



## AN ABSTRACT OF THE THESIS OF

Jill Marrone for the degree of Master of Science in Nutrition presented on September 9, 2009.

Title: Effect of Moderate Alcohol Consumption on Biochemical Markers of Bone Turnover in Postmenopausal Women

Abstract approved:

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Epidemiological studies have reported that postmenopausal women who consume moderate quantities of alcohol have higher bone mass than non-drinkers. However, the mechanism for the putative bone-sparing effect of alcohol is unknown. Postmenopausal bone loss is due, in part, to increased bone turnover. This study investigated the hypotheses that alcohol slows bone loss by reducing bone turnover. The serum bone formation marker, osteocalcin (OC) and serum bone resorption marker, C-terminal telopeptide (CTx) concentrations in response to alcohol withdrawal for two weeks and acute as well as chronic restoration of alcohol consumption were measured in postmenopausal women who consumed a moderate amount of alcohol (4-14 standard drinks/week on average) the year prior to the beginning of the study. A total of 40 non-osteoporotic postmenopausal women ( $56.3 \pm 0.5$  years, mean  $\pm$  SE) completed this study. There was an association between trochanter and total hip BMD and alcohol consumption. Compared to baseline values, serum OC and serum CTx were increased after the 14-day abstinence

interval ( $4.1 \pm 1.6\%$ ;  $p = 0.008$  and  $5.8 \pm 2.6\%$ ;  $p = 0.016$ , respectively).

Following abstinence, participants were administered alcohol and evaluated the following morning (day 15). The participants were re-evaluated for a final time two weeks following restoration of unsupervised alcohol consumption.

On day 15 vs. day 14, OC was significantly lower ( $3.4 \pm 1.4\%$ ;  $p = 0.01$ ), and CTx showed a similar but borderline significant decrease ( $3.4 \pm 2.1\%$ ;  $p=0.05$ ).

There was no significant difference found between OC and CTx on day 15 vs. baseline ( $0.1 \pm 1.6\%$ ;  $p =0.9$  and  $1.7 \pm 3.4\%$ ;  $p=0.56$ , respectively). CTx increased at the end of the study (day 28) compared to baseline ( $8.0 \pm 3.0\%$ ;  $p= 0.02$ ), but there was no significant difference found for OC between baseline and day 28 ( $14.8 \pm 7.9\%$ ;  $p=0.06$ ). In conclusion, BMD was positively associated with alcohol consumption, and abstinence from alcohol intake increased markers of bone turnover. These results support the hypotheses that moderate alcohol attenuates bone turnover in early postmenopausal women.

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Effect of Moderate Alcohol Consumption on Biochemical Markers of Bone  
Turnover in Postmenopausal Women

by  
Jill Marrone

A THESIS

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Presented on September 9, 2009.

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I understand that my thesis will become part of the permanent collection of Oregon State University libraries. My signature below authorizes release of my thesis to any reader upon request.

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Jill Marrone, Author

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## **Chapter 1 – Introduction**

Osteoporosis is characterized by low bone mass and deterioration of bone micro-architecture (1). The National Osteoporosis Foundation estimates that 10 million Americans currently have osteoporosis and that 34 million are at risk for osteoporosis due to low bone mass. The incidence of this condition is expected to increase as the number of Americans over the age of 65 years is expected to double and reach 69 million by 2050 (2).

Gonadal insufficiency is one of the most important risk factors for osteoporosis (3). After menopause, bone mineral density (BMD) rapidly declines which increases the risk of developing osteoporosis and suffering from osteoporotic fractures. Postmenopausal bone loss is due to accelerated but unbalanced bone turnover, in which bone resorption predominates over bone formation (1,2,4). Osteoclasts and osteoblasts release biochemical markers in blood, and levels of these markers reflect the rate of bone resorption and formation (4).

Although not all postmenopausal women will develop osteoporosis, bone loss is generally thought to be inevitable with age (3). Several approaches have been explored as potential ways in which to prevent bone loss and treat osteoporosis. One approach is the use of anti-resorptive pharmacological drugs, such as hormone replacement therapy, selective estrogen receptor modulators (SERMs), or bisphosphonates, but these therapies have the

potential for undesirable side effects and safety issues. Estrogen therapy has been found to prevent postmenopausal bone loss when initiated during menopause, but its use is associated with an increased risk for developing breast cancer (5). Oral bisphosphonates can cause gastrointestinal inflammation (6), and intravenous injections may cause fever and musculoskeletal pain (7). Lifestyle factors such as physical activity have also been explored as approaches to reducing bone loss (8).

Alcohol consumption is a lifestyle factor which influences bone mass. Chronic alcohol abuse has been found to reduce BMD (9,10), osteoblast numbers (11-13) and osteoblast function without affecting osteoclastic activity (9). In contrast, there have been several published studies showing a positive association between BMD and moderate alcohol consumption (8,14-18). Additionally, lower bone turnover has been reported with moderate levels of alcohol intake (8). Many factors, such as physical activity, certain medications, food intake, and seasonal variation can affect bone metabolism (19). As a consequence, it is uncertain whether the difference in BMD in these studies is due to alcohol or due to other accompanying unidentified factors. On the other hand, animal studies suggest that moderate alcohol consumption decreases bone turnover while maintaining the balance between bone formation and bone resorption (20). Since high bone turnover is one risk factor for osteoporosis (21) and plays an important role in postmenopausal bone loss, reducing bone turnover, a strategy used in pharmacological

interventions, may also be a useful lifestyle intervention. Therefore, examining the effects of alcohol consumption and withdrawal on bone turnover biomarkers can aid in assessing the influence of alcohol on bone loss and provide a mechanism by which moderate alcohol consumption may potentially reduce bone loss and increase BMD.

The purpose of this study was to examine the effects of moderate alcohol consumption and withdrawal on bone turnover biomarkers in postmenopausal women. Our central hypothesis was that moderate alcohol consumption would reduce bone turnover in postmenopausal women. The working hypothesis was that alcohol withdrawal would result in an increased level of biochemical markers for bone turnover and subsequent return to normal alcohol consumption would decrease these markers.

## Chapter 2 – Literature Review

### 2.1 Osteoporosis

Osteoporosis is the most common cause of fractures in the elderly, and it is estimated to result in 1.5 million fractures each year in the United States (22). Eighty percent of the 10 million Americans who have been diagnosed with osteoporosis are women (2). In premenopausal women, the coupled processes of bone resorption by osteoclasts and bone formation by osteoblasts are balanced, so the degraded old bone is replaced with approximately an equal amount of new bone. After menopause, when estrogen levels decline, this balance can be disrupted, and bone resorption increases to a greater extent than formation (22). Therefore, postmenopausal women are at the greatest risk for developing osteoporosis due to high bone turnover rates, with bone resorption exceeding bone formation (1,4).

One out of two women over the age of 50 will experience an osteoporotic fracture, and the cost associated with osteoporosis is high. Therefore, it is important to reduce the risk of developing osteoporosis by preventing bone loss (23). Osteoporotic fractures have several implications for people who are at risk for fracture. Hip and vertebral fractures can be painful, debilitating, and increase morbidity as well as cause a drastic reduction in quality of life and loss of independence. Osteoporotic fractures also have an economic impact. In 2002, health care costs in the U.S. for hip



fractures were \$18 billion, and it is estimated that the cost of osteoporosis could be as high as \$240 billion by 2050 in the U.S. (24).

Several approaches have been explored as potential ways in which to prevent bone loss. One approach is the use of pharmacological drugs, such as hormone replacement therapy, selective estrogen receptor modulators (SERMs), or bisphosphonates that work to inhibit osteoclasts and reduce bone turnover (25). Physical activity is also one approach to reducing bone loss (26). Dietary sources including dietary supplements such as calcium and vitamin D (27), soy products (28), and omega 3 fatty acids (DHA and EPA) (29) have been researched as factors that may preserve bone. Moderate alcohol intake has also been explored as a potential factor influencing bone loss in postmenopausal women (8,14-18). Moderate alcohol intake is considered approximately one or two standard drinks per day for women and men, respectively. A standard drink is defined as 5 oz (15g) of wine, 12 oz (13g) of beer, 1.5 oz (15g) of distilled spirits, or 2 oz (9g) of sherry (30).

## **2.2 Effect of Moderate alcohol consumption on BMD and bone turnover markers in postmenopausal women**

Several epidemiological studies have observed higher BMD in postmenopausal women who drank alcohol moderately (8,14-18). Ganry et al. evaluated 7,598 postmenopausal women who were 75 years or older and drank either one to two standard drinks a day or abstained from drinking. These researchers found higher trochanter and total body BMD in the moderate alcohol consumers compared to the women who abstained from drinking alcohol (15). Ilich et al. found that postmenopausal women, who consumed approximately  $\frac{3}{4}$  of a standard alcoholic drink a day, had higher lumbar spine BMD than nondrinkers (17). In a similar study, Feskanich et al. found that postmenopausal women between the ages of 50-74 years, who drank about one standard drink a day, had higher lumbar spine BMD than nondrinkers (14).

