1-1-2006

Relationship of trabecular and cortical bone to circulating total homocysteine and C-reactive protein in postmenopausal women

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by

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A thesis submitted to the graduate faculty
in partial fulfillment of the requirements for the degree of
MASTER OF SCIENCE

Major: Nutrition

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Iowa State University
Ames, Iowa
2006

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This is to certify that the master's thesis of
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REVIEW OF LITERATURE

RELATIONSHIP OF TRABECULAR AND CORTICAL BONE TO CIRCULATING TOTAL HOMOCYSTEINE AND C-REACTIVE PROTEIN IN POSTMENOPAUSAL WOMEN

LIST OF ABBREVIATIONS

BMC	Bone mineral content
BMD	Bone mineral density
BMI	Body mass index
CRP	C-reactive protein
CVD	Cardiovascular disease
DXA	Dual energy x-ray absorptiometry
Hcy	Homocysteine
HDL-C	High density lipoprotein cholesterol
HPLC	High performance liquid chromatography
IL-6	Interleukin-6
LDL-C	Low density lipoprotein cholesterol
MTHFR	Methylenetetrahydrofolate reductase
NHANES	National Health and Nutrition Examination Survey
oxLDL	Oxidized low density lipoprotein
PEPI trial	Postmenopausal Estrogen/Progestin Intervention trial
PLP	Pyridoxal 5'-phosphate
pQCT	Peripheral quantitative computed tomography
QCT	Quantitative computed tomography
SAM	S-adenosylmethionine
SD	Standard deviations
TNF-α	Tumor necrosis factor-alpha
WHI	Women's Health Initiative

ACKNOWLEDGEMENTS

This thesis would not have been possible without the support, guidance, trenchant critiques, probing questions, and the remarkable patience of my major professor Dr. D. Lee Alekel. I cannot thank her enough. I would like to gratefully acknowledge Dr. Manju Reddy for her many hours of help and expert advice during my graduate work. I am extremely grateful to Jeanne Stewart for her time and patience in teaching me invaluable laboratory techniques, trouble shooting, and for her help with experimental setup. I would also like to thank Laura Hanson for her help during the course of my graduate career and more importantly for pep talks when I needed one. I would like to acknowledge the help of Beth Westbrook for her assistance in the laboratory. I would also like to express my sincere thanks to Dr. Ulrike Genschel for her many hours in running the statistical analyses for this thesis as well as Ted Peterson's help with database management. I would like to thank my fellow graduate student Betsy Deardorff for her support and friendship. The expertise of my committee members, Dr. Manju Reddy, Dr. Kevin Schalinske, and Dr. Kenneth Koehler has been invaluable for the success of my thesis. I was especially fortunate to work with an outstanding group of researchers, including Dr. D. Lee Alekel, Dr. Manju B. Reddy, Dr. Kathy B. Hanson, Laura Hanson, Jeanne Stewart, Dr. Oksana A. Matvienko, Betsy Deardorff, and our undergraduate assistants who made my graduate career at Iowa State University an enjoyable success. They have also been my surrogate family during these past couple of years. I would like to acknowledge the help of Ann Perera, MRL Metabolomics for her expertise in the HPLC setup. My sincere appreciation is extended to each subject in the study for her cooperation and participation in our study.

Finally, I would especially like to thank my husband Phani, for his constant love and support, endless patience and for always keeping me focused. I am forever indebted to my parents, my grandfather, and my brother for their love, blessings, and their encouragement when it was most required.

ABSTRACT

Homocysteine (Hcy) and C-reactive protein (CRP) are established risk factors for atherosclerotic cardiovascular disease and are emerging as novel risk factors for osteoporosis. The primary purpose of this study was to determine whether total Hcy and CRP concentrations are associated with trabecular and/or cortical bone mineral content (BMC) or bone mineral density (BMD) in postmenopausal women. The secondary objective was to determine the body composition and nutritional status indices (dietary and circulating) and key biologic factors related to total Hcy and CRP. The tertiary purpose of this study was to examine changes in total Hcy and CRP over one year. We enrolled healthy postmenopausal women (N=242) as part of a randomized, double-blind, placebo-controlled multi-center clinical trial designed to examine the effect of two doses of soy isoflavones on bone loss over three years in early postmenopausal women. This study assessed volumetric BMD at the distal tibia and femur (1/3 site) using peripheral quantitative computed tomography (pQCT) in a subset of women (N=184 for distal tibia; N=237 for 1/3 femur site). Total Hcy and CRP did not contribute to the variability in trabecular BMC of the distal tibia or cortical BMC of the 1/3 femur site using pQCT. Approximately 22% of the variability in trabecular BMC was accounted for by weight, hemoglobin, serum uric acid, and blood glucose. Study site, weight, and age accounted for about 14% of the variability in cortical BMC. The overall variability (19%; $p \leq 0.0001$) in total Hcy was accounted for by serum vitamin B_{12} and creatinine; the overall variability (28%; $p \leq 0.0001$) in CRP was accounted for by serum iron, overall percent body fat, serum uric acid, triglycerides, and white blood cell count. Total Hcy and CRP increased, while serum vitamin B_{12}, serum folate, and intracellular folate decreased over a one year period. Total Hcy and CRP were not related to trabecular or cortical bone, but this may be because these women were healthy and non-osteoporotic. Since hemoglobin was a significant contributor to trabecular BMC, while iron was a significant contributor to CRP, it is possible that inflammation may mediate the relationship between iron and trabecular BMC.

GENERAL INTRODUCTION

Thesis Organization

This thesis begins with a general introduction describing the objectives, hypothesis, specific aims, limitations, and significance of this research project.

Objectives

The first objective of this study is to determine whether total homocysteine (Hcy) and C-reactive protein (CRP) concentrations are associated with trabecular and/or cortical bone mineral content (BMC) while taking into account key biologic factors in postmenopausal women. The second objective is to identify various factors (key biologic and nutritional status indices) related to total Hcy and CRP. The tertiary objective is to determine whether total Hcy and CRP change during the course of one year. The long-term objective is to pursue a non-pharmacologic approach to reducing disease risk in women after menopause.

Hypotheses

1) At baseline, total Hcy is inversely related to trabecular BMC in postmenopausal women, while taking into account key biologic factors.
2) At baseline, CRP is inversely related to trabecular or cortical BMC and BMD in postmenopausal women, while taking into account key biologic factors.
3) At baseline, adiposity, indices of nutritional status (dietary intake and circulating analytes), and key biologic factors are significantly related to total Hcy and CRP.
4) During the course of one year, total Hcy increases whereas CRP does not change in postmenopausal women.

Specific Aims

1) To determine whether plasma total Hcy is inversely related to trabecular BMC of the distal tibia using peripheral quantitative computed tomography (pQCT) in postmenopausal women, taking into account key biologic factors, particularly folate and vitamin B_{12} status.
2) To determine whether serum CRP is inversely related to trabecular BMC of the distal tibia and/or cortical BMC of the 1/3 femur site using pQCT in postmenopausal women, taking into account key biologic factors.

