loss achieved in this study was less likely to influence the precision errors of DXA measurements. Total fat loss did not exceed 2 kg of fat representing significantly less than the 6 kg excess fat surrounding the bone shown to produce measurements errors by DXA, but not when measuring volumetric BMD by pQCT (Yu et al., 2012). Moreover, by using pQCT measurements we were able to acquire additional information about bone
microarchitecture that areal BMD measurements alone cannot capture, and these measurements are especially important in studies of the obese who show evidence of compromised bone quality. Subjects in this study were recruited in the same season (winter) so the seasonal influence on 25OHD concentrations over 1 year was similar between individuals or groups. Also, we were able to measure DBP concentrations and free and bioavailable 25OHD metabolites and to determine whether DBP modulates the relationship between 25OHD and bone outcome.

In conclusion, higher doses of vitamin D₃ had a small effect on BMD and prevented a decline in cortical thickness over 1 year in healthy postmenopausal women. Whether this vitamin D effect on bone geometry indicates greater bone strength overall and could lead to fracture reduction remains to be determined.

**Acknowledgments and Funding Sources**

We thank Robert Zurfluh, RD and the other clinical staff for their invaluable clinical assistance and Lihong Hao, PhD for her laboratory assistance with vitamin D₃ analysis of the supplements. We are also grateful for the commitment of the volunteers. This study was supported by NIH-AG12161 and Busch Biomedical Award (BBGP2010695157).
Table 9. Body composition and aBMD over 12 months at three doses of vitamin D3

<table>
<thead>
<tr>
<th></th>
<th>600 (n=19)</th>
<th>2000 (n=20)</th>
<th>4000 (n=19)</th>
<th>P value</th>
<th>Group</th>
<th>Time</th>
<th>Group X Time</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Baseline</td>
<td>12 months</td>
<td>Baseline</td>
<td>12 months</td>
<td>Baseline</td>
<td>12 months</td>
<td></td>
</tr>
<tr>
<td>Age (years)</td>
<td>57.4 ± 6.3</td>
<td>58.4 ± 4.9</td>
<td>57.2 ± 5.5</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>29.5 ± 2.9</td>
<td>28.7 ± 3.3</td>
<td>31.5 ± 4.5</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Weight (kg)</td>
<td>79.2 ± 10.4</td>
<td>76.8 ± 10.4</td>
<td>85.9 ± 13.3</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fat mass (kg)</td>
<td>33.8 ± 6.5</td>
<td>32.8 ± 7.6</td>
<td>38.9 ± 8.9</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fat-free soft tissue (kg)</td>
<td>41.2 ± 4.2</td>
<td>41.4 ± 4.4</td>
<td>42.4 ± 5.5</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>BMD (g/cm²)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>UD radius</td>
<td>0.34 ± 0.04</td>
<td>0.34 ± 0.04</td>
<td>0.35 ± 0.04</td>
<td>0.35 ± 0.04</td>
<td>0.35 ± 0.05</td>
<td>0.35 ± 0.06</td>
<td>0.567</td>
</tr>
<tr>
<td>1/3 radius</td>
<td>0.66 ± 0.05</td>
<td>0.66 ± 0.04</td>
<td>0.69 ± 0.04</td>
<td>0.69 ± 0.04</td>
<td>0.66 ± 0.07</td>
<td>0.66 ± 0.07</td>
<td>0.715</td>
</tr>
<tr>
<td>Lumbar spine</td>
<td>1.13 ± 0.12</td>
<td>1.12 ± 0.13</td>
<td>1.28 ± 0.17</td>
<td>1.27 ± 0.18</td>
<td>1.18 ± 0.17</td>
<td>1.18 ± 0.17</td>
<td>0.040</td>
</tr>
<tr>
<td>Trochanter</td>
<td>0.75 ± 0.07</td>
<td>0.74 ± 0.07</td>
<td>0.79 ± 0.11</td>
<td>0.80 ± 0.12</td>
<td>0.79 ± 0.12</td>
<td>0.80 ± 0.12</td>
<td>0.443</td>
</tr>
<tr>
<td>Femoral neck</td>
<td>0.90 ± 0.11</td>
<td>0.88 ± 0.10</td>
<td>0.92 ± 0.15</td>
<td>0.92 ± 0.14</td>
<td>0.94 ± 0.10</td>
<td>0.94 ± 0.10</td>
<td>0.567</td>
</tr>
<tr>
<td>Total hip</td>
<td>0.94 ± 0.09</td>
<td>0.94 ± 0.09</td>
<td>0.98 ± 0.12</td>
<td>0.99 ± 0.13</td>
<td>0.99 ± 0.11</td>
<td>0.99 ± 0.12</td>
<td>0.493</td>
</tr>
<tr>
<td>Total body</td>
<td>1.14 ± 0.07</td>
<td>1.14 ± 0.08</td>
<td>1.18 ± 0.07</td>
<td>1.19 ± 0.07</td>
<td>1.14 ± 0.07</td>
<td>1.14 ± 0.08</td>
<td>0.568</td>
</tr>
</tbody>
</table>

Mean ± SD (all values). A mixed-model ANCOVA analysis was performed with time (0, 12 months) and group (600, 2000 or 4000 IU) as independent variables with age, years since menopause (YSM) and body weight as covariates. Abbreviations: BMI, body mass index; aBMD, areal bone mineral density; UD radius, ultra-distal radius; 1/3 radius, radial BMD at 1/3 of the distance from the distal end.
Table 10. Trabecular and cortical vBMD, geometry, strength and microstructure over 12 months at three doses of vitamin D3

<table>
<thead>
<tr>
<th></th>
<th>600 (n=19)</th>
<th>2000 (n=20)</th>
<th>4000 (n=19)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Baseline</td>
<td>12 months</td>
<td>Baseline</td>
<td>12 months</td>
</tr>
<tr>
<td>Total</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>vBMD (mg/cm³)</td>
<td>293.1 ± 32.5</td>
<td>297.2 ± 37.2</td>
<td>291.8 ± 30.2</td>
<td>292.3 ± 32.5</td>
</tr>
<tr>
<td>BMC (mg)</td>
<td>281.3 ± 40.7</td>
<td>277.1 ± 44.5</td>
<td>279.6 ± 36.2</td>
<td>275.7 ± 35.7</td>
</tr>
<tr>
<td>Area (mm²)</td>
<td>962.3 ± 144.5</td>
<td>957.2 ± 160.1</td>
<td>976.5 ± 157.9</td>
<td>965.4 ± 157.6</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Trabecular</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>vBMD (mg/cm³)</td>
<td>216.3 ± 33.2</td>
<td>212.0 ± 34.8</td>
<td>210.3 ± 27.5</td>
<td>207.2 ± 27.3</td>
</tr>
<tr>
<td>BMC (mg)</td>
<td>93.7 ± 20.5</td>
<td>89.3 ± 24.7</td>
<td>91.0 ± 17.8</td>
<td>88.5 ± 18.2</td>
</tr>
<tr>
<td>Number (mm⁻¹)</td>
<td>1.326 ± 0.088</td>
<td>1.304 ± 0.096</td>
<td>1.305 ± 0.106</td>
<td>1.309 ± 0.097</td>
</tr>
<tr>
<td>Thickness (mm)</td>
<td>0.356 ± 0.048</td>
<td>0.358 ± 0.049</td>
<td>0.346 ± 0.026</td>
<td>0.343 ± 0.025</td>
</tr>
<tr>
<td>Separation (mm)</td>
<td>0.401 ± 0.076</td>
<td>0.413 ± 0.077</td>
<td>0.424 ± 0.076</td>
<td>0.424 ± 0.072</td>
</tr>
<tr>
<td>BV/TV (%)</td>
<td>0.473 ± 0.073</td>
<td>0.467 ± 0.072</td>
<td>0.453 ± 0.057</td>
<td>0.450 ± 0.054</td>
</tr>
<tr>
<td>Cortical</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>vBMD (mg/cm³)</td>
<td>1145.1 ± 32.5</td>
<td>1138.5 ± 34.6</td>
<td>1141.8 ± 30.5</td>
<td>1136.6 ± 30.4</td>
</tr>
<tr>
<td>BMC (mg)</td>
<td>308.7 ± 32.4</td>
<td>305.0 ± 34.3</td>
<td>322.2 ± 47.7</td>
<td>324.6 ± 45.6</td>
</tr>
<tr>
<td>Area (mm²)</td>
<td>269.7 ± 28.4</td>
<td>268.7 ± 27.2</td>
<td>283.0 ± 41.7</td>
<td>285.5 ± 39.2</td>
</tr>
<tr>
<td>Thickness (mm)</td>
<td>4.9 ± 0.5</td>
<td>4.8 ± 0.6</td>
<td>5.0 ± 0.5</td>
<td>5.1 ± 0.4</td>
</tr>
<tr>
<td>Periosteal (mm)</td>
<td>71.3 ± 3.8</td>
<td>71.6 ± 4.0</td>
<td>72.1 ± 5.7</td>
<td>72.1 ± 5.3</td>
</tr>
<tr>
<td>Endosteal (mm)</td>
<td>41.1 ± 6.0</td>
<td>41.3 ± 6.2</td>
<td>39.9 ± 4.3</td>
<td>40.2 ± 4.6</td>
</tr>
<tr>
<td>Ip (mm⁴)</td>
<td>23,042 ± 4838</td>
<td>23,181 ± 4371</td>
<td>25,493 ± 8122</td>
<td>26,022 ± 7530</td>
</tr>
<tr>
<td>SSI (mm³)</td>
<td>1520 ± 225</td>
<td>1517 ± 212.2</td>
<td>1602 ± 349</td>
<td>1625 ± 329</td>
</tr>
</tbody>
</table>