A longitudinal study conducted by Rapuri et al. examined the relationship between alcohol intake and BMD, calcitropic hormones, and bone turnover markers (urinary N-telopeptide of type I collagen (NTx), osteocalcin, and alkaline phosphatase) in elderly women aged 65-77 years old. The results of this study observed higher BMD in alcohol consumers compared to nondrinkers. The group that drank between 28.6 and 57.2 grams of alcohol per week had the highest BMD, especially in the lumbar spine. There was a decrease in bone resorption and formation markers, urinary NTx to creatinine

ratio (NTx:Cr) and osteocalcin, respectively, suggesting a decrease in bone remodeling. Serum PTH concentrations decreased with alcohol intakes of >28.6 grams per week. A positive correlation between serum PTH and bone turnover biomarkers was observed, and bone turnover negatively correlated with BMD. The 25-hydroxyvitamin D3 levels were lower in the group consuming between 57.2 and 142.9 grams per week; whereas, the 1, 25-dihydroxyvitamin D3 remained the same for this group. Alkaline phosphatase was significantly higher in the group that consumed >28.6 grams of alcohol per week. These results demonstrate an association between moderate alcohol intake and decreased bone turnover in elderly postmenopausal women (8).

A prospective study that looked at BMD and alcohol consumption in men and women greater than 56 years of age reported that with increasing amounts of alcohol intake, BMD increased significantly in the radial shaft ( $p < 0.05$ ) and in the spine ( $p < 0.001$ ) for women. Overall, the results from this study found an association between social drinking and higher BMD in men and women (16). Examining data on 2,043 postmenopausal women from the Third National Health and Nutrition Examination Survey (NHANES III), 1988-1994, Wosje and Kalkwarf found postmenopausal women who consumed alcohol >29 occasions per month had a 3.8% higher BMD than abstainers (18). Williams et al. examined the effects of moderate alcohol consumption on BMD in 46 pairs of female monozygotic twins (31). Alcohol intake was

assessed through questionnaires, and BMD was evaluated by DXA. A positive association was noted between alcohol consumption and BMD. To examine the effects of alcohol on bone metabolism, the researchers looked at serum and urine bone formation markers, osteocalcin and bone specific alkaline phosphatase and urinary bone resorption marker, C-terminal cross links (CTx). There was not an association between bone turnover markers and alcohol or BMD (31).

### **2.3 Effects of alcohol on growing rats**

Chronic alcohol consumption has been shown to have detrimental effects on bone, especially during bone development in adolescents. Reaching optimal peak bone mass during adolescence is important for raising the fracture threshold and reducing the risk of fracture in adulthood. Several experiments using laboratory animals have found that chronic alcohol consumption during adolescence reduced bone density, volume, growth and strength (32-37) Sampson et al. found that young actively growing rats fed a diet containing 35% of their total daily caloric intake as ethanol had significantly reduced bone mass in both the proximal tibia and femur diaphysis which are primarily cancellous and cortical bone, respectively. The alcohol-fed rats also had significantly less percent ash than non-alcohol-fed animals. These results indicate that chronic alcohol consumption during bone growth reduces bone density and peak bone mass in both cortical and cancellous bone (35). In a study by Maddalozzo et al., rats consuming 35% of their daily

calories from alcohol for three months starting at 4-weeks of age were found to have a decreased tibia size compared to ad libitum-fed controls (38).

Likewise, cancellous bone volume/tissue volume (BV/TV) in the lumbar vertebrae and in the tibia was lower in alcohol fed rats. The alcohol-fed rats had lower trabecular number, trabecular separation, trabecular and cortical thickness, and cortical bone volume in the proximal tibia. Assessment of cortical bone in young actively growing female rats revealed that there was a reduction in cortical bone area in the alcohol-fed rats compared to controls fed a normal diet (34).

Growing alcohol-fed rats have reduced bone formation rates (34,38). Endocortical and periosteal bone formation rates and mineral apposition rates were greatly reduced in the alcohol-fed rats compared to the pair-fed (liquid diet) and *ad lib*-fed chow-fed rats (34). Whole bone mechanical properties, including stiffness, ultimate load, and energy absorbed were found to be 40%, 45%, and 53%, respectively, lower in alcohol-fed, growing female rats who began consuming alcohol chronically at 4 weeks of age compared to control-fed rats (34). Other studies also found whole bone mechanical properties to be reduced due to alcohol consumption (36,39). In one study conducted by Peng et al. female rats, who were 11-weeks old and consumed alcohol for 4 weeks had a 22% reduction in bone strength and 26% reduction in stiffness compared to controls, but no significant difference for energy absorbed to failure was observed (37). Turner et al. found that rats fed alcohol beginning

at 8 weeks of age had a 30% reduction in energy absorbed and a 25% reduction for strength compared to non-alcohol-fed controls, but there was not a significant difference in stiffness (36). The variations in the results from these three studies may be due to alcohol having a greater effect on the mechanical properties of younger rats due to its growth suppressive action (34).

## **2.4 Effects of chronic alcohol consumption on bone remodeling in adults**

### 2.4.1 Human Studies

Studies with cultured marrow cells obtained from humans found chronic alcohol consumption reduced osteoblast number (11-13) and activity (9-13). Klein et al. found a decreased number of osteoblasts in skeletal tissue of alcoholic patients due to inhibited osteoblastic cell proliferation (13). De Vernejoul et al. found a significant reduction in mean wall thickness in male subjects who consumed alcohol daily and had osteoporosis (10). In a study conducted on older men with cirrhosis and osteoporosis, histomorphometric analysis of transiliac bone after tetracycline labeling revealed significantly decreased bone mass and a reduction in osteoid parameters, lower mean wall thickness, and slower bone formation indicating impaired osteoblastic function. In addition to a decrease in osteoblast function, Chappard et al. found normal osteoclastic activity. These results may provide insight into alcoholic osteoporosis and decreased bone mass (9). One study compared biochemical markers of bone turnover in alcoholic male subjects after long-

term (abstainers), and short-term (alcoholics), withdrawal of ethanol with healthy control subjects. Alcoholic subjects who chronically consumed alcohol had a significantly lower serum level of osteocalcin ( $p < 0.001$ ) at baseline compared to alcoholic subjects who abstained from drinking alcohol for at least 5 years and compared to control subjects. After discontinuing alcohol consumption, osteocalcin levels normalized after 10 days, and there was no significant difference between the groups. Fasting urinary deoxypyridinoline levels were significantly increased in alcoholics compared to controls ( $p = 0.001$ ), and these levels did not normalize after 10 days. The abstainers also had a significant increase in deoxypyridinoline levels compared to controls ( $p = 0.022$ ). C-terminal telopeptide (ICTP) was significantly higher ( $p = 0.007$ ) among the alcoholics compared to abstainers. The ICTP levels for abstainers were similar to the controls. These results indicate that chronic alcohol consumption may decrease osteoblast function and increase bone resorption. This data further suggests that bone turnover may be imbalanced in alcoholics and could eventually result in bone loss (40).

#### 2.4.2 Animal Studies

In adults, chronic alcohol consumption is associated with disrupted bone remodeling associated with decreased osteoblast function and proliferation (41,42). Peng et al. looked at osteoblasts from rats *in vitro* and found that after alcohol consumption osteocalcin levels were decreased, which suggests a decrease in osteoblastic activity (43). Other studies conducted by Sampson et al. found that in addition to decreased trabecular bone volume,

chronic alcohol consumption in adult rats decreased osteoblast numbers and rates of bone formation. These results indicate that bone formation and mineralization rate is impaired (44,45). Histomorphometric analysis revealed that alcohol-fed rats had significant reductions in wall thickness, a measure of osteoblastic activity (44,46), and in the amount of active osteoblasts covering trabecular bone surfaces compared to controls (44). Reed et al. reported a 38% reduction in cancellous bone formation rates in male rats fed alcohol (35% of total caloric intake) compared to rats on a normal diet. A significant decrease in periosteal bone formation rate and in trabecular thickness in the tibial metaphysis was also found in the alcohol-fed rats (47).

## **2.5 Effect of alcohol consumption on hormones**

Excessive alcohol consumption has been shown to reduce parathyroid hormone (PTH) levels (48), and to decrease the effect of PTH to stimulate the synthesis of bone matrix proteins, such as type I collagen, osteocalcin, and osteonectin (49). Heavy alcohol consumption is also associated with lower vitamin D metabolites, which can lead to less calcium absorption and cause hypocalcemia (40). Chronic alcoholic male subjects had decreased levels of serum 25-hydroxy vitamin D compared to control subjects (50). Decreased testosterone levels are commonly found in male alcoholics. Whereas, in a study conducted by Gavalier and Van Thiel, postmenopausal women who consumed moderate amounts of alcohol had significantly higher serum estradiol levels compared to abstainers (51). One proposed theory of how



moderate alcohol consumption may have a positive effect on BMD in postmenopausal women is that alcohol increases circulating levels of estrogen (52). While estrogenic effects of alcohol have been seen, neither ethanol nor its metabolites have been shown to have estrogenic properties. Three possible estrogenic effects of alcohol have been proposed. The first possibility is that consumption of ethanol may increase estrogen receptor activity in tissues by increasing estrogen receptor alpha expression (53). The second way is that alcohol may promote aromatization of androgens to estrogens (54). The third mechanism is that ethanol may directly inhibit the catabolism of estradiol (55).