3) To identify the body composition, nutritional status indices (dietary and circulating), and key biologic factors related to total Hcy and CRP.
4) To examine whether total Hcy or CRP change over the course of one year.

Limitations

The first limitation is that we have only included for the pQCT data about half of the women from the UC-Davis site because of incorrect data acquisition. The second limitation is that there is more variability in some of the data from UC-Davis for the outcomes of interest and their corresponding contributing factors. The design of the study is to determine the effect of two doses of isoflavones (80 and 120 mg) on total Hcy concentration, taking folate and B_{12} into account. However, the third limitation of this study is that we will not be able to unblind the investigators until we have completed the intervention at both study sites in 2008. Thus, statistical analyses for the treatment effect will be completed in 2008.

Significance

The menopausal transition is characterized by a rapid decline in ovarian function and a subsequent decline in circulating hormones, including estradiol. This hormone-deficient state contributes to significant risk for developing osteoporosis (Scheiber and Rebar 1999) and CVD (Welty 2001) in postmenopausal women. Some research has related high total Hcy, common after menopause (Hak et al. 2000), to a decrease in BMD. However, no studies have examined the effect of total Hcy on trabecular and cortical bone separately. This is of primary importance as trabecular bone reflects metabolically active bone, which is preferentially lost during menopause and thus is of interest in assessing early osteoporotic risk (Takagi et al. 1995). Additionally, at the time these data were analyzed, no studies had been published on the association between CRP and bone loss associated with menopause. Similar to atherogenesis, osteoporosis has an inflammatory component, thus providing the biological plausibility for the potential use of CRP, a proinflammatory risk factor for CVD, as a marker of osteoporotic risk.

Data on the effect of soy isoflavones on total Hcy and CRP in postmenopausal women are limited and inconsistent. Published studies have examined the relationship between total Hcy and BMC and BMD, but have not distinguished between cortical and trabecular bone. Further, there are no long-term studies published that have assessed the

response of two doses of isoflavones on these two markers of CVD and osteoporotic risk (specifically trabecular bone) in postmenopausal women. This study will determine the effect of two doses (80 and 120 mg) of isoflavones on total Hcy and CRP concentrations in relation to change in trabecular bone.

REVIEW OF LITERATURE

A. Consequences of Menopause: Cardiovascular Disease and Osteoporosis

Atherosclerotic CVD and osteoporosis are major public health problems that often coexist and account for significant morbidity and mortality in postmenopausal women. The postmenopausal period typically occupies one-third of a woman's life, with more than 40 million in the United States now in the postmenopausal phase (Hargrove and Eisenberg 1995). Menopause is a transitional phase in a woman's life, where loss of ovarian function is marked by cessation of menstruation and reproductive capability (World Health Organization 1981). It is this decrease in estrogen that places postmenopausal women at increased risk of atherosclerotic CVD and osteoporosis. Although traditionally thought of as separate disease entities, accumulating evidence indicates that there are similar pathophysiological mechanisms underlying CVD and osteoporosis. In addition to age and menopause, other risk factors for CVD, such as dyslipidemia, oxidative stress, hyperhomocysteinemia, hypertension, and diabetes, have also been associated with low BMD (McFarlane et al. 2004). Thus, novel forms of therapy that effectively treat both CVD and osteoporosis hold considerable promise for the postmenopausal woman who is at an increased risk for these diseases.

In the United States, 500,000 women die each year due to atherosclerotic CVD; one out of nine women aged 45-63 years and one out of three women older than 65 years suffer from ischemic events caused by atherosclerosis (Gensini et al. 1996). The incidence of atherosclerotic CVD is much lower in premenopausal women compared to their male counterparts, suggesting that premenopausal women are somewhat protected. The rate of mortality in men from CVD is five to eight times higher than women during ages 25-55 years (Ryan 1976), with this difference narrowing considerably after menopause. This suggests that premenopausal women have protective vascular/endothelial factors that are lost after menopause (Dimitrova et al. 2002). Circulating 17β-estradiol, the most biologically active form of estrogen, declines during the menopausal transition. 17β-Estradiol selectively enhances endothelium-dependent coronary vasodilation by enhancing the bioavailability of nitric oxide (Guetta et al. 1997), a potent coronary vasodilator and a suppressor of several key

processes in atherosclerosis. 17β-Estradiol also acts as an antioxidant at the molecular level by improving the nitric oxide/free oxygen radical ratio and normalizing the expression of proatherosclerotic gene products in human endothelial cells (Wagner et al. 2001). The atheroprotective effect of estrogen is also partially attributable to its modulating effect on the lipid profile, with changes being evident in postmenopausal women. After menopause, women have significantly higher concentrations of total cholesterol, triglycerides, and low density lipoprotein cholesterol (LDL-C) (Stevenson et al. 1993) and lower concentrations of high density lipoprotein cholesterol (HDL-C). Additionally, changes in body composition with increased waist circumference and abdominal fat place menopausal women at increased risk by contributing to impaired lipoprotein profiles (Berg et al. 2004).