*Mean ± SD (all values). A mixed-model ANCOVA analysis was performed with time (0, 12 months) and group (600, 2000 or 4000 IU) as independent variables with age, years since menopause (YSM) and body weight as covariates. All scans passed quality control except for a subset examined for higher resolution Tb parameters (number, thickness, separation and BV/TV) that left n=16, 18 and 12 in the 600, 2000 and 4000 IU groups, respectively. Abbreviations: vBMD, volumetric bone mineral density; BMC, bone mineral content; BV/TV, Bone Volume/Total Volume; IP, polar moment of inertia; SSI, stress-strain index.
<table>
<thead>
<tr>
<th></th>
<th>600 (n=19)</th>
<th>2000 (n=20)</th>
<th>4000 (n=19)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Baseline</td>
<td>12 months</td>
<td>Baseline</td>
<td>12 months</td>
</tr>
<tr>
<td>Baseline</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>25OHD (ng/mL)</td>
<td>26.5 ± 4.5</td>
<td>30.5 ± 5.0</td>
<td>28.6 ± 4.7</td>
<td>36.0 ± 4.2</td>
</tr>
<tr>
<td>Free 25OHD</td>
<td>4.1 ± 0.6</td>
<td>5.1 ± 1.4</td>
<td>4.6 ± 1.0</td>
<td>6.2 ± 1.4</td>
</tr>
<tr>
<td>Bioavailable 25OHD</td>
<td>1.6 ± 0.2</td>
<td>2.1 ± 0.6</td>
<td>1.8 ± 0.4</td>
<td>2.5 ± 0.6</td>
</tr>
<tr>
<td>PTH (pg/mL)</td>
<td>36.0 ± 9.0</td>
<td>33.2 ± 8.3</td>
<td>41.8 ± 13.2</td>
<td>36.2 ± 8.5</td>
</tr>
<tr>
<td>E2 (pg/mL)</td>
<td>12.4 ± 4.6</td>
<td>11.5 ± 4.3</td>
<td>15.0 ± 5.7</td>
<td>13.2 ± 5.2</td>
</tr>
<tr>
<td>P1NP (ng/mL)</td>
<td>46.6 ± 12.3</td>
<td>54.1 ± 15.5</td>
<td>46.9 ± 9.9</td>
<td>54.1 ± 8.4</td>
</tr>
<tr>
<td>CTX (pg/mL)</td>
<td>161.4 ± 31.5</td>
<td>180.9 ± 45.0</td>
<td>164.2 ± 26.7</td>
<td>181.2 ± 52.9</td>
</tr>
<tr>
<td>Calcium (mmol/L)</td>
<td>9.02 ± 0.33</td>
<td>8.90 ± 0.82</td>
<td>8.79 ± 0.70</td>
<td>9.04 ± 0.46</td>
</tr>
<tr>
<td>Urinary Ca/creatinine</td>
<td>0.45 ± 0.16</td>
<td>0.45 ± 0.22</td>
<td>0.43 ± 0.19</td>
<td>0.44 ± 0.15</td>
</tr>
</tbody>
</table>

*Mean ± SD (all values). A mixed-model ANCOVA analysis was performed with time (0, 12 months) and group (600, 2000, and 4000 IU) as independent variables with age, years since menopause (YSM) and body weight as covariates.

Abbreviations: 25OHD, 25-hydroxyvitamin D; PTH, parathyroid hormone; E2, estradiol; P1NP, procollagen type 1 amino-terminal propeptide; CTX, C-terminal crosslinked telopeptide of type I collagen.
Table 12. Nutrient intake over 12 months at three doses of vitamin D3

<table>
<thead>
<tr>
<th></th>
<th>600 (n=19)</th>
<th>2000 (n=20)</th>
<th>4000 (n=19)</th>
<th>P value</th>
<th>Group</th>
<th>Time</th>
<th>Group X Time</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Energy (kcal)</strong></td>
<td>1511 ± 335</td>
<td>1300 ± 138</td>
<td>1680 ± 356</td>
<td>1337 ± 279</td>
<td>1546 ± 309</td>
<td>1350 ± 257</td>
<td>0.546</td>
</tr>
<tr>
<td><strong>Protein (g)</strong></td>
<td>69.5 ± 19.2</td>
<td>56.9 ± 12.0</td>
<td>72.6 ± 32.0</td>
<td>70.3 ± 13.9</td>
<td>59.1 ± 21.8</td>
<td>55.5 ± 20.6</td>
<td>0.132</td>
</tr>
<tr>
<td><strong>Carbohydrate(g)</strong></td>
<td>167.3 ± 36.8</td>
<td>159.1 ± 56.9</td>
<td>187.0 ± 39.8</td>
<td>171.3 ± 46.6</td>
<td>168.1 ± 57.7</td>
<td>149.2 ± 34.5</td>
<td>0.267</td>
</tr>
<tr>
<td><strong>Total Fat (g)</strong></td>
<td>67.7 ± 18.2</td>
<td>51.0 ± 5.5</td>
<td>65.4 ± 32.0</td>
<td>52.5 ± 20.8</td>
<td>63.63 ± 8.9</td>
<td>56.0 ± 15.9</td>
<td>0.798</td>
</tr>
<tr>
<td><strong>Vitamin D (IU)</strong></td>
<td>578.3 ± 51.0</td>
<td>591.5 ± 90.0</td>
<td>567.8 ± 78.1</td>
<td>2047.4 ±216.5</td>
<td>551.7 ± 66.3</td>
<td>4100.1 ± 55.3</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td><strong>Calcium (mg)</strong></td>
<td>1194 ± 74</td>
<td>1170 ± 99</td>
<td>1179 ± 94</td>
<td>1146 ± 206</td>
<td>1185 ± 47</td>
<td>1202 ± 98</td>
<td>0.514</td>
</tr>
<tr>
<td><strong>Phosphorus (mg)</strong></td>
<td>960 ± 333</td>
<td>778 ± 224</td>
<td>1136 ± 305</td>
<td>938 ± 254</td>
<td>988 ± 200</td>
<td>932 ± 256</td>
<td>0.049</td>
</tr>
<tr>
<td><strong>Magnesium (mg)</strong></td>
<td>320.3 ± 93.1</td>
<td>268.7 ± 47.9</td>
<td>416.8 ± 228.6</td>
<td>353.3 ±58.7</td>
<td>343.3 ±109.3</td>
<td>269.4 ± 53.8</td>
<td>0.035</td>
</tr>
<tr>
<td><strong>Sodium (mg)</strong></td>
<td>2317 ± 1076</td>
<td>2024 ± 487</td>
<td>2209 ± 1048</td>
<td>2014 ± 981</td>
<td>2290 ± 814</td>
<td>1975 ± 533</td>
<td>0.936</td>
</tr>
<tr>
<td><strong>Vitamin K (µg)</strong></td>
<td>74.3 ± 17.3</td>
<td>66.6 ± 15.8</td>
<td>75.9 ± 18.9</td>
<td>61.6 ± 22.5</td>
<td>73.1 ± 15.3</td>
<td>75.15 ± 39.6</td>
<td>0.974</td>
</tr>
</tbody>
</table>

*Mean ± SD (all values). A mixed-model ANCOVA analysis was performed with time (0, 12 months) and group (600, 2000, and 4000 IU) as independent variables. Daily nutrient intake is an average of nutrient intake over 12 month assessed at regular intervals (monthly 3-day food diaries). Baseline and intervention intake includes multivitamins (containing 400 IU of vitamin D, 200 mg calcium, 48 mcg of phosphorus, 100 mg of magnesium and 10 mcg of vitamin K) and individualized calcium supplement and intervention includes vitamin D tablets. Pre-stabilization Ca intake: 806 ± 158 mg/d, 809 ± 215 mg/d, 796 ± 155 mg/d in 600 IU, 2000 IU and 4000 IU groups, respectively.*
Table 13. Adverse events over 12 months at three doses of vitamin D3

<table>
<thead>
<tr>
<th></th>
<th>600 (n=19)</th>
<th>2000 (n=20)</th>
<th>4000 (n=19)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Baseline</td>
<td>Intervention</td>
<td>Baseline</td>
<td>Intervention</td>
</tr>
<tr>
<td><em>Serious, n (%)</em></td>
<td>Baseline</td>
<td>Intervention</td>
<td>Baseline</td>
<td>Intervention</td>
</tr>
<tr>
<td>Pain in the leg</td>
<td>0 (0.0)</td>
<td>1 (5.8)</td>
<td>6 (40.0)</td>
<td>5 (33.3)</td>
</tr>
<tr>
<td>Swelling in the leg</td>
<td>1 (5.8)</td>
<td>0 (0.0)</td>
<td>4 (26.6)</td>
<td>3 (20.0)</td>
</tr>
<tr>
<td>Headaches</td>
<td>0 (0.0)</td>
<td>0 (0.0)</td>
<td>3 (20.0)</td>
<td>1 (6.6)</td>
</tr>
<tr>
<td><em>Non-serious, n (%)</em></td>
<td>Baseline</td>
<td>Intervention</td>
<td>Baseline</td>
<td>Intervention</td>
</tr>
<tr>
<td>Pain or heaviness in the chest</td>
<td>6 (35.3)</td>
<td>3 (17.6)</td>
<td>6 (40.0)</td>
<td>6 (40.0)</td>
</tr>
<tr>
<td>Dizziness</td>
<td>2 (11.7)</td>
<td>2 (11.7)</td>
<td>2 (13.3)</td>
<td>1 (6.6)</td>
</tr>
<tr>
<td>Nausea</td>
<td>0 (0.0)</td>
<td>1 (5.8)</td>
<td>3 (20.0)</td>
<td>1 (6.6)</td>
</tr>
<tr>
<td>Fatigue</td>
<td>8 (47.1)</td>
<td>6 (35.3)</td>
<td>9 (60.0)</td>
<td>7 (46.6)</td>
</tr>
<tr>
<td>Muscle pain</td>
<td>2 (11.7)</td>
<td>0 (0.0)</td>
<td>2 (13.3)</td>
<td>3 (20.0)</td>
</tr>
<tr>
<td>Abnormal urinary frequency</td>
<td>6 (35.3)</td>
<td>3 (17.6)</td>
<td>9 (60.0)</td>
<td>7 (46.6)</td>
</tr>
<tr>
<td>Abdominal pain</td>
<td>1 (5.8)</td>
<td>0 (0.0)</td>
<td>2 (13.0)</td>
<td>2 (13.3)</td>
</tr>
<tr>
<td>Hair loss</td>
<td>3 (17.6)</td>
<td>0 (0.0)</td>
<td>1 (6.6)</td>
<td>0 (0.0)</td>
</tr>
<tr>
<td>Other (minor medical events)</td>
<td>1 (5.8)</td>
<td>0 (0.0)</td>
<td>1 (6.6)</td>
<td>0 (0.0)</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td>31 (14.0)</td>
<td>17 (7.7)</td>
<td>53 (27.2)</td>
<td>43 (22.1)</td>
</tr>
</tbody>
</table>

*aCumulative adverse events (frequency) at baseline and throughout intervention. There was no between-groups difference for cumulative or individual events.*
Figure 7. Flowchart of study participants
Figure 8. The percent change in bone parameters over 12 months at three doses of vitamin D3

A. Areal bone mineral density at the femoral neck (FN); trochanter (Troch); lumbar spine (LS), hip and total body (TB).

B. Cortical parameters for bone mineral density (BMD); bone mineral content (BMC), area and cortical thickness (Ct.Th)

Comparison between the groups was examined by mixed-model ANCOVA analysis with time (0, 12 months) and group (600, 2000, and 4000 IU) as independent variables. ap<0.05, for interaction effect, groups are significantly different from each other; bp<0.06 for interaction effect.
Figure 9. Changes in 25OHD and PTH over 12 months at three doses of vitamin D3.