## **2.6 Bone Turnover Biomarkers**

Bone turnover biomarkers provide a measure of bone resorption and formation and can be used as a surrogate for bone remodeling. Therefore, in addition to predicting fracture risk, bone turnover biomarkers are used to assess the rate of bone turnover. Biochemical markers of bone turnover can be measured for bone resorption and bone formation using immunoassay tests that are specific for a particular biomarker of bone turnover.

### **2.6.1 Bone Resorption Markers**

There are several bone resorption markers that are type I collagen breakdown products. C-terminal telopeptide of type I collagen (CTX), N-terminal telopeptide of type I collagen (NTx), deoxypyridinoline (DPD), and

pyridinoline (PYD) are examples of collagenous bone resorption biomarkers. During bone resorption, these collagen breakdown products are released into circulation and eventually cleared by the kidneys. Therefore, these markers can be assessed in blood and urine (56). CTx is a fragment of 8 amino acids from C-telopeptide of type I collagen, and it is released by cathepsin K activity. It can be measured in urine or serum samples (21). CTx is less specific to bone than NTx since CTx can be derived from other connective tissues (57). Specific immunoassays are used to assess CTx in serum or urine. NTx is another 8 amino acid fragment and breakdown product of type I collagen and can be measured in urine or serum (21). DPD and PYD are important in the formation of cross-links of adjacent polypeptides of collagen and found in mature type I collagen. These biomarkers can be measured in urine using specific immunoassays (58). PYD is more abundant compared to DPD in the skeleton, but DPD is more specific to bone (57).

### 2.6.2 Bone Formation Markers

Bone specific alkaline phosphatase (BSAP), osteocalcin (OC), C- and N-terminal pro-peptides of type I collagen (PICP and PINP) are commonly measured bone formation markers. BSAP is a marker of enzyme activity and is expressed by mature osteoblasts. It is associated with the mineralization of bone (59). Alkaline phosphatase is derived from various tissues, such as intestines, spleen, kidney, liver, bone, and placenta during pregnancy, but liver

and bone express the highest amounts of ALP. Specific assays have been formulated to identify the bone specific alkaline phosphatase (BSAP) (56).

Bone formation markers include OC, PICP and PINP. OC is a non-collagenous bone matrix protein and is secreted by osteoblasts. OC is a polypeptide of 49 amino acid residues (21). It is found in two forms—an uncarboxylated and carboxylated form. Vitamin K acts as a cofactor to the enzyme responsible for carboxylating OC (56,57). The carboxylated form binds calcium and therefore changes the conformation of OC. Glutamic acid residues in OC then promote mineralization (21). OC can be measured in plasma and serum using an immunoassay specifically designed to detect OC (60). PICP and PINP are globular peptides that are released by enzymes from newly synthesized pro-collagen before being incorporated into the extracellular matrix (21). These peptides are produced by bone and other tissues that synthesize type I collagen, such as skin. Since PICP and PINP are produced by non-bone specific tissue, they are not as useful in identifying bone formation (56).

CTx and OC are the two bone turnover biomarkers that are being assessed in this study. OC was selected because it is one of the most sensitive markers for bone formation (4). OC is considered a late biochemical marker of bone formation and used as a marker to determine rate of bone formation (57). CTx is one of the preferred resorption markers (57). It is found

to be highly correlated with the rate of bone loss, and its rate of release from bone is useful in assessing the resorbing activity by osteoclasts (21).

### **Chapter 3 – Specific Aims**

Several epidemiological studies have observed higher BMD in postmenopausal women who consume moderate amounts of alcohol (8,14-18), and measured bone turnover biomarkers in this population (8). To date, there are no studies that have assessed bone turnover biomarkers when alcohol is withdrawn and then restored in the diet in postmenopausal women who consume moderate alcohol. This study is designed to evaluate the effects of moderate alcohol consumption on bone turnover biomarkers in postmenopausal women who consume moderate amounts of alcohol (8,14-18).

The **long-term goal** of this research is to understand how alcohol intake affects the risk of developing osteoporosis. The **objective** of this study is to examine the effects of moderate alcohol consumption (1-2 standard drinks a day) on bone turnover biomarkers in postmenopausal women. Our **central hypothesis** is that moderate alcohol consumption reduces bone turnover and slows bone loss in postmenopausal women and thus reduces the risk of osteoporosis. The **rationale** for this study is that alcohol intake is achievable, socially acceptable, and that moderate levels of alcohol consumption will preserve bone mineral density. Assessment of alcohol

withdrawal on bone turnover biomarkers in postmenopausal women who have consistently consumed moderate levels of alcohol over the past year was selected for the design of this study. We evaluated the potential benefit of moderate alcohol consumption on bone in this group of women. The **working hypothesis in this study** is that alcohol withdrawal would result in an increased level of biochemical markers for bone turnover and subsequent return to normal alcohol consumption would decrease these markers. The following two specific aims were examined to test this hypothesis:

**Specific aim #1: Determine the effect of alcohol withdrawal on bone turnover biomarkers in postmenopausal women who consume moderate amounts of alcohol.**

We hypothesize that alcohol withdrawal will result in increased levels of biochemical markers of bone turnover.

**Specific aim #2: Determine the effect of restoring moderate alcohol consumption on biochemical markers of bone turnover in postmenopausal women.**

We hypothesize that restoring alcohol consumption will result in decreased levels of biochemical markers of bone turnover.

## **Chapter 4 – Materials and Methods**

### **4.1 Study Participants**

To be eligible for participation in the study, participants had to be 1) postmenopausal for 10 years or less, 2) under the age of 65 years, 3) not on hormone replacement therapy (HRT) within six months prior to the start of the study, and 4) a consumer of, on average, 4-14 standard alcoholic drinks/week over the year prior to and at the beginning of the study. Participants were disqualified from participating in the study if they were diagnosed as having osteoporosis (a T-score of -2.5 or less for total hip, lumbar spine, or whole body) or taking any medications that affect bone turnover, such as bisphosphonates or SERMs. Osteoporosis was determined by DXA scans of the lumbar spine, total hip, or whole body. Forty-four postmenopausal women were recruited to participate in this study, and forty completed the study. Three of the participants were diagnosed as having osteoporosis at the hip and spine. One participant withdrew from the study for personal reasons unrelated to the study. The study was approved by the Institutional Review Board at Oregon State University. Written informed consent was obtained from each participant prior to starting the study.

### **4.2 Experimental Design**

The experiment took place over a 28 day interval (figure 4.1). During the week

prior to intervention, participants maintained their normal alcohol intake and kept a daily record of alcohol intake. The type of alcoholic beverage and amount in ounces of alcohol were recorded as well as the time of day the alcohol was consumed. Participants submitted alcohol records at the first blood draw. In addition, the study participants completed a Block Food Frequency Questionnaire designed by the National Cancer Institute (61). Information about daily average dietary and supplemental calcium and vitamin D, total grams of protein, and kcalories were obtained at baseline of the study.

At the beginning of the study (day 0 of intervention), a blood sample was collected between 7:00 and 9:00am. From day 0 to day 14, participants abstained from consuming alcohol. On day 14, a blood sample was collected between 7:00 and 9:00am, and participants were given a container of a pre-determined amount of alcohol, which was approximately equivalent to their average daily consumption as determined from their one-week alcohol record. On the evening of day 14, participants consumed their container of alcohol, and the following morning (day 15), the participants returned to the laboratory for a blood draw between 7:00am and 9:00am. Participants then resumed drinking alcohol unsupervised. On day 28, participants came into the laboratory for a final blood sample collection between 7:00 and 9:00am.

### **4.3 Bone mineral density and body composition**

Dual-energy X-ray absorptiometry (DXA, Hologic QDR-4500 Elite A Waltham, MA) was used to measure total body mass,, lean mass, fat mass,

and bone mineral density (BMD, g/cm<sup>2</sup>) of the lumbar spine (L1-L4), and proximal femur (total hip, femoral neck, and greater trochanter). Body Mass Index (BMI) for each participant was calculated using weight from the DXA results and height that was self-reported by participants during baseline DXA scans. All scans were conducted at Oregon State University Bone Research Laboratory and analyzed using Hologic software version 9.80 D (Hologic, Inc., Waltham, MA). The DXA machine was calibrated daily against a phantom. The coefficient of variation (CV) for repeated DXA scans at Oregon State University Bone Research Laboratory are 1.0% for BMD of the hip and lumbar spine and 1.5% for whole body BMD.

#### **4.4 Assays for bone turnover biomarkers**

Blood samples were collected between 7:00am-9:00am for each participant in order to minimize diurnal variation in the levels of the bone turnover biomarkers as a confounding variable. Participants fasted for at least 10 hours prior to each blood collection. Blood collections took place at Oregon State University (OSU) by a trained phlebotomist, and 10 ml of blood was collected at each blood draw. Blood samples were centrifuged within two hours of collection. Serum was frozen at -20°C until analysis.

Serum samples were analyzed in duplicate for serum CTx and OC using ELISA at the Maine Medical Center Research Institute (Scarborough, ME). To avoid inter-assay variability, all four samples for each participant were



analyzed in the same assay test. The inter-assay and intra-assay variation for CTx were 2.5-10.9% and 1.7-3.0%, respectively, and the inter-assay and intra-assay variation for OC were 2.7-5.1% and 1.2-3.3%, respectively. Serum samples from all four blood collections during the study were batch-analyzed at the conclusion of the study.