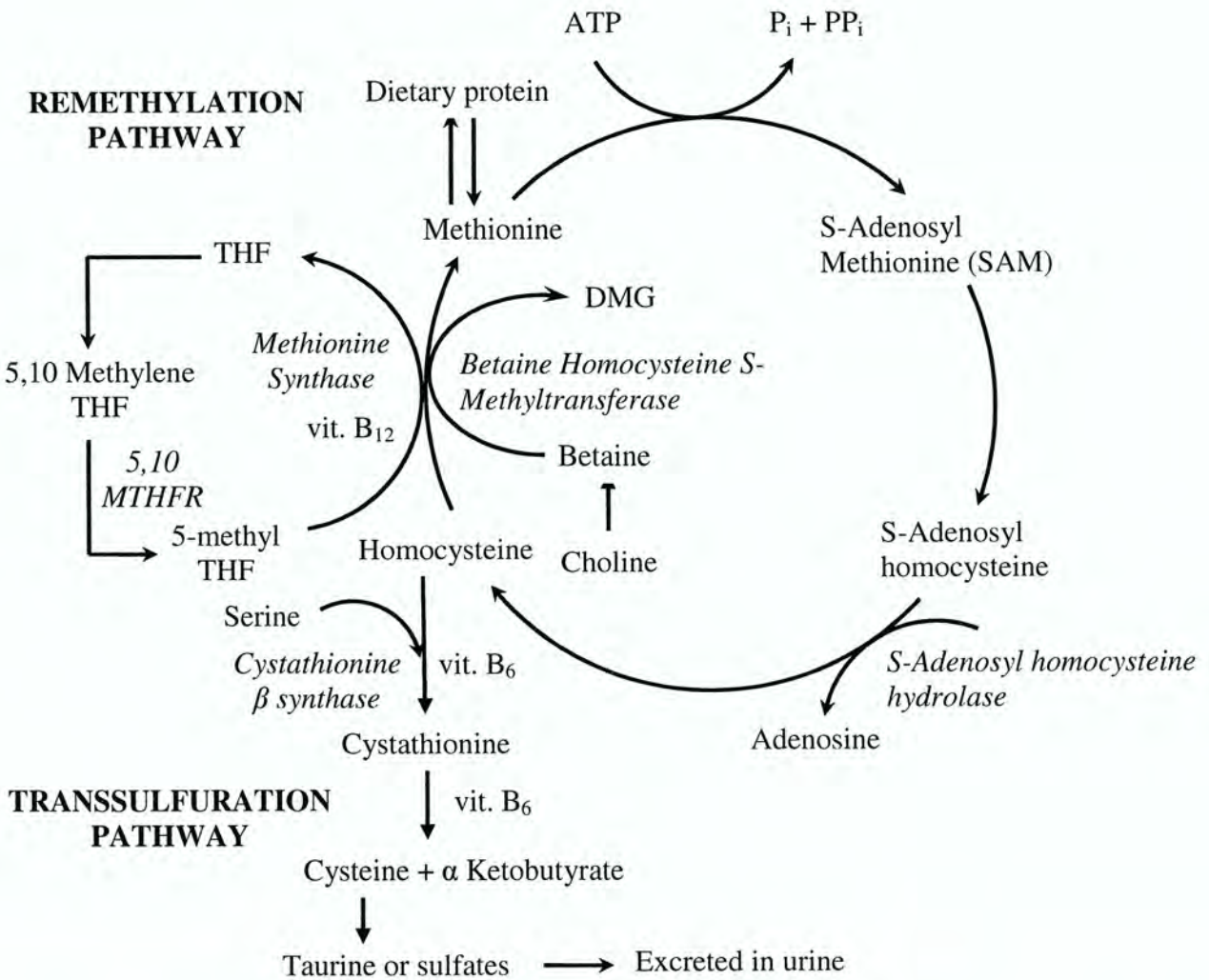
Osteoporosis and low BMD are currently a major public health threat to more than 44 million Americans aged 50 or older. Currently in the United States, 10 million people have osteoporosis and 80% of these are women (National Osteoporosis Foundation 2002). Osteoporosis is defined as a "disease characterized by low bone mass and microarchitectural deterioration of bone tissue leading to enhanced bone fragility and a consequent increase in fracture incidence" (Melton and Riggs 1983). The World Health Organization has developed an operational definition of osteoporosis based on the BMD of young adult Caucasian women (Melton 2000). The World Health Organization defines osteoporosis as a BMD less than 2.5 standard deviations (SD) below the mean for young women. The World Health Organization defines osteopenia as a BMD between 1 and 2.5 SD (also referred to as t-score) below the mean for young women. A t-score ranging from -1 SD below the mean or any value greater than this indicates normal BMD.

Menopause, which is essentially a hormone-deficient state, contributes to significant risk for developing osteoporosis in postmenopausal women (Scheiber and Rebar 1999). Bone loss occurs most rapidly during the years immediately after menopause (Ahlborg et al. 2001); in the five to seven years following menopause, women can lose up to 20% of their BMD (National Osteoporosis Foundation 2002). Several bone-protective actions exerted by estrogen include increased synthesis of $1,25(OH)_2$ vitamin D, control of bone-resorbing cytokine production, and decreased bone sensitivity to parathyroid hormone.

B. Homocysteine Metabolism

Homocysteine (Hcy) is a sulfur-containing amino acid and is a metabolite produced indirectly in the demethylation of methionine (Figure 1). Hcy is itself metabolized by two pathways: the re-methylation pathway, which regenerates methionine, and the transsulfuration pathway, which converts Hcy to cysteine and then to taurine. In the remethylation pathway, N-5-methyltetrahydrofolate or betaine donates a methyl group to Hcy to form methionine. The methionine synthase reaction with N-5 methyltetrahydrofolate is vitamin B_{12} dependent and occurs in all tissues, while the reaction with betaine occurs mainly in the liver and is vitamin B_{12} independent. After remethylation, methionine is reutilized to produce S-adenosylmethionine (SAM), a universal methyl donor. S-adenosylhomocysteine, the by-product of these trans-methylation reactions, is subsequently hydrolyzed, thus regenerating Hcy. In the transsulfuration pathway, Hcy condenses with serine to form cystathionine in an irreversible reaction catalyzed by cystathionine β-synthase, a vitamin B_6 dependent enzyme. Cystathionine is further metabolized to cysteine and α-ketobutyrate. Excess cysteine is then oxidized to taurine or inorganic sulfates and is excreted in the urine. The transsulfuration pathway effectively catabolizes excess Hcy, which is not required for methyl transfer (Selhub 1999). This pathway has a limited distribution and is found primarily in the liver, kidney, small intestine, and pancreas (Finkelstein 2000).

Several studies have shown that the Hcy metabolic pathways are nutritionally regulated. The remethylation and transsulfuration pathways are coordinated by SAM, which acts as an allosteric inhibitor of the methylenetetrahydrofolate reductase (MTHFR) and as an activator of cystathionine β-synthase enzymes. It is also thought that the utilization of SAM is regulated specifically by a reaction in which the methyl group of SAM is transferred to the amino group of glycine, forming sarcosine. This reaction is catalyzed by glycine N-methyltransferase, which is strongly inhibited by N-5-methyltetrahydrofolate polyglutamates (Selhub 1999). When dietary methionine is high, it is rapidly converted to SAM resulting in a rise in intracellular SAM concentrations. This causes (a) inhibition of MTHFR, resulting in suppressed N-5-methyltetrahydrofolate synthesis, thereby allowing full activity of glycine N-methyltransferase, and (b) activation of cystathionine β-synthase, thus increasing the rate of Hcy catabolism. Conversely, when dietary methionine is low, SAM is insufficient for



THF-Tetrahydrofolate; MTHFR-Methylenetetrahydrofolate reductase; DMG-Dimethylglycine

Figure 1: Homocysteine Metabolism

inhibition of MTHFR, resulting in increased concentrations of N-5-methyltetrahydrofolate production. These increased concentrations increase the inhibition of glycine N-methyltransferase and thereby conserve SAM and increase the availability of substrate for Hcy remethylation (Selhub 1999). Recent work in five healthy men showed that a diet containing low methionine (5 mg/kg/d; RDA for methionine = 21 mg/kg/d) and excess dietary cysteine (19 mg/kg/d; RDA for cysteine=11 mg/kg/d) reduced the transsulfuration pathway by 81% compared to a diet containing excess methionine (24 mg/kg/d). This suggests that at high methionine intakes, the methionine pool is regulated by high rates of transsulfuration. Replacement of dietary methionine with cysteine results in increased remethylation at the expense of the transsulfuration pathway (Di Buono et al. 2003).

Hcy concentrations are partly controlled by genetics. Polymorphisms in genes encoding for enzymes involved in the metabolism of Hcy may alter the factors affecting circulating total Hcy concentrations. For example, in homozygotes for the MTHFR C677T mutation, riboflavin status may affect Hcy metabolism, but only in those who have a low folate status (Jacques et al. 2002). In these individuals, approximately one to three percent of the US population, riboflavin status may be a major determinant in preventing hyperhomocysteinemia, while it does not appear to be a significant contributor in the general population. Unlike the MTHFR polymorphism, methionine synthase and methionine synthase reductase gene polymorphisms do not affect total Hcy concentrations (Jacques et al. 2003). Lifestyle modifications in populations with the different MTHFR genotype subgroups do not alter total Hcy concentrations (Husemoen et al. 2006).