Comparison between the groups was examined by mixed-model ANCOVA analysis with time (0, 12 months) and group (600, 2000, and 4000 IU) as independent variables. Values with different superscripts are significantly different from each other over 12 months of intervention (p<0.05 for interaction effect); *p<0.05 for time effect, the groups are not significantly different from each other.
Chapter 4.

Three doses of vitamin D on insulin resistance and osteocalcin measures in older women
Abstract

Short-term clinical studies suggest that vitamin D supplementation influences osteocalcin (OC) levels during caloric restriction, and this is associated with lower insulin resistance (IR). In mice, the undercarboxylated form of OC (ucOC) acts as a circulating hormone to regulate insulin production and sensitivity. This one-year long double blind randomized controlled weight control trial addresses whether vitamin D supplementation in healthy overweight/obese older women influences markers of insulin sensitivity and total OC or ucOC levels. **Design:** Fifty-eight women (age, 58 ± 6 years; body mass index, 30.1 ± 3.8 kg/m², serum 25-hydroxyvitamin D (25OHD), 27.1 ± 4.4 ng/ml, glucose, 86 ± 10.3 mg/dl, insulin, 7.2 ± 2.7 mIU/l) were randomly assigned to one of 3 doses of vitamin D (600 IU/d; 2000 IU/d; 4000 IU/d). Serum was analyzed for 25OHD, OC, ucOC, glucose and insulin. An oral glucose tolerance test (OGTT; glucose area under the curve, AUC) was performed at 12 month. Homeostasis Model Assessment (HOMA) and quantitative insulin sensitivity check index (QUICKI) were calculated. **Results:** At 1 year, serum 25OHD levels increased to 30.4 ± 5.2, 35.8 ± 4.5 and 41.5 ± 6.9 ng/ml in the 600, 2000 and 4000 IU, respectively, and levels differed between groups (p<0.05). Weight change was similar across groups (-3.0 ± 4.1%). There was an interaction between VitD group and time on glucose and QUICKI (p≤0.05). The decline in serum glucose and the increase in QUICKI was greater in the 2000 IU compared to the 600 IU group (p≤0.05) and did not differ significantly compared to the 4000 IU group. Insulin decreased to a similar extent between groups over time (p<0.05). There were no significant correlations between changes in serum 25OHD and glycemic markers or OC measures. **Conclusion:** Dietary vitamin D intake modestly affects glycemic markers in healthy older women, but ucOC does not predict these changes.
Introduction

Vitamin D is essential for bone health (IOM, 2011). Also, vitamin D status has been linked to various non-skeletal diseases including cancer, cardiovascular disease, and type 2 diabetes (IOM, 2011). Studies suggest that vitamin D improves beta cell survival, promotes synthesis and secretion of insulin and enhances insulin sensitivity in peripheral tissues (Cade and Norman, 1987; Pittas et al., 2007). However, the mechanism is not entirely clear. Observational studies in humans have found a correlation between vitamin D status and the prevalence of diabetes (Mitri et al., 2014; Seida et al., 2014), but intervention trials with vitamin D supplementation on insulin resistance (IR) and diabetes outcomes have been less conclusive. Most studies show no effect of vitamin D supplementation on glucose metabolism and IR in healthy individuals (Pittas et al., 2007; Tai et al., 2008), subjects with pre-diabetes (Davidson et al., 2013) or established diabetes (Jorde et al., 2009; Witham et al., 2010; Heshmat et al., 2012; Kampmann et al., 2014). A few studies have reported improved glucose tolerance with vitamin D supplementation in patients without a previous diagnosis of diabetes (Pittas et al., 2007; Belenchia et al., 2013; Dutta et al., 2014).

Osteocalcin (OC) is a small protein (49 amino acids) produced by mature osteoblasts during bone formation. OC has three potential γ-carboxyglutamic acid residues and the carboxylation of these residues by a vitamin K-dependent carboxylase results in greater affinity for calcium and hydroxyapatite (Hauschka et al., 1989). Carboxylated OC is deposited into the bone matrix while the amount of OC that is either not carboxylated or is partially carboxylated (undercarboxylated OC or ucOC) is released into the circulation (Lee et al., 2007). In mice, circulating ucOC increases insulin secretion and proliferation of β cells in pancreas via a presumed receptor GPRC6A (Lee
et al., 2007; Ferron and Lacombe, 2010). Human subjects with glucose intolerance or at high risk of type 2 diabetes (Díaz-López et al., 2013) have low serum OC levels while improved glycemic control normalizes OC (Rosato et al., 1998; Sayinalp et al., 1995). However, some failed to find a relationship between serum OC and insulin resistance (Zhou et al., 2013; Wang et al., 2013). Similarly, serum ucOC levels and IR have been inversely correlated in some studies (Hwang et al., 2009; Iki et al., 2012), but not in all (Shea et al., 2009; Wang et al., 2013).

In postmenopausal women at higher risk of obesity and metabolic diseases, a significant relationship between serum OC, fasting glucose and the homeostatic model assessment of IR (HOMA-IR) has been reported (Kim et al., 2013). Also, the odds ratio for metabolic syndrome was significantly lower with higher ucOC levels in this study (Kim et al., 2013). However, fasting plasma glucose and insulin, and IR measures do not significantly correlate with total OC in healthy postmenopausal women (Caglar et al., 2014).

While vitamin K regulates carboxylation of OC, vitamin D directly stimulates osteocalcin gene transcription (Kerner et al., 1989; Staal et al., 1996). In subjects with vitamin D deficiency, there are low levels of OC (Fonseca 1988). However, vitamin D supplementation alone has not been shown to affect serum OC (Je et al., 2010; von Hurst et al., 2011) or ucOC (O’Connor et al., 2010). Moreover, although vitamin D treatment increased insulin sensitivity and glucose metabolism in children with diabetes and vitamin D deficiency, it did not affect OC levels (Poomthavorn et al., 2014). Nevertheless, a six-week study from our lab conducted in overweight/obese postmenopausal women undergoing caloric
restriction showed that the interaction between short-term vitamin D supplementation and weight loss significantly affects total OC levels and insulin resistance, but ucOC levels were not measured (Sukumar et al., 2015). Whether long-term vitamin D supplementation on serum OC and ucOC will attenuate markers of IR is not known and is examined in this study.

**Subjects and Methods**

*Subjects*

Postmenopausal women (age 50-70 years old, BMI 25-40 kg/m2) were recruited for a 1-year long vitamin D supplementation to study bone health and insulin resistance/OC as primary and secondary outcomes, respectively. Women, who were less than two years postmenopausal, experiencing 5% body weight loss or gain within three months before recruitment, had serum 25-hydroxyvitamin D (25OHD) levels >30 ng/mL or a fasting blood glucose >126 mg/dL were not eligible. Exclusion criteria included metabolic bone disease, hyperparathyroidism, untreated thyroid disease, immune, hepatic, or renal disease, kidney stone in the last five yrs., significant cardiac disease, active malignancy or cancer therapy within the past year. Participants signed an informed consent form reviewed and approved by the Rutgers University Institutional Review Board before undergoing any study-related procedures. The trial was registered at clinicaltrials.gov as NCT01631292.

*Study design*

Subjects enrolled in the study were randomized in a double-blind, controlled manner to one of three treatment groups and were asked to consume vitamin D3 capsules or placebo during 5 consecutive weekdays with their largest meal of the day. The 600 IU group
received 5 placebo capsules/week; the 2000 IU group received 2 vitamin D3 capsules + 3 placebo/week and the 4000 IU group received 5 vitamin D3 capsules/week. All subjects also received 400 IU from a multivitamin/mineral supplement throughout the study duration. Total vitamin D intake (habitual dietary intake + supplements) was estimated at 600 IU, 2000 IU and 4000 IU per day in the three treatment groups, respectively. One month prior to treatment assignments, in all subjects vitamin D and Ca intake were stabilized to meet the requirements with a daily multivitamin/mineral (400 IU vitamin D/tablet; NatureMade Multi 50+) and calcium supplement (200 mg Ca/tablet; Citracal; Bayer) to total 600 IU/d vitamin D and 1.2 g Ca/d (diet and supplement) that continued throughout the study duration. Subjects followed a standard behavior modification nutrition education/weight control program and adhered to individualized moderate energy-restricted diets with modest weight-loss goals. Food diaries were filled in by the participants on a weekly basis to enhance compliance with the nutrition-education plan and were reviewed by a registered dietitian at the monthly counseling sessions. Food diaries were analyzed from 2 non-consecutive weekdays and one weekend day at baseline and regular intervals three times during the intervention using nutrient analysis software (Version 17, FoodWorks, Long Valley, NJ).

*Anthropometric measurements* - A balance beam scale and stadiometer (Detecto, Webb City, MO) were used to measure body weight and height to the nearest 0.25 kg and cm, respectively. At each monthly visit body weight was measured in all subjects wearing light clothing.

*Laboratory methods*
Fasting serum and urine samples collected at baseline, month 6 and 12 and were analyzed in batch analysis for: 25OHD (radioimmunoassay; DiaSorin) (coefficient of variation, CV <12.5%), intact PTH (immunoradioassay; Scantibodies) (CV < 6.8%) and ultrasensitive estradiol (radioimmunoassay; DSL) (CV <8.9%). Bone turnover markers were measured at baseline and after 12 months. Propeptide of type 1 collagen (P1NP) and C-telopeptide of type 1 collagen (CTX) were measured by ELISA (MyBiosource) (CV< 15%). Serum was analyzed for glucose (colorimetric reagent kit, Pointe Scientific, Canton, MI, USA, CV <11.3%) and insulin (ELISA, Mybiosource LLC, San Diego, CA, CV<7.0%).