#### **4.5 Statistical analysis**

The statistical analysis was performed using S-Plus, version 8.0. Since there were large individual differences in baseline serum levels of bone turnover markers, each woman was used as her own control and statistical analysis was performed on the calculated change in level of the biomarkers. To determine whether or not CTx and OC increased after two weeks of abstinence from alcohol compared to baseline, a one-sided t-test was performed. One-sided t-tests were also used to determine if CTx and OC decreased the morning after participants consumed alcohol on day 14 (12-30 g). Two-sided t-tests were performed to assess if CTx and OC differed on day 15 vs. 0, and to compare serum CTx and OC levels on day 28 versus day 0. The correlation between serum CTx and OC concentrations was determined by linear regression using log-transformed data. Linear regressions were performed to compare the change in serum CTx and OC against alcohol intake at day 14 vs. day 0 and to compare bone mineral density (BMD) with alcohol in grams at day 0. Differences were considered significant at  $p < 0.05$ . Data are reported as mean  $\pm$  SE.

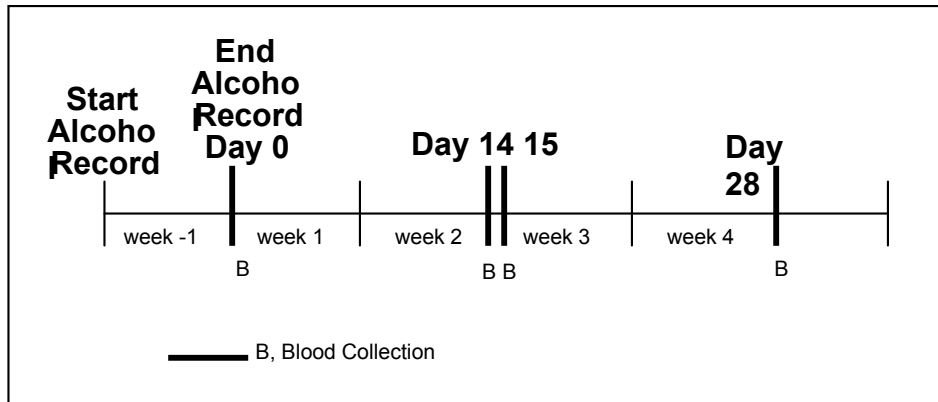


Figure 4.1 Study Design Timeline.

## Chapter 5 – Results

### 5.1 Characteristics of the study population

The characteristics of the study participants at baseline are shown in Table 5.1. At baseline, the mean age of the study participants was  $56.3 \pm 0.5$  years, and the mean number of years post menopause was  $4.7 \pm 0.4$  years. Body fat percent and weight in kilograms as determined by DXA was  $32.3 \pm 1.3$  percent and  $67.8 \pm 1.9$  kg, respectively.

DXA measurements were used as a screening tool prior to the start of the study. Three women were excluded from the study based on a BMD T-score of less than -2.5 SD in the hip or lumbar spine. One participant dropped out of the study prior to the first blood collection due to medical issues unrelated to the study. The mean BMD, T-scores, and Z-scores for the forty eligible participants who completed the study are shown in Table 5.2.

Based on the one-week alcohol records that participants kept during the week prior to the study initiation, the amount of alcohol consumed by participants was equivalent to approximately 9 standard drink/week (Table 5.3).

Daily dietary means for grams of protein, milligrams of calcium and IU of vitamin D, and the means for daily supplemental calcium and vitamin D were reported at baseline using Food Frequency Questionnaires. Mean protein, calcium, and vitamin D intake met or exceeded recommended values (Table 5.3).

## 5.2 Bone Mineral Density (BMD) and Alcohol Consumption

There was an association between amount of alcohol consumption at baseline and trochanter BMD ( $p=0.01$ ) (figure 5.1) and total hip BMD ( $p = 0.02$ ) (figure 5.2). A significant association was not detected between baseline alcohol and femoral neck BMD ( $p= 0.24$ ) (figure 5.3) or total lumbar spine BMD ( $p= 0.19$ ) (figure 5.4). Femur measurements were not available for one participant due to a double hip replacement.

## 5.3 Bone Turnover Biomarkers

Percent changes in serum OC and CTx are shown in Figures 5.5, 5.6, 5.7, 5.8). Serum OC and CTx increased significantly, compared to day 0, during the 14 day abstinence interval ( $4.1 \pm 1.6\%$ ;  $p= 0.008$ ) and ( $5.7 \pm 2.6\%$ ;  $p = 0.016$ ), respectively. OC decreased significantly on day 15 compared to day 14 ( $-3.4 \pm 1.4\%$ ;  $p = 0.01$ ). CTx showed a similar but marginally significant decrease on day 15 vs. day 14 ( $-3.4 \pm 2.1\%$ ;  $p=0.05$ ). Day 15 did not differ from day 0 for OC and CTx ( $0.1 \pm 1.6\%$ ;  $p = 0.9$  and  $1.7 \pm 3.4\%$ ;  $p = 0.5$ , respectively). After participants resumed consumption of alcohol on day 14, CTx significantly increased on day 28 compared to day 0 ( $8.0 \pm 3.0\%$ ;  $p = 0.02$ ). There was no significant difference in OC on day 28 vs. day 0 ( $14.8 \pm 7.9\%$ ;  $p = 0.06$ ).

#### **5.4 Baseline Bone Turnover Markers and Alcohol Consumption**

The change in bone turnover biomarkers was not associated with alcohol intake. The change in CTx from day 0 to day 14 (figure 5.9) was not associated with alcohol intake ( $p=0.13$ ), nor was the change in osteocalcin from day 14 to day 0 (figure 5.10) associated with alcohol intake ( $p=0.77$ ). A linear association between OC and CTx was observed ( $r= 0.71$ ;  $p < 0.0001$ ) (Figure 5.11).

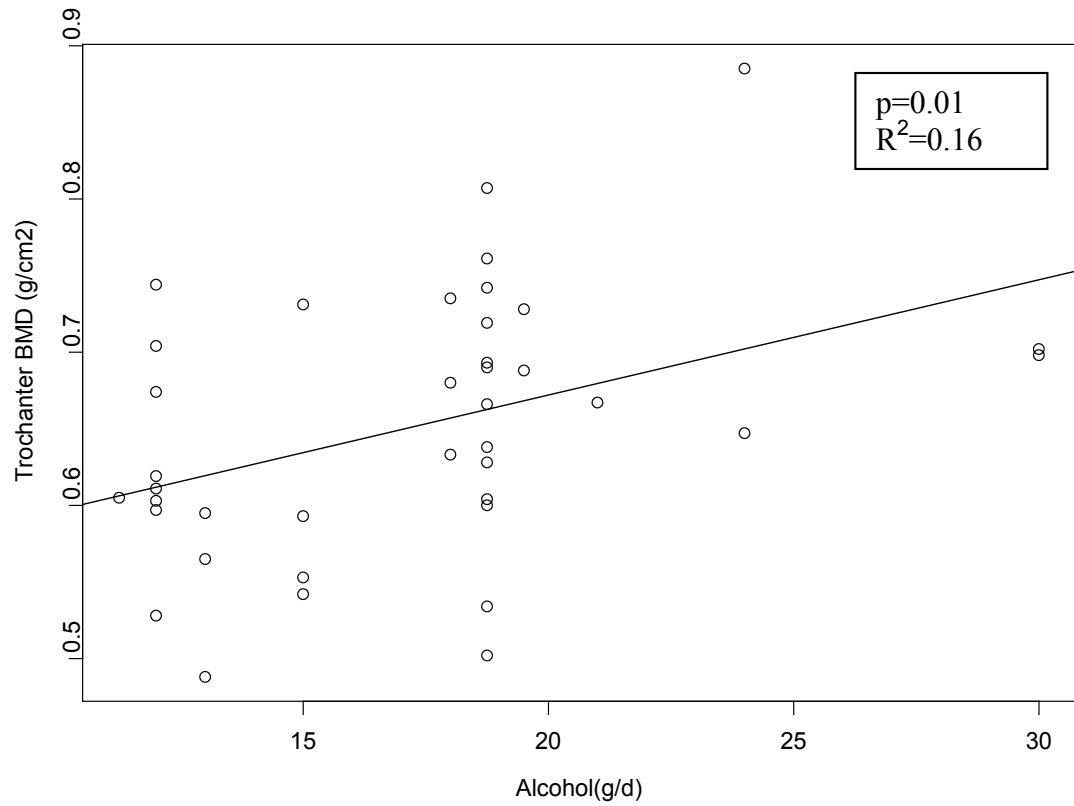


Figure 5.1. Trochanter BMD vs. baseline alcohol consumption. There was a significant association between trochanter BMD and alcohol intake at baseline.