C. C-Reactive Protein Metabolism

C-reactive protein (CRP) is an acute phase protein produced by hepatocytes, stimulated by systemic inflammation, and is marker of inflammation. CRP is a member of the pentraxin family of innate immune response proteins (Bassuk et al. 2004). Although it is synthesized as a monomer, the endoplasmic reticulum assembles CRP into a pentamer. In the resting state, CRP is bound to carboxylesterases in the endoplasmic reticulum of hepatocytes. Upon stimulation, binding to carboxylesterase sites is decreased and the transit time from the endoplasmic reticulum to secretion is substantially diminished. As a result of enhanced

synthesis and secretion, an acute elevation in CRP is apparent. The concentration of CRP increases from less than 1 to as high as 600-1000 mg/L during an acute phase response (Du Clos and Mold 2004). In contrast to an acute phase response, results from autopsies of 302 men and women without inflammatory conditions other than atherosclerosis, during an acute rupture of plaque, revealed that the serum CRP was 3.2 compared with 2.5 μg/mL in those who had stable plaque (Burke et al. 2002).

Although traditionally thought to be produced in the liver in response to interleukin-6 (IL-6), mounting evidence now shows that key inflammatory markers, including CRP and associated complement proteins, are synthesized in human coronary artery smooth muscle cells (Calabro et al. 2003) and atherosclerotic lesions (Yasojima et al. 2001). Recently, Meuwissen and others (2006) stated that CRP, macrophages, and oxidized low-density lipoprotein (LDL) are present in a higher proportion of plaque from patients with myocardial infarction and unstable angina compared to patients with stable angina. This suggests that these surrogate markers have important proinflammatory effects within atherosclerotic plaques. Calabro and coworkers (2005) demonstrated that human adipocytes also produce CRP after stimulation with inflammatory cytokines IL-1β and IL-6 and by a specific adipokine, resistin, thereby suggesting a link between obesity and vascular inflammation.

D. Cardiovascular Disease Risk Factors

1. Established Risk Factors

Risk factors for atherosclerotic CVD have been long established and are often known to occur in combination. Apart from age and gender, the well established risk factors for CVD are hypertension (systolic blood pressure >140 mmHg and/or diastolic pressure >90 mmHg), cigarette smoking, hypercholesterolemia (>220 mg/dL), impaired glucose tolerance (two hour plasma glucose ≥140 mg/dL and < 200 mg/dL with a glucose challenge) or diabetes (fasting blood glucose ≥126 mg/dL), hypertriglyceridemia (>150 mg/dL), and obesity (Aizawa et al. 2004; Wilson et al. 1987). According to the Adult Treatment Panel III of the National Cholesterol Education Program, the recommended LDL-C goal is <100 mg/dL in high-risk persons (patients who have coronary heart disease or underlying blood vessel disease to the brain or extremities, or diabetes, or multiple risk factors [e.g., smoking,

hypertension] that imposes a greater than 20% chance of experiencing a heart attack within 10 years; Grundy et al. 2004). The Framingham Heart Study has shown that a decline of 5 mg/dL in HDL-C increases the risk of CVD in women by 26% and in men by 21% (Wilson et al. 1987). Cigarette smoking doubles the risk of CVD in men (56%) but not in women (5%). Another risk factor, cardiac enlargement (defined as left ventricular hypertrophy as detected by an electrocardiogram) or enlarged heart as determined by a chest x-ray, imposes an increased risk of 55% and 110% in women and men, respectively. Other potentially modifiable risk factors include abdominal obesity (waist/hip ratio cut-offs of 0.90-0.95 for men and 0.83-0.90 for women); psychosocial factors; irregular consumption of fruits or vegetables (daily consumption associated with a 30% reduction in relative risk) and/or high levels of alcohol (≥3 times per week); and irregular physical activity (Yusuf et al. 2004). Most often, these established risk factors act synergistically to increase CVD risk. For example, when the three risk factors of cigarette smoking, increased systolic blood pressure, and increased cholesterol concentration are present, the risk increases by 40% in women and 100% in men (Wilson et al. 1987).

2. Homocysteine and the Cardiovascular System

A substantial proportion of CVD events occur in individuals without the established risk factors discussed above. Thus, the search for additional etiologic agents or novel risk factors still continues. Moreover, while the population-attributable risk of the major established risk factors is substantial, it is often difficult to distinguish those individuals with a moderate baseline risk who might benefit from aggressive risk reduction strategies. Therefore, additional tests for novel risk factors to assist in the prediction of CVD risk in these individuals may be warranted. In 1969, McCully was the first to propose that an elevated Hcy concentration was responsible for widespread vascular lesions in infants with hyperhomocysteinemia. He also hypothesized that moderate hyperhomocysteinemia may be causally linked to CVD (McCully 1969). It was later postulated that mild to moderate elevations of Hcy in the general population predispose individuals to atherosclerosis in a manner akin to the classic risk factors (Hackam and Anand 2003). This is of particular importance as this risk can be partially ameliorated by a simple therapy with B-vitamins. High concentration of Hcy is now recognized as an independent risk factor for CVD.

Total Hcy concentrations in postmenopausal women are 7% higher than in premenopausal women (Hak et al. 2000) due to decreased circulating estrogen. Recently, Morris et al. (2000) reported an association of high estrogen concentration with decreased mean serum total Hcy concentration, independent of nutritional status or muscle mass. Hcy concentrations are lower in pregnant and premenopausal women and in postmenopausal women who are on estrogen therapy, compared to postmenopausal women who are not on estrogen therapy. Estrogen impacts methionine/Hcy metabolism likely by interfering with the transsulfuration pathway. Estrogen prevents Hcy accumulation by enhancing the net production of glutathione, which in turn may stabilize nitric oxide, contributing to its beneficial effects (Dimitrova et al. 2002). A Canadian study (Massé et al. 2005) in healthy women found that the mean fasting plasma total Hcy concentration of the postmenopausal group was two-fold higher than that of the premenopausal group ($P<0.0001$), which remained highly significant after adjustment for confounding variables (dietary intake and blood concentrations of vitamin B_{12}, vitamin B_6, and folate). This further reiterates that the postmenopausal increase in total Hcy is due to estrogen deficiency and not due to dietary factors.

a. Epidemiologic evidence for homocysteine and cardiovascular disease

Numerous observational studies have reported on the association between Hcy concentration and CVD risk in both the general population and in those with preexisting CVD. Two meta-analyses have appeared in recent years in an attempt to summarize the evidence (Wald et al. 2002; Homocysteine Studies Collaboration 2002). Lowering Hcy concentrations by 3 μmol/L reduces the risk of ischemic heart disease by 16%, deep vein thrombosis by 25%, and stroke by 24% (Wald et al. 2002). In a meta-analysis of 30 prospective studies involving a total of 5,073 ischemic heart disease events and 1,113 stroke events, after adjustments for known CVD risk factors and regression dilution bias, authors demonstrated that a 25% lower Hcy concentration was associated with an 11% lower ischemic heart disease risk and 19% lower stroke risk (Homocysteine Studies Collaboration 2002). Proportional hazards modeling in a large population-based cohort of 1,933 elderly (mean age 70 ± 7 years; 58.9% women) participants from the original Framingham Study cohort revealed that total Hcy concentrations in the upper quartile (≥14.26 μmol/L) compared

to the lower three quartiles (<14.2 μmol/L) were associated with similar relative risk estimates of 2.18 for all-cause mortality and 2.17 for CVD mortality. Adjustments for various risk factors attenuated these associations but they remained significant (Bostom et al. 1999).