**Serum osteocalcin and undercarboxylated osteocalcin**

Total serum OC was measured using an automated immunoassay (Elecsys 170; Roche Diagnostics). This assay has a sensitivity of 0.5mg/L, with an intra-assay precision of 1.3%. Serum ucOC was measured by the same immunoassay after adsorption of carboxylated OC on 5mg/mL hydroxylapatite slurry, following the method described by Gundberg et al. (Gundberg et al., 1998). The inter assay CV for total OC is 8.3% and the inter assay CV for ucOC is 5.7%. This method is based on a lower affinity of ucOC for hydroxyapatite compared to the carboxylated osteocalcin. The total amount of osteocalcin is reported as OC in ng/ml and undercarboxylated OC as ucOC in ng/ml. ucOC is also reported as a percentage of total OC (%ucOC) using the formula:

\[(\text{ucOC}/\text{OC}) \times 100\]

**Measures of insulin sensitivity**
Insulin resistance and sensitivity surrogates were calculated from samples collected at baseline and after 12 months of vitamin D treatment: Homeostasis model assessment of insulin resistance (HOMA-IR) (Matthews et al., 1985) and Quantitative insulin sensitivity check index (QUICKI) (Katz et al., 2000).

HOMA-IR = (fasting Glucose (mg/dL) x fasting Insulin (µU/mL)/405)

QUICKI = 1/ (log (µU/mL) +log (G mg/dL))

Oral glucose tolerance test (OGTT)

After overnight fasting, women consumed 75 g glucose administered as a drink (Trutol 75, Fisher Scientific) and blood samples were collected at times 0, 60 and 120 min at 12 month. Samples were analyzed for glucose and area under the curve (AUC) was calculated.

Compliance and safety

Adherence to treatment protocol was assessed by pill count at each counseling session. Dietary Ca and vitamin D intake was estimated from food records collected at baseline and throughout the study duration from all the participants. Adverse events forms were filled in at baseline and during monthly counseling sessions by all subjects. Study outcomes and adverse events were reviewed periodically by an internal Data Safety Monitoring Committee and Rutgers University IRB.

Statistical analysis

In a six-week study conducted in our lab in overweight postmenopausal women, the interaction between weight loss (WL) and vitamin D supplementation (D) or placebo (Pl)
resulted in greater improvement in glycemic indices and explained changes in OC compared to individual effects of treatment or WL alone (Sukumar et al., 2015). To be able to detect a similar difference for HOMA-IR changes (-18.8 ± 26% vs. 4.6 ± 28.7% in the WL-D and WL-Pl groups, respectively) and to allow for two covariates a sample size of 19 per group (power of 80% and α set at 0.05) was calculated. To account for potential dropouts, we recruited additional subjects in each treatment group.

Descriptive statistics were used for demographic and baseline characteristics. Differences between groups for baseline characteristics and percent changes over 12 months were examined with one-way ANOVA. Two-way ANOVA analysis was conducted to analyze the interaction between treatment group (dose) and time (0 and 12 months) on all biomarkers. If model F ratio was significant, Tukey’s post-hoc analysis was performed. Pearson correlation was used to assess the relationship between study parameters.

Analyses were conducted using the SPSS statistical software (IBM, version 23.0). Values are reported as mean ± SD, and graphs with SEM. P-value of less than 0.05 was considered significant.

**Results**

Figure 10 shows the study participant’s flowchart and baseline characteristics are presented in Table 14. Fifty-eight women (age, 57.6 ± 5.6 years; BMI, 30.2 ± 3.7 kg/m2), predominantly Caucasian (85%) were analyzed at the end of the 12 months vitamin D intervention. Six women had fasting glucose between 100-126 ng/dl (n=1, 3, and 2 in the 600, 2000 and 4000 IU groups, respectively). Serum 25OHD concentration was 22.8 ± 6.4 ng/mL at screening and increased to 27.2 ± 4.3 ng/mL after one month of stabilization with a multivitamin/mineral pill with no significant differences in baseline values.
between groups. After 12 months, subjects lost $3.0 \pm 4.1 \%$ body weight (p-value for time $< 0.02$), and this did not differ significantly between groups. This weight loss occurred in the first six months of intervention ($-3.1 \pm 3.8 \%$ body weight), and there was no further weight change in the 2nd six months of the intervention ($-0.2 \pm 2.4 \%$). The increase in serum 25OHD in the 600, 2000 or 4000 IU groups, respectively was $3.8 \pm 4.1$, $7.4 \pm 6.5$, and $14.1 \pm 8.1$ ng/ml (p-value for interaction between time and treatment $<0.01$). After 12 months of vitamin D supplementation, PTH decreased to a similar extent between groups (p-value for time $< 0.02$). Serum CTX and P1NP levels increased from baseline to 12 months (p-value for time $<0.05$) and the groups were not significantly different. The change in total, undercarboxylated or % ucOC measures over 12 months of treatment and the interaction between treatment and time did not significantly differ between groups (Figure 11). However, insulin sensitivity assessed by QUICKI differed between groups after 12 months of vitamin D treatment (p $\leq 0.05$), and post-hoc analysis showed a greater increase in QUICKI in the 2000 IU compared to the 600 IU group (p $<0.05$) (Figure 12). Insulin decreased to a similar extent between groups after 12 months of vitamin D treatment (p-value for time $< 0.01$). Similarly, there was an effect over time to attenuate insulin resistance assessed by HOMA-IR in all the groups (p value for time $<0.01$). Results of the OGTT after treatment, indicated that serum glucose and insulin levels and AUC did not differ significantly between groups (Figure 13). Pearson’s correlations indicated no significant relationship between changes in serum 25OHD, glycemic markers and changes in total and ucOC levels. Also, the relationship between change in ucOC and CTX (a marker of bone resorption) was not significant.
Discussion

In this 1-year long intervention in overweight/postmenopausal women, there was a dose-response effect of vitamin D3 supplementation on serum 25-hydroxyvitamin D concentrations. While there was a modest effect of vitamin D treatment (2000 IU/d vs. 600 IU/d) on insulin sensitivity, we did not find an effect of vitamin D on OC measures at any dose. Also, we did not find an effect of vitamin D supplementation on the other bone markers measured in this study. In addition, there was no relationship between OC or its undercarboxylated form and changes in glycemic indices.

Studies with vitamin D supplementation on glucose homeostasis or diabetes prevention have been inconsistent. In healthy subjects, no improvement in glucose and insulin metabolism (Seida et al., 2014) and no changes in insulin secretion and β-cell function in adults at risk of type 2 diabetes (Gagnon et al., 2014) were found. However, a positive relationship between serum 25OHD and insulin sensitivity, independent of changes in weight in healthy individuals (Chiu et al., 2004) and patients with prediabetes (Gagnon et al., 2014) has been previously reported. Furthermore, the combination of higher vitamin D supplementation (2500 IU/d vs. 400 IU/d) and caloric restriction resulted in significantly lower serum glucose and insulin levels and improved glycemic indices in a six-week intervention trial conducted in our lab (Sukumar et al., 2015). Moreover, in this previous short-term study in overweight/obese postmenopausal women undergoing significant weight loss in a short interval, changes in serum 25OHD levels correlated with total OC levels (Sukumar et al., 2015). The possibility that the weight-loss-induced increase in OC levels explains its role as a glucose regulator, independently
of any changes in bone formation, has been previously suggested (Hinton et al., 2011). It is well known that weight reduction can increase bone turnover markers (Shapses and Sukumar, 2012). In addition, a significant increase in OC levels has been reported in studies with approximately 10% body weight reduction (Rector et al., 2009, Riedt et al., 2005). In our study the amount of weight loss was considerably lower at ~ 3% weight reduction in the first six months of intervention and no weight change after that. Although in contrast to OC levels, serum P1NP and CTX increased in all groups, independently of the vitamin D treatment, this may be due to a difference in the age-dependent pattern of change between bone markers (Seibel, 2005).

Osteocalcin is a vitamin K dependent-protein (Hauscka et al., 1989), which is primarily known as a marker of bone formation. Carboxylation of osteocalcin requires vitamin K and results in increased levels of carboxylated OC and reduced levels of undercarboxylated osteocalcin (ucOC) (Cranenburg et al., 2007). A high percentage of undercarboxylated OC (%ucOC) indicates a poor vitamin K status (Vermeer et al., 2012). In our study, there were no differences in vitamin K intake between groups that would be expected to affect differences in OC carboxylation, and all subjects met the requirements at ~ 90 µg/d.

In the last decade, some researchers have suggested that OC, possibly via the ucOC form, plays a role in the regulation of glucose metabolism (Kanazawa, 2015). However, whether or not this is the active form in humans (as found in mice) is less clear. Total OC levels are inversely associated with glucose levels and insulin sensitivity in healthy subjects (Kindblom et al., 2009; Fernandez-Real
et al., 2009) as well as insulin secretion and sensitivity in patients with type 2 diabetes (Kanazawa et al., 2011). Similar findings for ucOC and glucose metabolism were reported (Hwang et al., 2009). Nevertheless, not all studies report significant results and whether ucOC is the active form of OC in humans remains controversial (Booth et al., 2013; Zwakenberg et al., 2015). Reasons for this discrepancy between studies range from a lack of effect in healthy populations in the absence of glucose intolerance (Booth 2013, Gower 2013) to differences in assay methods between studies (Zwakenberg et al., 2015). Often, measurements precision and sensitivity do not allow to differentiate between various degrees of OC carboxylation (Li et al., 2016). Also, structural differences between mouse and human OC molecules such as the size and some amino acids, as well as the presence of a single OC encoding gene in humans vs. three in mice, contribute to the controversy regarding specific OC function in humans (Kanazawa, 2015).

A vitamin D responsive element in the promoter region of the OC gene has been identified, and a role of 1,25-dihydroxyvitamin D3 (1,25(OH)2D3) or calcitriol to increase OC gene expression has been shown (Kerner et al., 1989; Staal et al., 1996). However, some found no effect of vitamin D supplementation on total OC (Je et al., 2011; Von Hurst et al., 2010) or ucOC levels (O’Connor 2010) and correction of vitamin D insufficiency had no effect on OC measures (Poomphavorn et al., 2014). Furthermore, no correlations were found between vitamin D receptor polymorphisms and serum OC levels (Ozaydin et al., 2010). Consistent with the previous reports, in our study, we found no effect of vitamin D treatment on OC measures. It is possible that 1,25(OH)2D3 (calcitriol) rather than 25OHD modulates serum OC, and may require a critical level of
calcitriol to detect a significant effect on serum OC, but we did not measure serum calcitriol in our study.