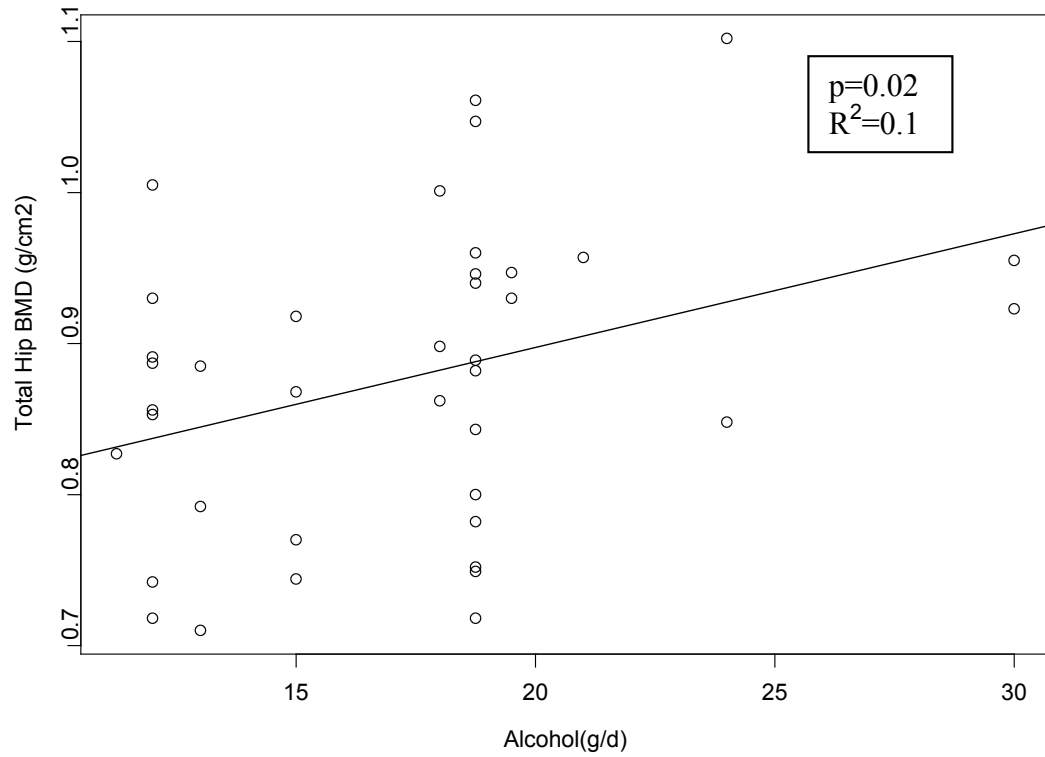


Figure 5.2. Total Hip BMD vs baseline alcohol consumption. There was a significant association between total hip BMD and alcohol intake at baseline.

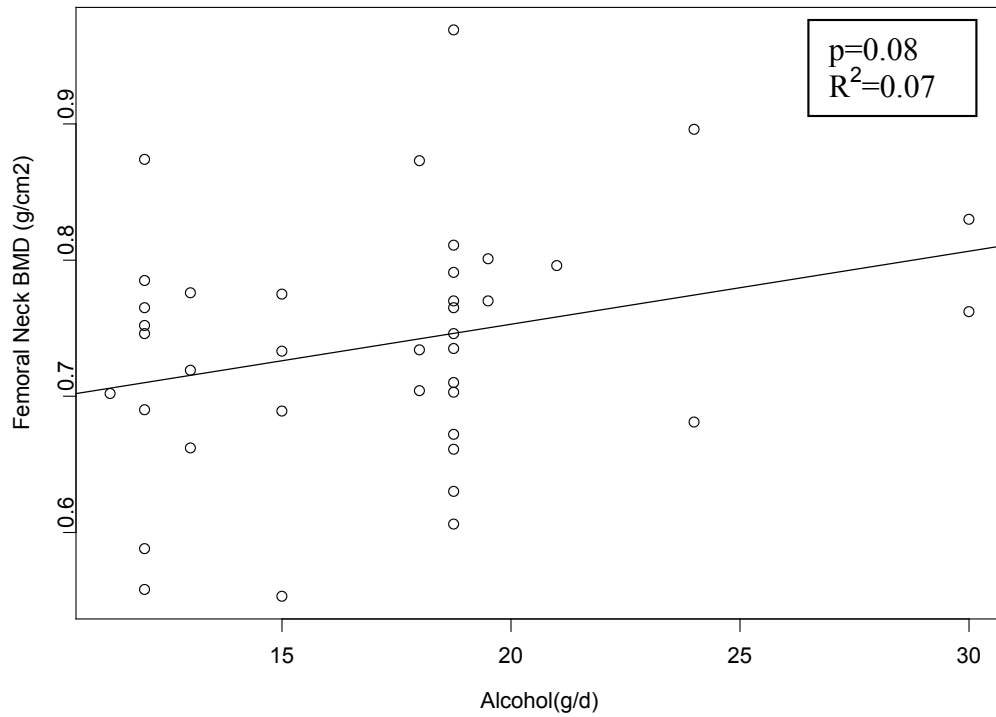
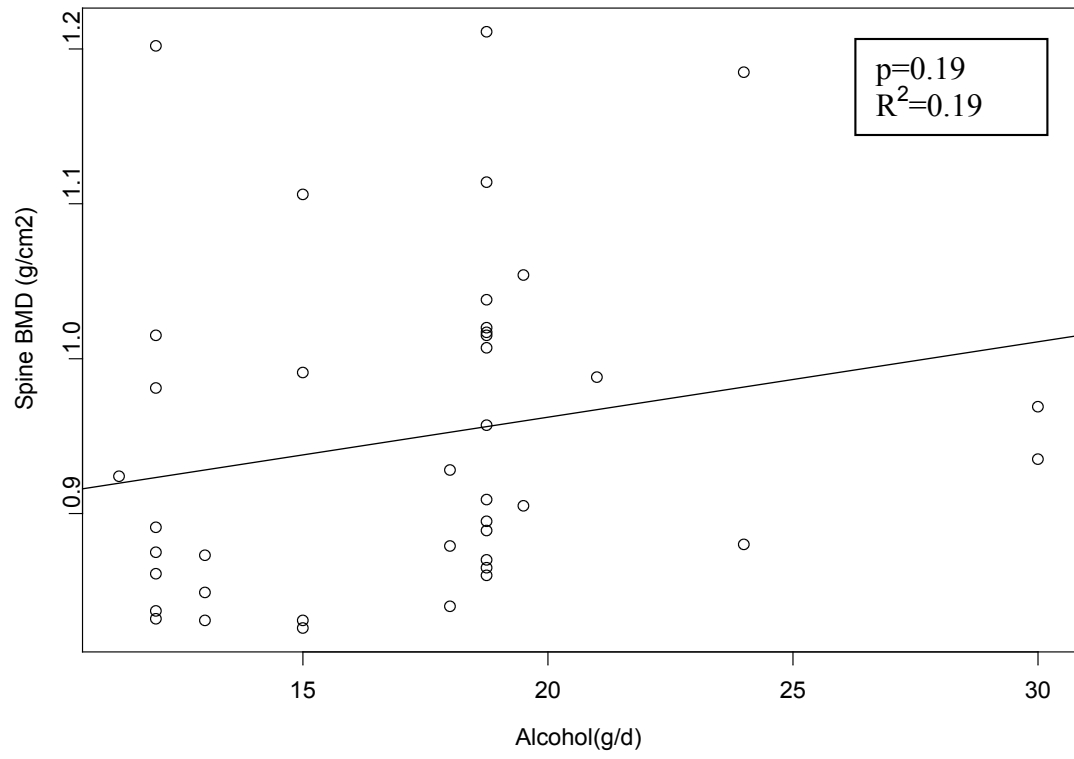


Figure 5.3. Femoral Neck BMD vs. baseline alcohol consumption. There was no association between femoral neck BMD and alcohol intake at baseline.