Data from the third National Health and Nutrition Examination Survey (NHANES) III, a nationally representative survey of US adults (N=3,173 in this analysis), revealed that there was a two-fold increase in the likelihood of myocardial infarction among persons with a total Hcy concentration of ≥15 μmol/L and this association did not differ by race or ethnicity (Giles et al. 2000). The Physician's Health Study involving 14,916 male physicians, aged 40-84 years revealed that hyperhomocysteinemia was associated with increased prospective risk of myocardial infarction (Stampfer et al. 1992). In a study of 941 adolescents aged 12-16 years in the NHANES III trial, serum total Hcy was significantly and positively associated with blood pressure in boys (beta=0.21, P=0.03). After adjustment for confounders, parental history of high blood pressure or stroke before age 50 was very strongly associated with the subjects' serum total Hcy (beta=0.16, P=0.003) (Gillum 2004).

Although there is an established link between increased Hcy and atherosclerotic CVD risk, relatively few studies have been conducted on the effect of Hcy-lowering therapy in decreasing risk in patients with CVD. An interesting study by Bonaa et al. (2006) showed that Hcy-lowering treatment with folic acid, vitamin B_6, and vitamin B_{12} in 3,749 men and women who had experienced an acute myocardial infarction was not effective and in fact there was a trend toward increased risk. However, a recent study by Yang et al. (2006) revealed that there was an improvement in stroke mortality across all population strata from 1998 to 2002 (overall change from -0.3% to -2.9% per year; P=0.0005) after folic acid fortification in the United States, but not in England and Wales where there was no folic acid fortification. This is consistent with the hypothesis that folic acid fortification helps to reduce deaths from stroke by reducing Hcy concentrations.

b. Pathogenesis of hyperhomocysteinemia

Hyperhomocysteinemia or increased concentration of Hcy could be due to defects in genes encoding for the enzymes involved in Hcy metabolism (Table 1). Homocystinuria due to cystathionine β-synthase deficiency, inherited as an autosomal recessive trait, is the most prevalent inborn error of methionine metabolism (Kluijtmans et al. 1999). Homozygotes with

cystathionine β-synthase deficiency have severe hyperhomocysteinemia with Hcy concentrations ranging from 100-500 μmol/L (Yang et al. 2005). A common polymorphism in the gene coding for the 5,10-MTHFR (C677T, Ala → Val) is associated with a decreased activity of the enzyme due to thermolability (Cortese and Motti 2001).

Table 1: Classification of Hyperhomocysteinemia

Severe hyperhomocysteinemia
High total Hcy concentrations at all times; caused by deficiencies in CBS, MTHFR, or enzymes of B_{12} metabolism
Mild hyperhomocysteinemia
Moderately high total Hcy concentrations under fasting conditions; reflects impaired Hcy methylation (folate, B_{12}, or moderate enzyme defects such as thermolabile MTHFR)

Adapted from Selhub (1999); CBS – Cystathionine beta synthase, MTHFR – methylenetetrahydrofolate reducatse

The effect of MTHFR thermolability on total Hcy concentration is attenuated in premenopausal females and is not significant in subjects who exhibit serum concentrations of folate and/or vitamin B_{12} above the 50^{th} percentile of the general population (Cortese and Motti 2001). Kang et al (1993) demonstrated that MTHFR thermolability is present in 17% of patients with established coronary artery disease, but it is not associated with other coronary risk factors such as age, sex, diabetes, smoking, hypercholesterolemia, or hypertension. Data from 293 Physicians' Health Study participants who developed myocardial infarction through eight years of follow-up and 290 control subjects showed that MTHFR polymorphism was not associated with risk of myocardial infarction but with a higher Hcy concentration (Ma et al. 1996). However, in a case-control comparison of 711 vascular disease cases and 747 controls from nine European countries, there was a strong graded association between Hcy and vascular risk in all MTHFR genotypes (CC and TT). MTHFR genotype is a key determinant of plasma total Hcy concentrations (Meleady et al. 2003).

In addition to polymorphisms in genes encoding enzymes involved in Hcy metabolism, the less severe forms of hyperhomocysteinemia could also be due to deficiencies in vitamins involved in the Hcy metabolic pathway (Table 1) (Selhub 1999). Ebbesen and colleagues (2006) found that hyperhomocysteinemia due to folate deficiency caused changes in the gene expression of buffy coat cells, characterized by increased platelet activation and impaired fibrinolysis. This mechanism explains the increased risk of hyperhomocysteinemic patients for thrombosis. In a rat model, folate deficiency increased hepatic glycine N-methyltransferase activity and decreased liver SAM (Uthus et al. 2006). Several other studies demonstrated that hyperhomocysteinemia can be ameliorated by folic acid supplementation (Huemer et al. 2005; Racek et al. 2005), while others induced hyperhomocysteinemia in experimental animals by feeding a folate deficient diet (Yeh et al. 2006; Ebbesen and Ingerslev 2005; Zhang et al. 2004).

c. Purported mechanisms for homocysteine in atherogenesis

Elevated circulating Hcy is an independent risk factor for atherosclerotic CVD and has an effect over and above that of inflammatory markers and the major cardiovascular risk factors (Woodward et al. 2006). A large body of research has demonstrated several different biological mechanisms by which hyperhomocysteinemia may be pathologic. Although several mechanistic studies have demonstrated significant effects caused by hyperhomocysteinemia, several plausible mechanisms have been hypothesized with five major ones elaborated upon below.

1) Smooth muscle cell proliferation: In the mid 90's, Tsai and colleagues (Tsai et al. 1994) demonstrated in rat aortic smooth cells that Hcy concentration of 100 μmol/L and 1000 μmol/L caused DNA synthesis to increase by 25% and 450%, respectively. In contrast, elevated total Hcy caused a dose-dependent decrease in DNA synthesis in human umbilical vein endothelial cells. This growth-promoting effect of Hcy on vascular smooth muscle cells, together with its inhibitory effect on endothelial cell growth, represents an important mechanism to help explain Hcy-induced atherosclerosis. High Hcy concentrations (>100 μmol/L) increase vascular smooth muscle proliferation by increasing the synthesis and release of matrix metalloproteinase-2, which is integrally involved in vascular remodeling and atherosclerotic plaque destabilization (Doronzo et al. 2005). Cell

culture work in rat vascular smooth muscle cells revealed that extracellular folic acid (Guo et al. 2006) and copper, but not zinc (Guo et al. 2005), decreased Hcy-induced synthesis of matrix metalloproteinase-2 and thus may have a beneficial effect on the pathogenesis of atherosclerosis. Qureshi and coworkers (2005) have added that N-methyl D aspartate receptors typically found in rat cerebrovascular cells and involved in Hcy uptake also exist in vascular smooth muscle cells and appear to mediate, at least in part, Hcy-induced dysregulation of vascular smooth muscle cell function.