Vitamin D promotes osteoblasts differentiation into mature osteoblasts which produce osteocalcin. In contrast to other bone formation markers such as P1NP, OC is secreted into the circulation during later phases of osteoblast differentiation. This delay suggests that vitamin D promotes OC synthesis in the more mature osteoblast without affecting early markers of osteoblast differentiation (i.e. P1NP). However, in our study, serum OC levels did not increase in response to vitamin D treatment. Animal studies have shown that OC is activated following bone resorption which results in acidification of bone matrix and decarboxylation of OC to ucOC, the active glucose regulating hormone (Ferron and Lacombe, 2010). Although in our study, CTX increased by ~ 10% after 12 months of intervention in all groups with no differences between vitamin D doses, we did not find a significant relationship between CTX and serum ucOC levels. Whether a greater extent of bone resorption to produce a notable effect on serum ucOC levels is needed, is not clear.

The present study has several strengths and limitations. The study strength is that it employed a randomized controlled double-blinded design to test the effect of three doses of vitamin D in daily doses, representing the current RDA and two higher doses, on OC measures and glycemic indices. The subjects’ recruitment was conducted in the same season (winter) to minimize the seasonal influence on 25OHD concentrations. Measurements of serum ucOC using a validated method represent another strength of this study. Multiple cohorts in this
study can be a limitation; however batch analysis was conducted for all the assays. The sample size consisted of healthy subjects, and these findings may not be generalized to individuals with glucose intolerance or diabetes. Also, we did not perform an OGTT at baseline, so we were not able to evaluate changes in response to vitamin D treatment. Another limitation is that the baseline serum 25OHD in this study was relatively high, and results may differ in vitamin D deficient populations.

Although vitamin D supplementation was efficient at increasing serum 25OHD levels, it had only a modest effect on glycemic indices. In addition, our findings do not support the hypothesis that the vitamin D effect on markers of IR is modulated by OC or its undercarboxylated form. A different mechanism of action to explain the improve glycemic outcomes should be examined in a future study.

Acknowledgments and Funding Sources

We thank the clinical staff for their invaluable support and assistance and we are grateful for the commitment of the volunteers. This study was supported by NIH-AG12161, Busch Biomedical Award (BBGP2010695157).
Table 14. Baseline characteristics in the three vitamin D treatment groups

<table>
<thead>
<tr>
<th>Variable</th>
<th>600 IU Baseline</th>
<th>600 IU 12 months</th>
<th>2000 IU Baseline</th>
<th>2000 IU 12 months</th>
<th>4000 IU Baseline</th>
<th>4000 IU 12 months</th>
<th>Treatment</th>
<th>Time</th>
<th>T*T</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age</td>
<td>57.4 ± 6.3</td>
<td>58.4 ± 4.9</td>
<td>57.2 ± 5.5</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>BMI</td>
<td>29.5 ± 2.9</td>
<td>28.7 ± 3.3</td>
<td>31.5 ± 4.5</td>
<td>30.8 ± 4.6</td>
<td>29.4 ± 3.5</td>
<td>28.6 ± 3.3</td>
<td>0.195</td>
<td>0.003</td>
<td>0.991</td>
</tr>
<tr>
<td>Weight</td>
<td>79.2 ± 10.1</td>
<td>76.8 ± 10.4</td>
<td>85.9 ± 13.3</td>
<td>83.5 ± 12.9</td>
<td>78.6 ± 9.9</td>
<td>76.0 ± 10.1</td>
<td>0.108</td>
<td>0.004</td>
<td>0.965</td>
</tr>
<tr>
<td>25OHD</td>
<td>26.5 ± 4.5</td>
<td>30.5 ± 5.1</td>
<td>28.6 ± 4.7</td>
<td>36.0 ± 4.2</td>
<td>26.7 ± 3.7</td>
<td>40.8 ± 7.4</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>PTH</td>
<td>36.0 ± 9.0</td>
<td>33.1 ± 8.1</td>
<td>41.8 ± 13.2</td>
<td>35.9 ± 8.4</td>
<td>41.4 ± 22.8</td>
<td>31.1 ± 7.2</td>
<td>0.431</td>
<td>0.001</td>
<td>0.288</td>
</tr>
<tr>
<td>E2</td>
<td>12.4 ± 4.6</td>
<td>11.8 ± 4.66</td>
<td>14.6 ± 5.8</td>
<td>13.2 ± 5.2</td>
<td>15.4 ± 6.3</td>
<td>13.8 ± 3.8</td>
<td>0.749</td>
<td>0.045</td>
<td>0.241</td>
</tr>
<tr>
<td>CTX</td>
<td>161.4 ± 31.5</td>
<td>180.9 ± 45.0</td>
<td>164.1 ± 26.7</td>
<td>181.2 ± 52.9</td>
<td>161.2 ± 22.6</td>
<td>174.2 ± 3.6</td>
<td>0.898</td>
<td>&lt;0.001</td>
<td>0.773</td>
</tr>
<tr>
<td>P1NP</td>
<td>46.3 ± 12.6</td>
<td>54.1 ± 15.5</td>
<td>46.9 ± 9.9</td>
<td>54.1 ± 8.4</td>
<td>45.0 ± 6.1</td>
<td>51.7 ± 7.1</td>
<td>0.799</td>
<td>&lt;0.001</td>
<td>0.994</td>
</tr>
<tr>
<td>OC</td>
<td>6.6 ± 2.1</td>
<td>6.3 ± 2.2</td>
<td>5.7 ± 1.7</td>
<td>5.9 ± 2.2</td>
<td>5.1 ± 1.4</td>
<td>6.1 ± 1.7</td>
<td>0.346</td>
<td>0.257</td>
<td>0.178</td>
</tr>
<tr>
<td>ucOC</td>
<td>2.1 ± 0.8</td>
<td>2.1 ± 0.6</td>
<td>1.9 ± 0.9</td>
<td>2.2 ± 1.2</td>
<td>1.8 ± 0.8</td>
<td>2.1 ± 1.1</td>
<td>0.138</td>
<td>0.550</td>
<td>0.802</td>
</tr>
<tr>
<td>%ucOC</td>
<td>0.347 ± 0.151</td>
<td>0.364 ± 0.114</td>
<td>0.356 ± 0.178</td>
<td>0.390 ± 0.148</td>
<td>0.382 ± 0.211</td>
<td>0.353 ± 0.158</td>
<td>0.764</td>
<td>0.562</td>
<td>0.916</td>
</tr>
<tr>
<td>Glucose</td>
<td>80.9 ± 11.1</td>
<td>87.3 ± 8.1</td>
<td>91.5 ± 8.4</td>
<td>89.4 ± 11.9</td>
<td>86.4 ± 11.5</td>
<td>89.9 ± 11.8</td>
<td>0.998</td>
<td>0.063</td>
<td>0.181</td>
</tr>
<tr>
<td>Insulin</td>
<td>6.7 ± 2.6</td>
<td>5.9 ± 1.8</td>
<td>7.6 ± 2.7</td>
<td>6.6 ± 3.4</td>
<td>7.3 ± 2.8</td>
<td>6.3 ± 2.0</td>
<td>0.594</td>
<td>&lt;0.001</td>
<td>0.966</td>
</tr>
<tr>
<td>HOMA-IR</td>
<td>1.369 ± 0.694</td>
<td>1.306 ± 0.518</td>
<td>1.747 ± 0.707</td>
<td>1.498 ± 0.882</td>
<td>1.588 ± 0.751</td>
<td>1.445 ± 0.605</td>
<td>0.438</td>
<td>0.007</td>
<td>0.438</td>
</tr>
<tr>
<td>QUICKI</td>
<td>0.371 ± 0.022</td>
<td>0.372 ± 0.021</td>
<td>0.355 ± 0.019</td>
<td>0.368 ± 0.026</td>
<td>0.362 ± 0.024</td>
<td>0.366 ± 0.021</td>
<td>0.352</td>
<td>0.005</td>
<td>0.053</td>
</tr>
</tbody>
</table>

*Mean ± SD (all values). A two-way repeated measures ANOVA analysis was performed with time (0, 12 months) and group (600, 2000 or 4000 IU) as independent variables and age, body weight and menopausal status as covariates. Abbreviations: 25OHD, 25-hydroxyvitamin D; PTH, parathyroid hormone, E2, estradiol; P1NP, procollagen type 1 amino-terminal propeptide; CTX, C-terminal crosslinked telopeptide of type I collagen; OC, osteocalcin; ucOC, undercarboxylated osteocalcin; HOMA-IR, homeostatic model assessment of insulin resistance; QUICKI, quantitative insulin sensitivity check index.
Assessed for eligibility  
\( n = 210 \)

- Non eligible, \( n = 108 \)

Eligible post-complete screening  
\( n = 102 \)

- Not enrolled, \( n = 21 \)

Randomized  
\( n = 61 \)

- Allocated to 600 IU/d group  
\( n = 24 \)
  - Completed  
\( n = 19 \)

- Allocated to 2000 IU/d group  
\( n = 28 \)
  - Completed  
\( n = 20 \)

- Allocated to 4000 IU/d group  
\( n = 29 \)
  - Completed  
\( n = 19 \)

Figure 10. Study flowchart
Figure 11. Changes in serum 25-hydroxyvitamin D (25OHD), osteocalcin (OC), undercarboxylated OC (ucOC) and ucOC/OC after 12 months of vitamin D treatment

Figure 12. Changes in markers of insulin resistance and sensitivity after 12 months of vitamin D treatment

A two-way repeated measures ANOVA analysis was performed with time (0, 12 months) and group (600, 2000 or 4000 IU) as independent variables and age, body weight and menopausal status as covariates. Letters with different superscripts are significantly different.
Figure 13. Oral glucose tolerance test and computed area under the curve after 12 months of vitamin D3 treatment
Chapter 5.

Conclusions
There are definite health benefits in older adults undergoing weight loss (Darmon, 2013). However, bone and muscle loss also occur with dieting in older individuals (Shapses and Sukumar, 2012). Moreover, aging-induced hormonal changes affect both men and women and influence skeletal health (Khosla, 2013). The two clinical interventions presented in this dissertation examine whether bone quality changes with weight loss in older men, and with vitamin D supplementation in postmenopausal women undergoing weight control. Because the involvement of vitamin D in insulin resistance and other non-skeletal diseases has been of interest lately, we also addressed the effect of vitamin D supplementation on markers of IR and OC, a bone-specific vitamin D- responsive protein and a newly reported glucose regulator.