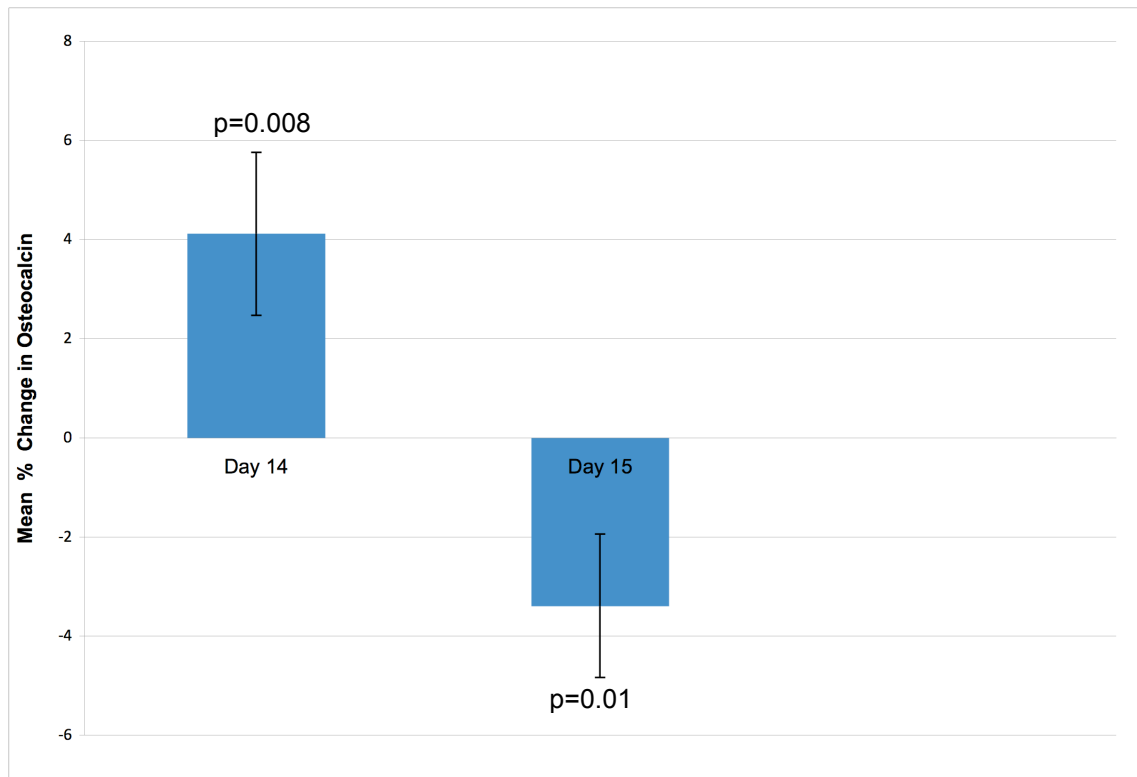


Figure 5.5. Mean % change in OC on day 14 vs. day 0 and day 15 vs. 14. OC significantly increased on day 14 vs. day 0. On day 15 vs. day 14, OC decreased significantly. Values are in means  $\pm$  SE.

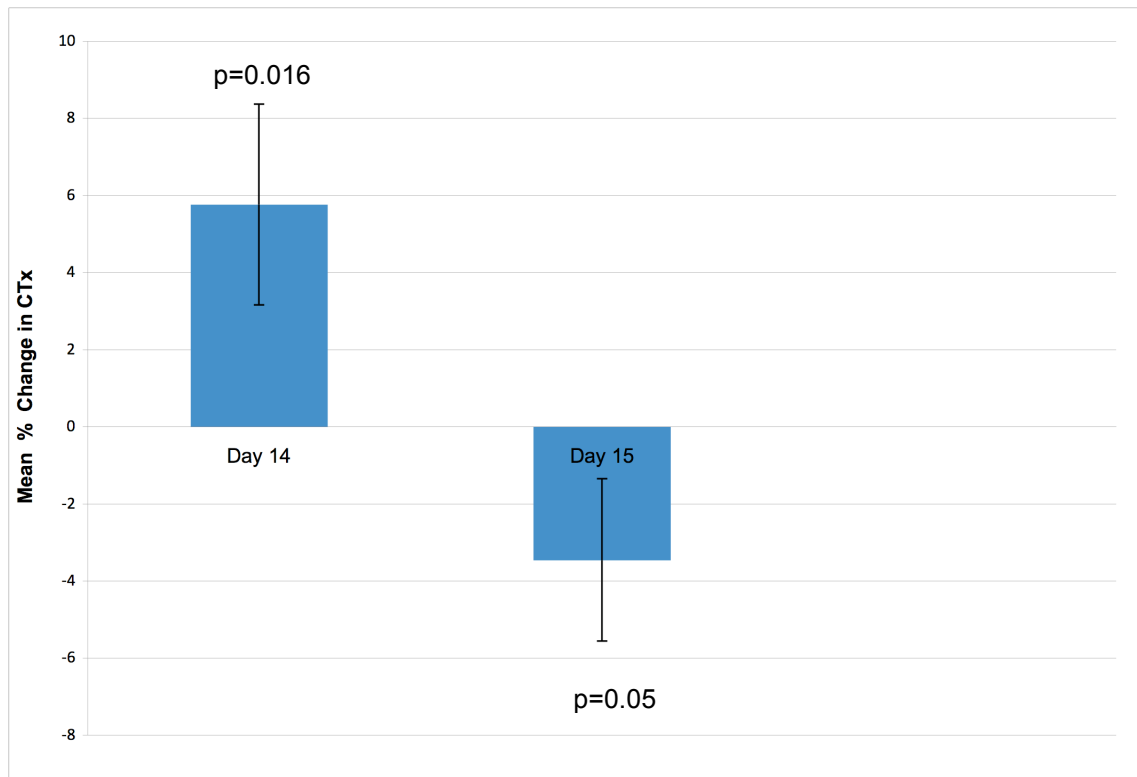


Figure 5.6. Mean % change in CTx on day 14 vs. day 0 and day 15 vs. day 14. CTx significantly increased on day 14 vs. day 0. On day 15 vs. day 14, there was a borderline significant decrease in CTx. Values are in means  $\pm$  SE.

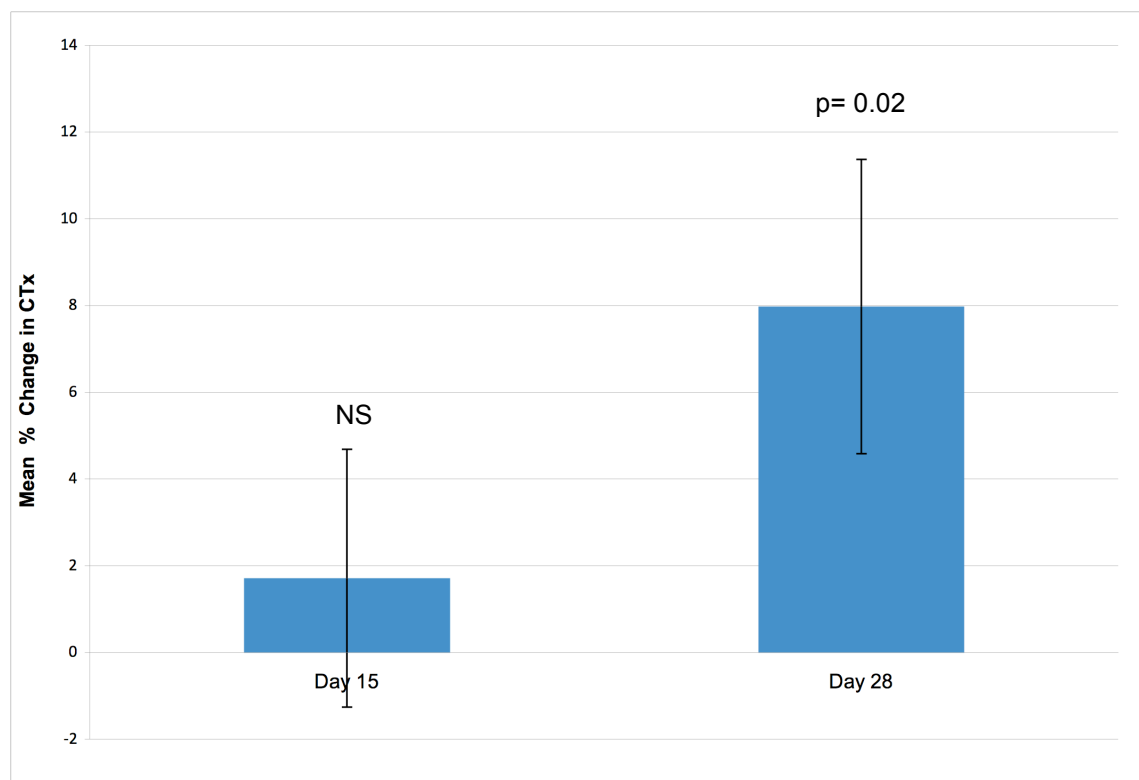


Figure 5.7. Mean % change in CTx on day 15 vs. day 0 and on day 28 vs. day 0. No significant difference on day 15 vs. day 0 in CTx. On day 28 vs. day 0, CTx significantly increased. Values are in means  $\pm$  SE. NS = not significant.