2) Endothelial cell damage and dysfunction: Wang et al. (1997) found that 10-50 μmol/L Hcy but not cysteine inhibited DNA synthesis in vascular endothelial cells and arrested their growth. Later, Zhang and coworkers (Zhang et al. 2001) showed that Hcy induced apoptosis in human umbilical vein endothelial cells; the apoptosis was specific to Hcy and was not mediated by oxidative stress. In patients with hyperhomocysteinemia, there is a decreased number and activity of endothelial progenitor cells, which are involved in ongoing endothelial repair (Junhui et al. 2006). This decrease is due to accelerated senescence of endothelial progenitor cells through telomerase inactivation (Zhu et al. 2006). In a mouse model of carotid injury, Hcy had a profound inhibitory effect on endothelial cell proliferation and migration at physiologically relevant concentrations. Also, Hcy inhibited endothelial cell adhesion at concentrations of ≥200 μmol/L, causing impaired reendothelialization and increased neointimal formation in severely hyperhomocysteinemic mice (Tan et al. 2006). The capacity of Hcy to inhibit proliferation and migration of endothelial cells may be responsible for impaired reendothelialization and healing, contributing to atherosclerosis in hyperhomocysteinemia. Results from Hcy-treated mouse and human aortic endothelial cells suggest that hyperhomocysteinemia impairs endothelial function and endothelial nitric oxide synthase activity, primarily through protein kinase C activation (Jiang et al. 2005).

3) Monocyte activation: Cell culture work in primary human monocytes (Zeng et al. 2003) and human aortic endothelial cells (Poddar et al. 2001) revealed that elevated Hcy concentrations (>10 μmol/L) increase protein secretion, mRNA expression, as well as activity of monocyte chemoattractant protein-1 and IL-8, chemokines found in atheromatous plaques. Homocysteine contributes to CVD risk through monocyte

chemoattractant protein-1 by increasing monocyte chemotaxis into the intima of the arterial wall (Sung et al. 2001).

4) Reactive oxygen species/oxidative stress: Recent work in human umbilical vein endothelial cells has shown that the production of reactive oxygen species under hyperhomocysteinemic conditions may induce a proinflammatory situation in the vessel wall that initiates and promotes atherosclerotic lesion development (Postea et al. 2006). Incubation of endothelial cells with 100 μmol/L Hcy produced a marked elevation of superoxide anion in these cells (Au-Yeung et al. 2004) and was regulated by the phosphorylation of two subunits of NADPH oxidase (Siow et al. 2006). Although oxidative stress is suggested as one of the pathophysiologic conditions underlying the association of total Hcy with CVD, work by Huerta et al. (2004) in 123 healthy elderly subjects revealed no significant differences in the antioxidant enzymes glutathione peroxidase or superoxide dismutase. Also, there were no differences in serum lipid-soluble antioxidants or a marker of lipid peroxidation between subjects with elevated plasma total Hcy (≥15 μmol/L) as compared to those with lower plasma total Hcy.

5) Vasodilation: Recent work in perfused coronary arteries of rats suggests that adenosine plays a role in both the negative inotropic and vasodilatory actions of Hcy (Kennedy et al. 2006). Supplementation with B_6, B_{12}, and folate reduced plasma Hcy in elderly patients with heart failure and decreased mean arterial blood pressure and pulse rate. These results suggest that B vitamin supplementation may improve the vasodilatory capacity in patients with underlying disease (Andersson et al. 2005).

d. Factors affecting homocysteine concentration

Epidemiological data from the NHANES III showed that total Hcy was positively correlated with male sex, age, race-ethnicity (higher in non-Hispanic whites than Mexican Americans), serum creatinine, systolic blood pressure, BMI, hard-liquor consumption, and smoking, but inversely associated with vitamin/mineral supplement use, serum folate, intracellular folate, and serum vitamin B_{12} (Ganji and Kafai 2003). The Hordaland Homocysteine Study examined whether changes in lifestyle influence total Hcy over time in a population-based, prospective study conducted in 7,031 subjects from western Norway. Changes in plasma vitamin status and vitamin supplement use, quitting smoking, weight

changes, baseline history of CVD or hypertension, and cardiovascular events during follow-up were significantly associated with changes in total Hcy (Nurk et al. 2004). In a multivariate analysis of lifestyle determinants of plasma total Hcy concentration in the Hordaland Homocysteine Study, sex, age, folate intake, smoking status, and coffee consumption emerged as the strongest determinants and were associated with a high median total Hcy concentration and a pronounced skewness toward high total Hcy values (Nygard et al. 1998). Alcohol consumption (30 ml ethanol/day) was associated with an odds ratio of 2.79 for hyperhomocysteinemia in a population of 974 middle aged Japanese men (Sakuta and Suzuki 2005).

In a cross-sectional study of Hispanic and non-Hispanic elders, high fruit and vegetable consumption was related to lower total Hcy and CRP concentrations (Gao et al. 2004), suggesting that lower total Hcy is associated with particular nutrients, such as folate and fiber. Contrary to the popular belief that a vegetarian diet protects against CVD, a Taiwanese study (Su et al. 2006) showed that plasma total Hcy and soluble vascular adhesion molecule concentrations were significantly higher in healthy postmenopausal vegetarians, placing these vegans at an increased risk for atherosclerotic CVD. However, further multivariate analysis revealed that age and systolic blood pressure, but not vegetarianism per se, as determinants of common carotid artery intima media thickness (Su et al. 2006). Multivariate analysis of dietary and lifestyle factors revealed that intakes of monounsaturated fatty acids, fiber, calcium, magnesium, folate, and vitamins A, E, C, B_1, and B_6 were positively associated with serum folate concentration in a population of healthy Greek adults (n=486) (Hatzis et al. 2006). Intake of betaine-rich foods such as wheat, spinach, shellfish, and sugar beets (Zeisel et al. 2003) possibly decreases plasma total Hcy. In the Framingham Offspring Study, higher intakes of dietary choline and betaine were related to lower total Hcy independent of other determinants, including folate and other B vitamins (Cho et al. 2006). In a cross-sectional study using data from NHANES (1999-2000), total Hcy concentration was significantly lower in those consuming fortified cereals (Song et al. 2005a), suggesting that the universal fortification of grains with folic acid effectively reduced total Hcy concentration.