Weight loss and bone quality in aging men

The majority of studies show that a moderate reduction in body weight improves health outcomes in overweight and obese individuals, but may result in bone loss, especially in older individuals. Specifically in postmenopausal women, at high risk of bone loss, a 1-2.5% bone loss occurs with voluntary moderate weight reduction when compared to women who maintain body weight (Shapses and Sukumar, 2012). However, in men, the effect of weight reduction on bone loss at specific sites and bone quality have not been conducted. In addition, although obesity has been associated with higher BMD, recent studies suggest that bone quality, a predictor of fracture risk, is altered in the obese (Sukumar et al., 2011). Undergoing frequent caloric restriction regimens or weight cycling may negatively impact bone in the obese individuals (Shapses and Riedt, 2006). Also, measurements of areal BMD by DXA at various bone sites is a relative tool to predict the risk of fracture. In our study we used pQCT to further capture geometrical
changes at the cortical and trabecular bone compartments, which may respond differently to physiological and pathological factors (Shapses and Sukumar, 2012).

Hence, understanding the effects of weight loss on bone quality is important to better predict osteoporosis risk in this population. In this dissertation, the effect of weight loss following caloric restriction on bone mineral density (BMD), geometry and strength were examined in middle-aged and older obese/overweight men. Also, we addressed whether endocrine changes associated with weight loss predicted bone changes.

There was an 8% body weight reduction over six months of intervention in this sample of healthy men who followed a calorie restricted diet. However, it was not associated with a loss of areal or volumetric BMD or changes in bone geometric properties. Bone loss that is associated with weight loss might be greater if there is a lower initial weight, or greater loss of lean body mass such as when there is lower protein intake or reduced physical activity (Shapses and Sukumar, 2012). In our study, the absence of femoral neck BMD loss with WL differed from previous WL findings in women and mixed-sex populations (Shapses and Riedt, 2006; Shapses and Sukumar, 2012). In contrast, in the overweight/obese men who maintained their body weight, there was a trend to decrease cortical thickness and area, consistent with previous studies where metabolic abnormalities associated with excess adiposity are detrimental to cortical bone. In our study, bone differences between groups were not related to hormonal changes, except a negative association between free estradiol and cortical bone size consistent with other studies (Lorentzon et al., 2005). However, men who lost weight in our study showed a 35% increase in testosterone that could be considered physiologically significant (Ensrud et al., 2006; Cauley et al., 2010). However, this rise did not differ from the weight stable
men. Nevertheless, a rise in serum testosterone possibly combined with the rise in 25OHD (Riedt et al., 2005) might have attenuated bone changes in our study.

Aging men are not protected against osteoporosis. Previous studies indicate that bone quality is compromised in obese populations, and moderate weight loss results in bone loss in women. Our findings suggest that moderate weight loss is not detrimental to bone in middle-aged and older men. In contrast, in men who maintained their body weight during the six-month intervention period, there was a trend to decrease bone quality measures. Based on our result, achieving a healthy body weight in aging men is beneficial not only for improving metabolic health but also for attenuating detrimental effects of excess weight on bone quality. The extent of bone quality changes that are due to hormonal alterations and other obesity-related factors and the exact mechanism explaining these changes should be addressed in longitudinal studies.

*Vitamin D supplementation, BMD and bone quality in postmenopausal women*

Postmenopausal women, often experience weight gain and undergo weight control plans designed to improve health outcomes. Even with modest weight loss goals (<4% body weight reduction), bone loss occurs (Ramsdale et al., 1994; Salamone et al., 1999) in this population already vulnerable to bone loss due to a sharp decline in estrogen production with menopause (Shapses and Sukumar, 2012). Adequate vitamin D intake potentiates Ca absorption, promotes bone mineralization and enables PTH to regulate serum Ca levels, with beneficial effects for skeletal health (IOM, 2011). Serum 25 hydroxyvitamin D (25OHD) represents a good biomarker of vitamin D intake. However, serum 25OHD response to vitamin D supplementation differs with age, ethnicity, baseline serum 25OHD, type, and level of vitamin D intake, as well as body adiposity (Rosen, 2011). It
has been shown that excess adiposity prevents an adequate response to vitamin D supplementation in the obese compared to lean population, and this is due to adipose tissue acting as a metabolic sequester and vitamin D impaired bioavailability (Gallagher et al., 2012; Wortsman et al., 2000; Ekwaru et al., 2014; Chun et al., 2013). Therefore, higher doses would be needed to raise the serum 25OHD levels to sufficient range. However, the optimum vitamin D dose and serum 25OHD concentrations for best skeletal outcomes are less clear. The relationship between vitamin D and BMD has been controversial, and the association between vitamin D supplementation and fracture prevention has been inconsistent across studies (Reid et al., 2014). Observational studies have found a positive relationship between serum 25OHD and BMD (Ooms et al., 1995; Garnero et al., 2007) but this relationship is not clear. Moreover, clinical trials have yielded inconsistent results and the association between changes in vitamin D status and bone health in older adults remains equivocal. Many times clinical trials with vitamin D intervention have used inadequate doses to raise serum 25OHD to sufficient levels, had high baseline serum 25OHD or did not adjust for dietary intake and sun exposure (Reid et al., 2014).

It is well established that weight loss (3-10%) decreases BMD at various bone sites when compared to weight stable women especially at older ages (Shapses and Sukumar, 2012). This loss in bone mass is attributed to at least a few factors including a decrease in nutrient intake and in calcium absorption (Shapses et al., 2013), reduced mechanical loading (Villareal et al., 2011) and reduced estradiol or insulin-like-growth factor levels accompanying weight reduction (Shapses and Sukumar, 2012). In our study, we found no bone loss over time which may be attributed to the modest weight
loss while consuming the recommended intake of Ca and vitamin D (or higher intakes) in all treatment groups. However, there was an increase in bone turnover possibly due to aging or the slight energy restriction, but this did not differ between groups.

In weight-stable women, there are many studies examining the effect of supplemental vitamin D intake on bone (Chapuy et al., 1992; Ooms et al., 1995; Dawson-Hughes et al., 1995; Cooper et al., 2003; Aloia et al., 2005; Grimnes et al., 2012; MacDonald et al., 2013; Hansen et al., 2015). While some trials, found a positive effect with 700-800 vitamin D IU/d and calcium supplements (500-1,200 mg) on bone (Meier et al., 2004), others, using similar or higher doses found no effect of vitamin D on BMD (Aloia et al., 2005; Jorde et al., 2010). Nevertheless, some reported higher BMD at the lumbar spine and hip with 5000 IU D3/d and 320mg calcium/d compared to placebo (Mocanu et al., 2009). However, previous studies with vitamin D supplementation did not included measures of bone quality. By using pQCT measurements we were able to assess changes in bone geometry and consequently bone strength that are independent of changes in areal bone mineral density. Our study showed that supplemental vitamin D3 did not affect areal BMD, but the higher intakes influenced bone geometry, specifically an increase in cortical thickness, which may translate to improved biomechanical strength and may reduce the risk of fragility fracture. So far, few studies have examined the relationship between 25OHD and the cortical and trabecular bone of the tibia in older women. One study reported a positive association between 25OHD and cortical vBMD (that was negative with PTH), but no relationship of either 25OHD or PTH with trabecular vBMD (Lauretani et al., 2008). However, some reported that high levels of PTH could result in increased endocortical resorption, cortical thinning and porosity.
(Dempster et al., 2007). Therefore, is important to address whether suppression of PTH that can be achieved by vitamin D supplementation, may improve cortical vBMD in obese women. Moreover, our study was the first to examine the effect of vitamin D supplementation on bone quality in a model of energy restriction. Weight loss has been shown to decrease total vBMD with a trend to decrease cortical vBMD in postmenopausal women (Sukumar et al., 2011) but not in premenopausal women (Uusi-Rasi et al., 2009). Energy restriction in rodent models with more severe weight loss also shows a decrease in cortical and trabecular bone parameters including vBMD, BMC, cortical thickness, BV/TV and TbTh (Hamrick et al., 2008; Mardon et al., 2008). The study presented in this dissertation suggests that weight loss causes a decrease in trabecular vBMD, BMC and BV/TV and increase in separation with no significant effect on cortical bone. However, additional intake of vitamin D above the recommended levels had no effect in preventing the trabecular decline with modest weight loss over 1 year. Also, even at high intakes of vitamin D3, cortical vBMD was unaffected, but there was a positive effect on cortical thickness.

The rise in serum 25OHD in our study was 0.19 and 0.27 ng/mL per μg intake when consuming 2000 or 4000 IU/d, respectively. This increase was slightly higher compared to previous studies using similar doses (0.16 ng/mL/μg/day) and may be due to a lower BMI in our study at ~30 kg/m2 vs. >35 kg/m2 in this earlier study (Dhaliwal et al., 2014). Free and bioavailable 25OHD also increased with increasing doses of vitamin D intake. Compared to serum 25OHD, neither free nor bioavailable 25OHD were better predictors of bone or PTH due to obesity or weight loss. This is in contrast to a previous study in a leaner population who were not taking supplements. In this study of 265
postmenopausal women with low BMD, total 25OHD correlated with PTH, whereas only free and bio-available 25OHD was associated with BMD (Johnsen et al., 2014). It is possible that adjusting for DBP gene polymorphism could help clarify how free and bioavailable 25OHD influence the relationship with bone and other outcomes (Johnsen et al., 2014). Also, the interaction with other factors including the type of adiposity, glomerular filtration rate or dietary intake, should also be considered.

Studies with vitamin D supplementation on bone turnover in postmenopausal women are scarce and have yielded mixed results. In our study, the rise in bone turnover was likely due to the combined effect of aging (Ensrud et al., 1995) and weight loss (Riedt et al., 2005; Villareal et al., 2006).

Overall, in our 1-year long study in healthy postmenopausal women, higher vitamin D3 intakes had a modest effect on BMD and attenuated the decline in cortical thickness. Whether this vitamin D effect on bone geometry indicates greater bone strength and could lead to fracture reduction remains to be determined.