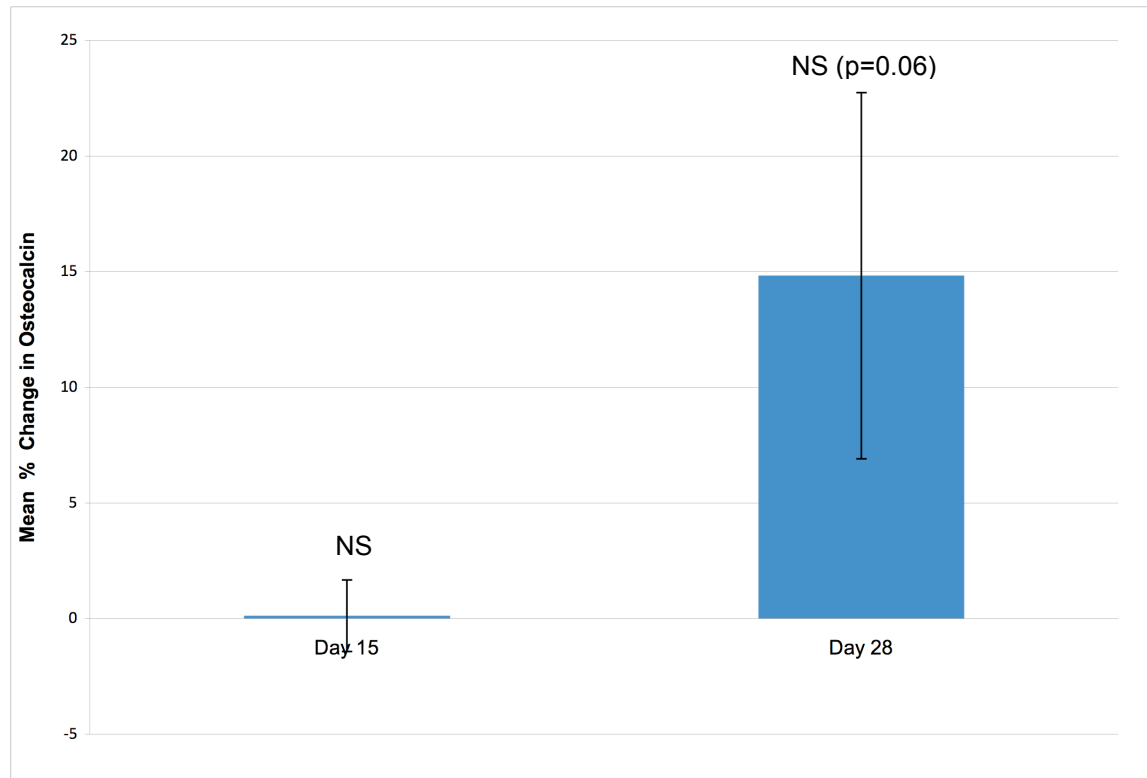


Figure 5.8. Mean % change in OC on day 15 vs. day 0 and on day 28 vs. day 0. No significant difference on day 15 vs. day 0 in OC. On day 28 vs. day 0, a strong trend for an increase in OC was detected. Values are in means  $\pm$  SE. NS = not significant.

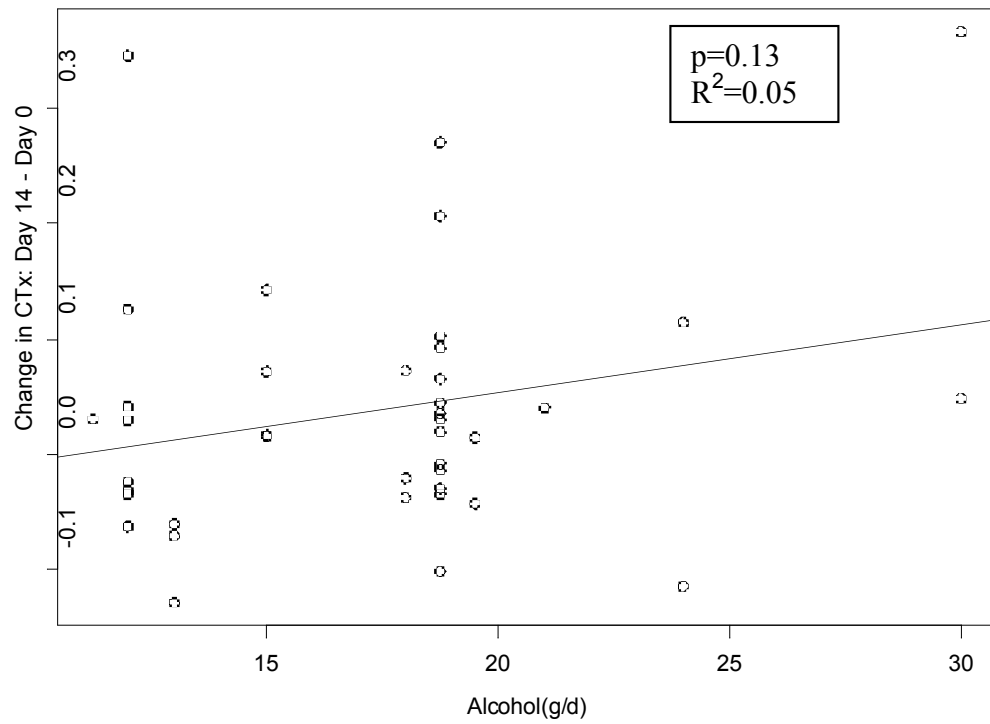


Figure 5.9. Change in serum CTx vs. alcohol in grams on day 0 vs. day 14. There was no association between the change in serum CTx from day 0 vs. day 14 with alcohol intake.

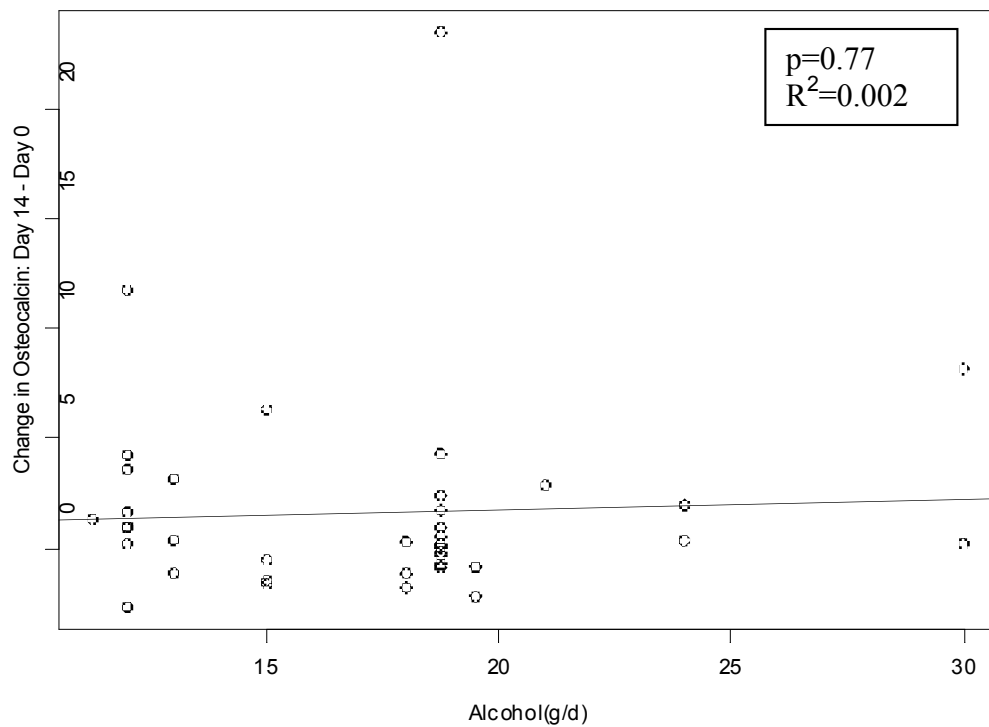


Figure 5.10. Change in serum OC vs. alcohol in grams on day 0 vs. day 14. There was no association between the change in serum OC from day 0 vs. day 14 with alcohol intake.

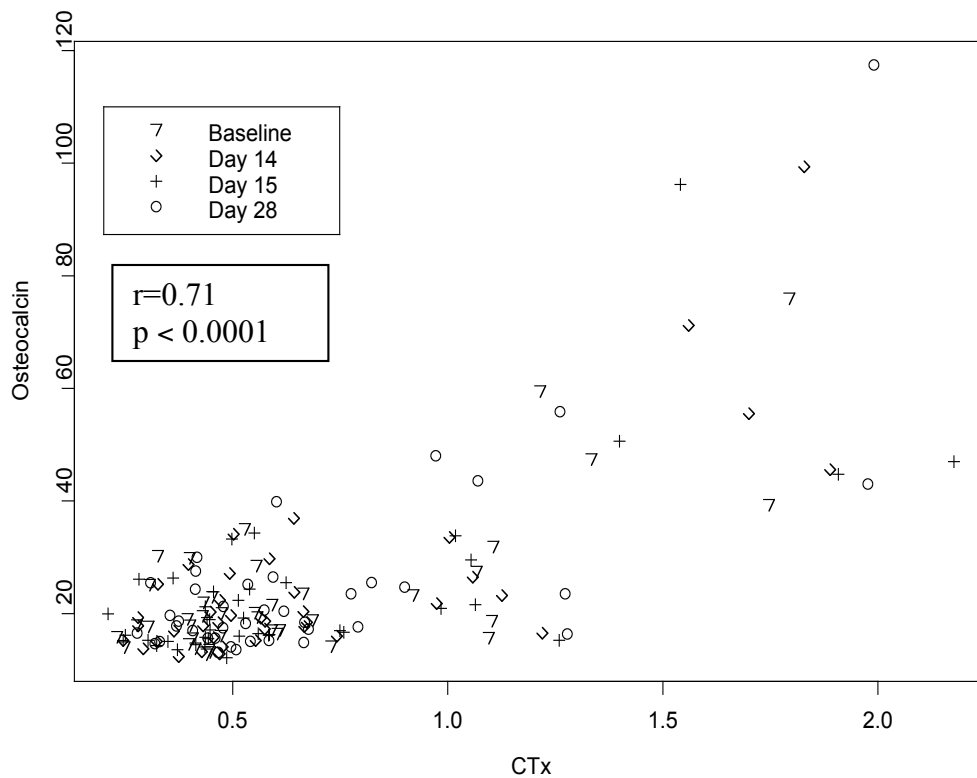


Figure 5.11 OC vs. CTx Correlation. There was a linear association between OC and CTx at all time points during the study.