Since the B vitamins riboflavin, B_6, and B_{12} are involved as coenzymes in the Hcy metabolic pathway, multivitamin supplementation effectively reduces Hcy. Multivitamin supplementation with physiological doses of B vitamins reduced total Hcy in healthy elderly women (Wolters et al. 2005). A single oral dose of betaine lowered plasma Hcy within two hours in healthy subjects (Schwab et al. 2006), as betaine donates its methyl group to Hcy to convert it back to methionine. Folic acid supplementation increases betaine concentrations (Melse-Boonstra et al. 2005) and thus indirectly reduces Hcy. Folic acid also reduces Hcy directly as evidenced by the decrease in Hcy concentration after the universal fortification of grains with folic acid.

3. C-Reactive Protein and Cardiovascular Disease Risk

C-reactive protein has received substantial attention in recent years as a promising biological predictor of atherosclerotic CVD. This stems in part from a recent shift in thinking about the pathogenesis of CVD, a disease once considered to be primarily a lipid storage disease. Inflammation is now widely accepted as a central etiologic factor in the development and progress of CVD (Ross 1999). Local inflammatory processes may trigger the occurrence of vascular events by mediating plaque instability. An evolving body of work suggests that even a small increase in CRP within the normal range is predictive of future vascular events in apparently healthy, asymptomatic individuals (Hackman and Anand 2003). Prospective data have shown that CRP is a stronger predictor of CVD than LDL-C. CRP is only minimally associated with LDL-C (Ridker et al. 2002) and cannot be predicted from total cholesterol concentration (Ridker 2003a). CRP adds to the predictive value of lipid parameters in determining the risk of first myocardial infarction (Ridker et al. 1998). To differentiate among patients at low, moderate, and high risk of future cardiovascular events, the corresponding CRP values are less than 1, 1-3, and greater than 3 mg/L, respectively (Ridker et al. 2003b).

a. Epidemiologic evidence for C-reactive protein

Epidemiological data from adults (N=4,900) in the NHANES III revealed that there was a significant and more robust relationship between concomitant elevations of CRP plus Hcy and a history of myocardial infarction, heart failure, and any CVD event compared to an elevation in only one of these biomarkers (Cummings et al. 2006). According to some

authorities, measuring CRP alone better predicts a myocardial infarction than measuring total cholesterol or HDL-C (Ridker et al. 1998). Results from 15,745 women enrolled in the Women's Health Study revealed that CRP was a stronger predictor of future cardiovascular events than LDL-C (Ridker et al. 2002). Similar results were noticed in the Physician's Health Study where CRP and the total cholesterol/HDL-C ratio emerged as the strongest independent predictors of peripheral arterial disease (Ridker et al. 2001).

Results from 6,051 men and women in Finland showed that both CRP and tumor necrosis factor-alpha (TNF-α) were significant, independent predictors of coronary heart disease and CVD events and total mortality among men (Tuomisto et al. 2006). Findings from this study provided further support for the role of inflammation in the pathogenesis of CVD. However, contrary to the above findings, results from the Framingham study showed that elevated CRP concentration provided no further prognostic information beyond that of traditional risk factor assessment to predict major cardiovascular events (Wilson et al. 2005). Similarly, in very elderly men (mean age=75 years) from the same study, CRP was not associated with CVD. Further, nonsteroidal anti-inflammatory drug use did not influence cytokine or CRP concentration in this population (Haider et al. 2004). In a prospective, nested, case-control analysis involving 97 cases of sudden cardiac death among apparently healthy men enrolled in the Physician's Health Study, CRP but not Hcy or lipid concentration, was significantly associated with risk of sudden cardiac death (Albert et al. 2002). In the Rotterdam study, CRP was found to be positively associated with the severity of atherosclerosis (van der Meer et al. 2002), further lending evidence to the role of inflammation in atherosclerosis.

b. Purported mechanisms for C-reactive protein

As we now understand atherosclerosis to be an inflammatory process, much data are evolving to suggest that CRP promotes atherogenesis. CRP is detectable in the early stages of plaque formation and is thus believed to be an integral part of the atherogenic process, facilitating events from the initial recruitment of leukocytes to the arterial wall, to the eventual rupture of the plaque (Ridker et al. 2003b). The broad mechanisms underlying the effect of CRP on atherosclerotic CVD risk are elaborated upon below.

1) Endothelial dysfunction: The endothelium is a single cell protective lining for blood vessels, valves, and numerous body cavities. A balance between the relaxing and contracting factors secreted by the endothelium results in vascular homeostasis (Verma and Anderson 2002). Endothelial cell dysfunction, an early and central event in lesion formation, results in vasoconstriction, leukocyte adherence, platelet activation, mitogenesis, pro-oxidation, thrombosis, impaired coagulation, vascular inflammation, and atherosclerosis (Verma and Anderson 2002). Although traditionally thought to be a passive downstream marker in the inflammatory process, a growing body of evidence implicates CRP as a direct mediator of endothelial dysfunction. CRP induces the expression of adhesion molecules, such as intercellular adhesion molecule-1, vascular cell adhesion molecule-1, and E-selectin in human endothelial cells (Pasceri et al. 2000). Additionally, culturing human umbilical vein endothelial cells with CRP has revealed an increase in production of another adhesion molecule, chemokine monocyte chemoattractant protein-1, further strengthening the role of CRP in the pathogenesis of vascular inflammation (Pasceri et al. 2001). CRP causes a marked and sustained increase in native LDL uptake by macrophages through increased secretion of endothelium-derived vasoactive factor endothelin-1 and the inflammatory cytokine IL-6 (Verma et al. 2002a). The number of circulating endothelial progenitor cells is positively associated with endothelial function and inversely associated with cardiovascular risk (Hill et al. 2003). When endothelial progenitor cells are cultured with CRP, there is inhibition of cell differentiation and survival, partly mediated through reduced endothelial nitric oxide synthase expression produced by endothelial progenitor cells (Verma et al. 2004).

A widely accepted definition of endothelial dysfunction is a reduction in endothelial nitric oxide, which may result from reduced expression of endothelial nitric oxide synthase (Moncada and Higgs 2006). Nitric oxide is known for its vasodilatory and platelet inhibitory actions and was found to inhibit vascular smooth muscle proliferation (Moncada and Higgs 2006). Less endothelial nitric oxide synthase activity reduces the bioavailability of nitric oxide, resulting in inhibition of vasodilatation, stimulation of LDL oxidation (oxLDL), smooth muscle proliferation, and monocyte adhesion (de Maat and Trion 2004). CRP causes a marked downregulation of endothelial nitric oxide synthase (Venugopal et al. 2002). This

occurs in part through posttranscriptional effects of endothelial nitric oxide synthase on mRNA stability (Verma et al. 2002b), thereby resulting in monocyte adhesion (Venugopal et al. 2002; Mineo et al. 2005). Numerous studies have shown that oxLDL plays a pivotal role in endothelial dysfunction. Li et al (2004) demonstrated that CRP increased human monocyte adhesion to endothelial cells and oxLDL uptake by these cells *in vitro*, both through increased expression of lectin-like oxLDL receptor-1.