Vitamin D supplementation, glycemic indices and OC measures
Besides skeletal health, vitamin D has been associated with non-skeletal outcomes such as IR, cardiovascular events, and cancer, however, uncertainty regarding a role for vitamin D at improving these conditions persists (IOM, 2011; Rosen, 2011). In our 1-year long intervention in overweight/postmenopausal women, we found a modest effect of vitamin D treatment (2000 IU/d vs. 600 IU/d) on insulin sensitivity, but there was no effect of vitamin D on OC measures at any dose. Also, vitamin D supplementation did not influence any of the other bone markers measured in our study. In addition, there was
no relationship between OC or its undercarboxylated form and changes in glycemic indices.

Previously, in our lab a short-term vitamin D intervention in overweight/obese postmenopausal women undergoing significant weight loss in a short interval, changes in serum 25OHD levels correlated with total OC levels (Sukumar et al., 2015). It is possible that the weight-loss-induced increase in OC levels explains its role as a glucose regulator which is not apparent in the absence of a significant weight change. It is well known that weight reduction increases bone turnover markers (Shapses and Sukumar, 2012). However, at least 5% body weight reduction is necessary for a significant increase in OC levels (Rector et al., 2009; Riedt et al., 2005). In our study the amount of weight loss was considerably lower at ~ 3% weight reduction that occurred mostly in the first six months of intervention. A difference in the age-dependent pattern of change between bone markers may be responsible for the increase in the other bone markers compared to osteocalcin (Seibel, 2005).

In the last decade, emerging data has suggested that OC via the ucOC form plays a role in the regulation of glucose metabolism (Kanazawa, 2015). However, the results across studies remain controversial and whether ucOC is the active form of OC in humans is debatable (Booth et al., 2013; Zwakenberg et al., 2015). The discrepancy between studies arise from a lack of effect in healthy individuals in the absence of glucose intolerance (Booth et al., 2013; Gower et al., 2013). Also, differences in assay methods between studies (Zwakenberg et al., 2015) affect measurements precision and sensitivity and do not allow to differentiate between various degrees of OC carboxylation (Li et al., 2016). Structural differences between mouse and human OC molecules and the
presence of a single OC encoding gene in humans vs. three in mice contribute to the controversy regarding the exact OC function in humans (Kanazawa, 2015). Consistent with the previous reports, in our study, we found no effect of vitamin D treatment on OC measures. Whether serum OC changes in response to an absolute serum 1,25(OH)2D3 level, rather than 25OHD was not addressed in our study. Although vitamin D supplementation was efficient at increasing serum 25OHD levels, it had a modest effect on glycemic indices. Overall, our results do not confirm the hypothesis that vitamin D effect on markers of IR is modulated by OC or its undercarboxylated form. A different mechanism of action to explain the attenuation in glycemic outcomes should be examined in future studies.

**Summary**

Obesity and osteoporosis epidemics are rising worldwide. Although weight loss is recommended to reduce comorbidities, in older men and women, it is known to increase fracture risk. In addition, while a higher body weight has been associated with higher bone mass, there is evidence of compromised bone quality in the obese. In the studies reported here, it was found that unlike in women, in men who voluntarily lose a moderate amount of weight, there is no detrimental effect on bone compartments and quality, and this was confirmed by others in a recent study. We attribute this to a different hormonal response to caloric restriction and possibly the higher baseline BMI and BMD in males compared to women. However, in studies with greater weight loss or in men older than in these studies, bone loss is compromised. Among strategies targeting bone health during aging and weight loss in women, higher than recommended vitamin D intakes are
of interest since it can attenuate the decrease in calcium absorption associated with caloric restriction. In the studies reported in this dissertation, we found no effect on BMD or a trend for vitamin D to attenuate cortical thickness, but it is not expected that this alone would significantly affect bone strength or fracture risk. Importantly, these are relatively short term studies to examine bone, and it is possible that it doesn’t reflect long term response of bone. Factors that attenuate bone quality during moderate caloric restriction are important to address since weight loss has a significant effect on fracture risk. Similarly, clarifying the non-skeletal effects of vitamin D in conjunction with understanding the bone endocrine role in glucose and insulin metabolism could provide therapeutic avenues in the future.
APPENDIX
Empirical estimation of free hormone from total hormone and binding-protein immunoassays

Aim 1: Free and bioavailable testosterone

Principle

The majority of total testosterone circulates bound to SHBG, a glycoprotein produced in the liver, with high affinity for testosterone (Bjornerem et al., 2004). Albumin and other low-affinity binding proteins bound the remaining testosterone and about 1–2% is not bound to any circulating protein and is considered free. These physiological events led to the belief that the small free fraction is the most biologically active fraction of circulating testosterone due to its greater availability to tissues (Mendel, 1989). Therefore, measuring this free fraction could provide valuable information about the hormonal bioactivity in the cell. However, the free fraction may also be readily accessible to sites of metabolism and undergo further degradation. Whether the free fraction represents the biologically active hormone and has a similar action in all tissues requires practical evaluation and the development of efficient methods for measurement of free testosterone that can be used in clinical interventions.

Therefore, alternative derived testosterone measures created either by additional assay steps or by calculations based on total testosterone, and SHBG has been developed. The additional assay approach includes the Free Testosterone Analog Assay and the bioavailable testosterone (which measures loosely albumin-bound testosterone as well as free testosterone. The calculations use theoretical equilibrium binding equations based on the Law of Mass Action, which can be solved for free testosterone from a second-
degree equation in total testosterone and SHBG (Vermeulen et al., 1999). However, these equilibrium binding equations are not free of several assumptions including the number of binding proteins to include in these equations, whether the SHBG-binding affinity is equal in all samples and which affinity constants for all the possible binding proteins to use in the equations.

Using a regression modeling approach with minimal assumptions about theoretical binding equilibria, Vermeulen et al. developed simple empirical equations to estimate free testosterone from measurements of total and SHBG in the same sample (Vermeulen et al., 1999).

**Aim 1**

**Assays**

After a 12 hour overnight fast, venous blood and a spot urine sample was taken from each participant in the study at baseline, and at 1, 3 and 6 months. Samples were analyzed in batch analysis for: ultra-sensitive estradiol (E2) (RIA; DSL, Webster, TX, CV<8.9%), total testosterone and free testosterone (enzyme-linked immunosorbent assay, ELISA; Alpco Diagnostics, Salem, NH, CV<9.6% and <12.4 respectively) and sex hormone binding globulin (SHBG) (ELISA; Alpco Diagnostics, Salem, NH, CV< 12.1%). Albumin concentrations were measured using colorimetric assays on an automated analyzer.
Calculations

Concentrations of total testosterone, total E2, SHBG and measured albumin were used to calculate bioavailable testosterone, and free and bioavailable E2 according to the algorithm described previously by Vermeulen et al (Vermeulen et al., 1999). The algorithm for free sex hormone calculation assumes that the concentration of the free sex steroids in blood is the result of the interaction between SHBG/albumin, and total hormone concentration (testosterone or E2) through different affinity constants of the peptides for these sex hormones, without any interaction with other hormones in the blood that could influence the equilibrium described below:

\[ [FT] = ([T] − (N \times [FT]))/(Kt\{SHBG − [T] + N[FT]\}), \text{ respectively} \]

\[ [FE2] = ([E2] − (N \times [E2]))/(Kt\{SHBG − [E2] + N[FE2]\}), \]

[T] and [E2] are total testosterone and total E2 concentrations, respectively.

[fT] and [fE2] are free T and free E2 concentrations.

KsT and KsE2 are the affinity constants of SHBG for T and E2

\[ N1 = KaT \text{ Ca+ 1} \]

\[ N2 = KaE2 \text{ Ca+ 1} \]

Ca is the albumin concentration

KsT and KsE2 are the affinity constants of albumin for T and E2.
Compared to the average albumin’s affinity constant for T of $\sim 3 \times 10^4$ L/mol, the association constant between SHBG and T is $1 \times 10^9$ L/mol. A greater affinity constant translates into several-fold stronger bound between SHBG and its ligand (testosterone).

**Aim 2: Free and bioavailable vitamin D**

**Assays**

After a 12 hour overnight fast, venous blood was taken from each participant in the study. Serum samples were analyzed in duplicates for the following hormones: 25OHD (radioimmunoassay, RIA; DiaSorin, Stillwater, MN, CV <12.5%), intact PTH (immunoradioassay, IRMA; Scantibodies, Santee, CA, CV <6.8%), and ultra-sensitive estradiol ($E_2$) (RIA; DSL, Webster, TX, CV <8.9%). The laboratory uses both internal and external standards, and also participates in the international Vitamin D External Quality Assessment Scheme to ensure quality and accuracy of 25OHD analysis. Calcium and albumin concentrations were measured using colorimetric assays on an automated analyzer. Concentrations of serum DBP were determined using a commercial ELISA kit (ALPCO, Salem, NH). Concentrations of DBP were interpolated from a standard curve after measuring absorbance on an Elx808 plate reader using Gen5 data analysis software (BioTek Instruments, Inc). The intra- and inter-assay coefficients of variation for this assay were 5.0% and 12.7%, respectively. The published normal reference range for DBP concentrations (7) is 300–600 µg/ mL (30-60 mg/dL).

**Calculations**

Free, bioavailable, and DBP-bound 25OHD were calculated using an adapted algorithm previously described (Vermeulen et al., 1999). This algorithm has been validated for
calculation of free and bioavailable testosterone based on measured amounts of serum total testosterone, sex hormone–binding globulin (SHBG), and albumin and application of the known binding-affinity constants of testosterone for albumin and SHBG. The method defines bioavailable hormone as the fraction that is both free and albumin-bound, that is, the fraction not bound to circulating binding proteins, such as vitamin D binding protein. In this study, the equations were adapted by replacing the variables for testosterone, SHBG, and albumin and their respective binding constants with those of 25OHD, DBP, and albumin (Powe et al., 2013). The formulas used to calculate free and bioavailable 25OHD are shown below:

\[
[\text{Total}] = \frac{\text{concentration of 25OHD in } g/mole}{400.5 \ g/mole}
\]

\[
[\text{Alb}] = \frac{\text{serum albumin concentration in } g/L}{66,430 \ g/mole}
\]

\[
[\text{Total DBP}] = \frac{\text{concentration of serum DBP in } g/L}{58,000 \ g/mole}
\]

\[
[D] = \frac{([\text{Total}] - (K_{\text{alb}} \cdot [\text{Alb}] + 1) \cdot [D]) \div K_{\text{DBP}} \div ([\text{Total DBP}] - ([\text{Total}] - (K_{\text{alb}} \cdot [\text{Alb}] + 1) \cdot [D]))}{[\text{Total}] - (K_{\text{alb}} \cdot [\text{Alb}] + 1) \cdot [D]}
\]

\[
[\text{Bio}] = [D] + [D_{\text{alb}}] = (K_{\text{alb}} \cdot [\text{Alb}] + 1) \cdot [D]
\]

\[
[\text{Total}] = \text{Total 25OHD}; \ [D] = \text{free vitamin D}; \ [\text{Bio}] = \text{bioavailable vitamin D}; \ K_{\text{alb}} = \text{affinity constant between vitamin D and albumin} = 6 \times 10^5 \ M^{-1}; \ K_{\text{DBP}} = \text{affinity constant between vitamin D and DBP} = 0.7 \times 10^9 \ M^{-1}.
\]
Physical activity assessment in Aim 1 and Aim 2

Increased exercise during weight loss attenuate bone loss (Villareal et al., 2006). We assessed physical activity level (PAL) in Aim 1 and Aim 2 to assure it does not influence our results. In addition, PAL was used as a covariate in the statistical analysis.