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**Table 5.1 Characteristics of participants at baseline**

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n	40
Age (y)	56.3 $\pm$ 0.5
# y Postmenopausal	4.7 $\pm$ 0.4
BMI (kg/m <sup>2</sup> )	25.1 $\pm$ 0.7
Total Body Fat (%)	32.3 $\pm$ 1.3
Fat Mass (kg)	22.1 $\pm$ 1.4
Lean Mass (kg)	43.5 $\pm$ 0.7
Lean Mass + BMC	45.7 $\pm$ 0.7
Weight (kg)	67.8 $\pm$ 2.0
Height (cm)	164.5 $\pm$ 0.8

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*Data are means  $\pm$  SE*

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**Table 5.2 Dual-Energy x-ray Absorptiometry (DXA)**


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Total hip	
Bone Mineral Density (BMD) (g/cm <sup>2</sup> )	0.88 ± 0.02
T-Score	-0.54 ± 0.13
z-Score	0.22 ± 0.13
Trochanter	
Bone Mineral Density (BMD) (g/cm <sup>2</sup> )	0.65 ± 0.01
T-Score	-0.5 ± 0.14
z-Score	0.22 ± 0.13
Femoral Neck	
Bone Mineral Density (BMD) (g/cm <sup>2</sup> )	0.74 ± 0.01
T-Score	-1.00 ± 0.13
z-Score	0.12 ± 0.13
Total Lumbar Spine	
Bone Mineral Density (BMD) (g/cm <sup>2</sup> )	0.95 ± 0.02
T-Score	-0.95 ± 0.15
z-Score	0.29 ± 0.16

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*Data are means ± SE*

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**Table 5.3 Food Frequency Questionnaire & Alcohol Record Results**

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## FFQ

Kcals	1747 $\pm$ 75
Dietary Protein (g)	67 $\pm$ 4
Dietary Calcium (mg)	814 $\pm$ 46
Dietary vitamin D (IU)	128 $\pm$ 12
Supplemental Calcium (mg)	748 $\pm$ 76
Supplemental vitamin D (IU)	383 $\pm$ 41

## Alcohol Records

Alcohol Consumption (g/week)	135 $\pm$ 8
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*Data are means  $\pm$  SE*

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## Chapter 6 – Discussion

The present study examines the effect of alcohol withdrawal and subsequent restoration of alcohol consumption on bone turnover in healthy postmenopausal women. To our knowledge, this is the first study that investigated the effect of alcohol removal from the diet on bone turnover biomarkers. We found a significant increase in bone turnover markers, OC and CTx, when alcohol was excluded for two weeks from the diets of our participants who consumed between 4-14 alcoholic beverages a week. Within 24 hours of resuming alcohol consumption, OC and CTx returned to values which did not differ from baseline. However, when participants resumed unsupervised alcohol intake for two weeks, there was a significant increase in CTx (resorption) compared to baseline but no significant difference in OC (formation) compared to baseline.

Prior epidemiological research has shown an association between moderate alcohol intake and decreased bone turnover in elderly postmenopausal women compared to non-drinkers (8). Rapuri et al. reported a significantly lower concentration of serum OC in alcohol drinkers consuming between 28.6 to 142.9 g/wk of alcohol compared to non-drinkers. Urinary NTX to creatinine ratio (NTx:Cr) was also significantly lower in alcohol drinkers who consumed >28.6g/wk of alcohol compared to non-alcohol drinkers (8). The age of the subjects in the Rapuri et al. study were 65 years and older whereas

our subjects were under 65 years. Rapuri et al. compared bone turnover levels in alcohol drinkers and nondrinkers. However, the similarities of the findings suggest that there is an association between alcohol consumption and biochemical markers of bone turnover in early and elderly postmenopausal women. Our results are also consistent with an animal study conducted which found decreased bone turnover in rats consuming alcohol. The groups consuming alcohol comparable to a moderate drinker (3 and 6% of total caloric intake) had reductions in both osteoblast as well as osteoclast numbers (20).

We observed a reduction in bone turnover markers following acute administration of alcohol (12-30 g). Similar acute effects of alcohol were reported in a recent study that evaluated bone turnover markers in young to middle-aged adults (20-47 years) after short-term consumption of alcohol (62). In these studies, Sripanyakorn et al. found a significant reduction in serum CTx within 6 hours after moderate beer ingestion (27.6 g alcohol), but they did not find any significant change in OC. When ethanol alone was ingested CTx decreased, but the decrease was not as great as was seen after beer ingestion. Their results suggested that other components of beer, such as silicon or calcium, might play a role in CTx suppression in addition to ethanol (62). In our study, we observed a borderline significant ( $p=0.05$ ) reduction in CTx after alcohol withdrawal. One potential explanation for the smaller magnitude of change in our study is the type of alcoholic beverage ingested.

In our study, the majority of participants consumed red wine while only a few participants consumed white wine or beer in that 24-hour period. Additionally, Sripanyakorn et al. measured serum, urine, and plasma CTx regularly within 6 hours of alcohol and ethanol consumption (62). In our study, serum samples were measured after a longer period of time, approximately 10-12 hours after alcohol consumption. Potentially the effect of ethanol on CTx may be seen to a greater extent after a shorter period of time after alcohol administration.

There were no significant differences in OC and CTx between day 15 and day 0. These results suggest that after moderate alcohol consumption, OC and CTx levels were similar to initial values, but lower than at the end of the abstinence period on day 14.

Surprisingly, there was a significant increase in CTx and a tendency for OC to be increased from day 28 vs. baseline. We anticipated that bone turnover markers would be similar to baseline values at the end of the study. During days 15-28 of the study, participants were asked to consume their usual amount of alcohol but were not asked to record their alcohol intake. Potentially participants consumed a different quantity of alcohol than their usual consumption prior to baseline. Therefore, alcohol consumption during the last 14 days of the study was less controlled for than the first 14 days.

Diurnal variation plays an important role in bone turnover markers, especially resorption markers. Bone formation markers are less affected by diurnal variation than resorption markers, and CTx is especially susceptible to diurnal variation (19). Bone resorption has been reported to peak during early morning (63,64) with a nadir in the afternoon (63). We controlled for diurnal variation by collecting blood samples from participants between 7 and 9am.

We found that moderate alcohol consumption (9-34 g/day) at baseline was associated with BMD at the total hip and at the trochanter. These findings are similar to the EPIDOS and Framingham studies (15,65). The EPIDOS study found that moderate alcohol use, 11-29 g/day, was associated with a significant increase in trochanter BMD in elderly women, 75 years or older (15). The Framingham study also found a significant association between alcohol intake and BMD in elderly women at the trochanteric site (65). Several studies found a relationship between higher lumbar spine BMD and alcohol intake (8,14,17). Ilich et al. found that postmenopausal women who consumed approximately  $\frac{3}{4}$  of a standard drink/day had higher lumbar spine BMD (17). Feskanich et al. found that postmenopausal women between the ages of 50-74 years, who drank about one standard drink/d also had higher lumbar spine BMD than nondrinkers (14). Rapuri et al. found that elderly women who consumed between 28.6 and 57.2 g alcohol/week had higher BMD at the lumbar spine than nondrinkers (8).

Although this study demonstrated a significant change in bone turnover markers with changes in alcohol consumption, there are several potential limitations in this study that should be noted. First, it was not a randomized clinical study. Second, eligibility was based on self-reported alcohol records. Third, participant reliability in following the study protocol, such as keeping alcohol records during the first week of the study, fasting prior to blood collections, and abstaining from alcohol for a two-week period is important, and improperly following any of the directions could affect the results. Fourth, ninety-seven percent of the study population was Caucasian, and 3% was Asian. Therefore, the homogeneity of the sample with regard to ethnicity limits the generalizability and study findings to all populations of postmenopausal women under the age of 65 years. Fifth, there are several factors that affect bone turnover such as age, gender, diurnal variation, food, and disease state (19). Sixth, the study may underestimate the effect of alcohol on bone turnover markers. A two-week abstinence interval may have been too short to guarantee that prior alcohol consumption had no residual effects. Never the less, the study demonstrates that moderate alcohol consumption has measurable effects on bone metabolism.



## Chapter 7– Conclusion

Several epidemiological studies have observed higher BMD in postmenopausal women who consumed moderate amounts of alcohol (8,14-18), but few studies have investigated decreased bone turnover as a potential mechanism for this observation (8,62). Our study design was a novel way of assessing the change in bone turnover markers by looking at the change after alcohol was withdrawn from the diet of healthy postmenopausal women and then restored. Our study was not conducted in order to promote alcohol consumption, but to understand the potential link between the observed higher BMD and moderate alcohol consumption that has been found in previous studies (8,14-18).

Our study shows that the withdrawal of moderate levels of alcohol consumption increased bone turnover in postmenopausal women, and the subsequent resumption of moderate alcohol consumption reduced bone turnover. The current study provides cause and effect evidence for an association between a reduction in bone turnover with moderate alcohol consumption. These findings suggest that moderate alcohol consumption may slow bone loss by suppressing bone turnover in postmenopausal women.

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