Methods

At baseline and throughout the intervention the type, duration and frequency of activity was recorded in all subjects. Physical activity questionnaires were used. In addition, PAL was recorded in a separate section of the food diary form. Physical activity level was reported as numerical scores ranging from 0 to 3 (0-inactivity, 1-low activity, 2-moderate activity and 3-high activity). The scores resulted from the estimation of energy expenditure in metabolic equivalent-minutes per week (MET mins/week) (Cawthon et al., 2009) (Table 15).

Results

Subjects were given a baseline and an averaged intervention cumulative score. A mixed model ANCOVA was conducted with time and treatment as main effects. There were no differences over time between groups (Tables 16 and 17).
### Table 15. Physical activity level scoring

<table>
<thead>
<tr>
<th>PAL</th>
<th>MET</th>
<th>Time/session</th>
<th>Sessions/week</th>
<th>Score</th>
</tr>
</thead>
<tbody>
<tr>
<td>Inactivity</td>
<td>&lt;180 METs*min/week</td>
<td>&lt;20 min/day</td>
<td>3 days/week</td>
<td>0</td>
</tr>
<tr>
<td>Low</td>
<td>180-449 METs*min/week</td>
<td>&gt;20 min/day</td>
<td>5 days/week</td>
<td>1</td>
</tr>
<tr>
<td>Moderate</td>
<td>450-750 METs*min/week</td>
<td>=30 min/day</td>
<td>5 days/week or 3 days/week</td>
<td>2</td>
</tr>
<tr>
<td>High</td>
<td>750 METs*min/week</td>
<td>&gt;30 min/day</td>
<td>5 days/week or 3 days/week</td>
<td>3</td>
</tr>
</tbody>
</table>

MET, metabolic equivalent-minutes/week.

### Table 16. Physical activity level in Aim 1

<table>
<thead>
<tr>
<th>Aim 1</th>
<th>WL</th>
<th>WM</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Baseline</td>
<td>Intervention</td>
<td>Baseline</td>
</tr>
<tr>
<td>Cumulative score</td>
<td>1.1 ± 0.9</td>
<td>1.0 ± 1.1</td>
<td>1.1 ± 0.9</td>
</tr>
</tbody>
</table>

### Table 17. Physical activity level in Aim 2

<table>
<thead>
<tr>
<th>Aim 2</th>
<th>600 IU</th>
<th>2000 IU</th>
<th>4000 IU</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Baseline</td>
<td>Intervention</td>
<td>Baseline</td>
<td>Intervention</td>
</tr>
<tr>
<td>Cumulative score</td>
<td>2.1 ± 0.8</td>
<td>2.0 ± 0.8</td>
<td>1.9 ± 0.8</td>
<td>1.9 ± 0.7</td>
</tr>
</tbody>
</table>
Standard operating procedures

In Aim 1 and 2, pQCT and High Resolution scans were performed to assess bone microarchitecture. After the measurement, the following procedure is conducted to export and analyze a scan from XCT 3000.

1. Measurement analysis

**Region of interest (ROI)-Bone area**

Start XCT550→Licensed→NO→ “user/user” (user name/password) → Perform backup (no)→Esc →QA scan (no) →Esc →Esc →Analyze → Select patient (by name/birth/ID/etc) [or select Previous patient or CT number (if you don’t choose any option and you press Enter all patients will show up in alphabetical order)] →Enter →List of measurements →Choose any RESMG measurement (except high res) or Tibia 4S→ Enter → You will see the screen with all the slices →ANALYSIS →Enter →Results →Enter →ROI →New → Enter →Use the up-down arrows to place the cursor in the center of the ROI (if the image looks clean, a lot of bright red means movement) and press F followed by M to minimize the region of interest.

If the ROI cannot be drawn automatically and the quality of the measurement is not good, from top left of the screen choose “Set irregular ROI” which allows you by multiple clicking on small areas to draw the ROI around the bone as close as possible and use M to minimize it when done. →OK →ROI name (ex. Tibia 4%) →OK→LOOP → Start “SUELOOP” →Enter →Comment (only if you want, usually same as ROI name, i.e. Tibia 4%) →now press Esc →Esc →Esc → END →Esc →Esc →END Program →Y.
To go back and analyze another slice or another subject, go to ANALYZE patient screen and repeat the previous steps.

**Muscle area/Fat area and Fat/Muscle ratio**

Follow the same steps mentioned before to select a subject → Screen with all the slices → Analysis → Macros → Select → Use up-down arrows to select “Muscle” → Start Muscle → Done (ideally you would print the screen or save it in the computer). Data are entered in an excel file as: Fat area, Fat/Muscle ratio and calculated Muscle area = Total area - (Fat + Bone area).

2. **Data export (pQCT)**

Open any excel file in the computer → Open → Computer → OS(C) drive → XCT550 → TZ → choose “all files” option (change from excel or any other type of file to “all files” so you can see your file) → open SUES.DBF → your subject will be the last raw in the excel file and that is the data you need. You can save the excel file with the date you are analyzing the measurements. Open this file once you are done with analyzing all your measurements for the day (so you don’t save multiple files on the same day).

3. **Data export (High resolution scan)**

Before starting the protocol, CT numbers (CT-NO) that need to be exported (i.e. 21221) are written down. The CT-NO is found in the list of measurements for each subject and the CT-NO allocated to the high res measurement is used. Procedure is done one by one.

Log into the XCT550 with “9999/service” (do not use “user/user” in this case) → OPTIONS → EXPORT → Select the drive location (best to use drive C) → Select CT
number → Enter the CT number to export (i.e. 21221) → This will create an AV002 directory in C drive → Esc (don’t worry about inserting a disk, just type ok to download onto C drive). Go to Computer → OS (C) drive and you will see the AV002 folder → copy all there is in this folder onto a memory stick and send out for analysis.

**Assessment of skin color**

Vitamin D is synthesized in the skin through sunlight exposure. Solar UVB radiation is the primary source of vitamin D for most persons (Holick, 2007). Therefore, when assessing vitamin D status, individual sunlight exposure needs to be considered. Noninvasive method, using diffuse reflectance spectroscopy are considered to be the gold standard for objective quantitative measurement of skin color and many types are available. The instrument is used to direct lights of specific wavelengths onto the area of interest, and then the wavelength and intensity of light reflected back from the skin to the instrument’s sensors are quantified.

**Method**

In Aim 2 we used a hand held tristimulus reflectance spectrophotometer (250–750 nm; Konica Minolta Optics Inc., Osaka, Japan) to assess skin color changes with the season. Skin reflectance is reported using Yxy color-indexing system (Takiwaki, 1998). The Y measures range from 0 (total black) to 100 (total white). Two different locations were measured: middle of the outer underarm (exposed) and waist area (unexposed), giving a total of 6 values (Y, x and y for each of these two sites). All women were measured in the same room and under similar lighting conditions.
Analysis

Three sets of measurements were taken at each time point (summer and winter months) for each subject by the same operator and averaged to ensure accuracy. Data were analyzed using one-way ANOVA to compare the changes in skin color with season between groups.

Results

Skin reflectance (Y) values at the beginning of the study (winter) averaged 47 ± 16 cd/m² at the exposed area and 67 ± 14 cd/m² at the unexposed area (p<0.01) and there were no significant differences between groups. Similarly, after the summer months, skin reflectance was lower (42 ± 11 cd/m²) at the exposed compared to the unexposed area (58 ± 21 cd/m², p<0.01) and the groups did not differ significantly. The difference between skin reflectance at the unexposed areas compared to exposed areas remained significant in all subjects throughout the study (p<0.01) (Figure 14).

Figure 14. Changes in skin reflectance from winter to summer in all subjects (Shapses lab)
In a previous study, MacDonald et al. assessed skin color at the exposed areas (cheekbones) and reported differences in the spectrophotometer readings between winter and summer (MacDonald et al., 2011).

**Figure 15. Comparison for changes in skin reflectance from winter to summer**

A similar change between the two studies from winter to summer was observed in skin reflectance at the exposed area. In our study we advised the women to use sunscreen and there were no reports of holidays abroad. MacDonald at al. analyzed skin reflectance in sunscreen user and non-users. In this graphs, data from the non-users is shown (MacDonald 2011) (Figure 15). However, in our study the unexposed areas were also measured and the differences in skin reflectance between exposed and unexposed areas remained unchanged throughout the study duration.
In our study, serum 25OHD levels at 6 months of vitamin D supplementation (measured after the summer months) were significantly higher compared to baseline values. During the second part of the study, from month 6 to month 12, representing the winter months, serum 25OHD levels stayed elevated or slightly increased in all groups (Figure 16). This suggests that serum 25OHD in our study are mainly due to vitamin D supplementation and not to sun exposure.

Figure 16. Serum 25OHD at 6 months and 12 months of vitamin D supplementation
Literature cited


110. Holick MF, Biancuzzo RM, Chen TC, 2008. Vitamin D2 is as effective as vitamin D3 in maintaining circulating concentrations of 25-hydroxyvitamin D. J Clin Endocrinol Metab. 93:677-81.


restriction-induced weight loss or exercise-induced weight loss: a randomized controlled trial. Arch Intern Med. 25;166:2502-10.