2) Monocyte recruitment: CRP seems to play a key role in monocyte recruitment, infiltration into the vessel wall, and finally formation of foam cells. Cell culture work in human endothelial cells showed that CRP upregulated the IL-8 gene in a time- and dose-dependent pattern and also enhanced monocyte adhesion to the endothelial cell layer (Wang et al. 2005). Inhibition of IL-8 significantly decreased (31%) monocyte-endothelial cell adhesion induced by CRP (Devaraj et al. 2004). CRP promoted monocyte chemoattractant protein-1 mediated chemotaxis receptor-2 expression in human monocytes (Han et al. 2004). Monocytes trafficked to the site of an atherosclerotic lesion may take up excess lipid, transform into foam cells, and be incorporated into the atheroma itself. CRP is chemotactic for freshly isolated human blood monocytes and CRP deposition precedes the appearance of monocytes in early atherosclerotic lesions (Torzewski et al. 2000). Moreover, monocytes have a receptor for CRP, which appears to be important in binding CRP and monocytes being taken up by an atherosclerotic lesion (Torzewski et al. 2000). Additionally, elevated blood CRP concentration increases the expression of a dominant chemokine receptor in monocytes, CC chemokine receptor 2, at both the protein and transcript levels. This receptor in turn enhances chemotaxis mediated by monocyte chemoattractant protein-1 up to two-fold (Han et al. 2004), thus playing a pivotal role in atherosclerotic plaque formation.

3) Complement activation: Complement activation in early atherosclerotic lesions is in part mediated by CRP in the atherosclerotic intima and may be interpreted as evidence for CRP-mediated complement activation in the arterial wall (Torzewski et al. 1998). Human CRP and complement activation are key mediators of ischemic injury (Griselli et al. 1999). A very important property of CRP is its ability to bind to the complement to form complement-CRP complexes and activate the classical

complement cascade (Marnell et al. 2005; Wolbink et al. 1996). Elevated concentration of the CRP-complement complexes is noted in clinical samples, indicating that CRP may induce activation of the complement *in vivo* (Wolbink et al. 1996). Griselli et al. (1999) showed that there was a 40% increase in infarct size in rats injected with CRP, suggesting that CRP and complement activation are major mediators of ischemic myocardial injury. Complement activation by CRP may increase CRP binding in the developing plaque by exposing more CRP binding sites on complement-attached smooth muscle cells (Li et al. 1994).

4) Thrombosis: CRP has been recently suggested to directly contribute to CVD by inducing a prothrombotic state. CRP inhibits the activity of tissue plasminogen activator via generation of proinflammatory cytokines (IL-1 beta and TNF-α) and is thus a procoagulant having implications for atherothrombosis (Singh et al. 2005). CRP also increases the expression and activity of the main inhibitor of fibrinolysis, plasminogen activator inhibitor-1, in human aortic endothelial cells (Blann et al. 2003), thus promoting atherothrombosis. Nakakuki et al. (2005) demonstrated that the Rho/Rho-kinase signaling pathway, a pathway integrally involved in the development of atherosclerosis and in signal cascades related to inflammation, is responsible for increased plasminogen activator inhibitor-1 expression in bovine aortic endothelial cells. Danenberg and coworkers (2003) observed that in human-CRP transgenic mice (mice carrying a fragment of human genomic DNA consisting of the CRP gene), 28 days after transluminal injury, 75% of the femoral arteries were occluded compared to 17% in the wild type. Thus, CRP is not merely a marker of atherosclerosis, but also a mediator, as it contributes to underlying lesion formation, plaque rupture, and coronary thrombosis (Verma and Yeh 2003).

c. Factors affecting C-reactive protein concentration

With both high and low levels of inflammation, CRP concentrations vary dramatically with diet, lifestyle, genetics, and disease state. In Spanish women, multiple regression analysis revealed that body mass index (BMI), triglycerides, and fasting glucose were independent predictors of CRP in women and together explained 42% of its variance. In Spanish men, CRP was independently and positively associated with waist circumference, smoking, diastolic blood pressure, uric acid, and triglycerides, and negatively associated with

HDL-C (r= -0.51) (Garcia-Lorda et al. 2006). Hoekstra et al. (2005) reported similar associations with BMI, serum insulin, and CRP. However, these relationships weakened in overweight women and lost significance, suggesting that the role of CRP in metabolic syndrome is modified by the amount of body fat. Excess body weight and body composition have strong effects on CRP concentrations. Measures of both total (fat mass, BMI) and abdominal adiposity (waist circumference, waist-to-hip ratio) were highly correlated with CRP in a population of 641 men and 597 women. In women, fat mass explained 18.2% of the variability in CRP, whereas waist-to-hip ratio explained 6.2% of the variability (Thorand et al. 2006). Physical activity is also associated with reduced CRP in older adults (Colbert et al. 2004), although this inverse relation may be partly mediated through body weight and adiposity.

In addition, CRP is affected by dietary intake, especially dietary fiber and whole grains. An inverse association between dietary fiber intake and serum CRP concentration was noted in 524 subjects (Ma et al. 2006). Consuming a carbohydrate restricted diet leads to a subsequent decrease in CRP (-8.1%) but not in IL-6 in overweight/obese men who are otherwise healthy (Wood et al. 2006). Conversely, intake of whole grains and dietary fiber decreased CRP in 902 diabetic women in the Nurse's Health Study (Qi et al. 2006). Recent work by Desroches et al. (2006) showed that both a low-fat diet and a high-monounsaturated fatty acid diet did not affect plasma CRP concentrations. Additionally, a decrease in dietary fat without a concurrent change in body weight does not affect CRP concentration in overweight healthy subjects (Koren et al. 2006), demonstrating that body weight and adiposity are stronger determinants of CRP concentration than diet alone. Dietary supplementation with polyunsaturated fatty acid had no effect on serum concentration of CRP in healthy subjects (Madsen et al. 2003). On the other hand, consumption of omega-3 fatty acids decreased CRP and IL-6 concentrations in the general population (Zampelas et al. 2005). An intake of dietary flavonoids (Song et al. 2005b) or green tea (Sung et al. 2005), each of which have antioxidant properties, does not have any effect on CRP or IL-6 concentration. However, an Italian study reported that dietary total antioxidant capacity is inversely related to CRP concentration, especially in subjects with hypertension (Brighenti et al. 2005). In a survey of older adults, fruit and vegetable intake was inversely associated with

CRP concentration (Gao et al. 2004), which could potentially be due to the fiber content rather than the antioxidant content. Analysis of data from 891 participants from the population-based Framingham Heart Study cohort showed that low circulating pyridoxal 5'-phosphate (PLP), the active form of vitamin B_6, was associated with higher CRP independently of total Hcy. These results may reflect utilization of vitamin B_6 during an underlying inflammatory process and may represent a possible mechanism to explain the decreased vitamin B_6 concentration observed in those with CVD (Friso et al. 2001).

In summary, although CRP is a strong predictor of CVD, the utility of CRP alone as a tool in global risk assessment has some important limitations. These include the poor specificity of CRP in the setting of coexisting inflammatory states (e.g., rheumatoid arthritis, chronic pulmonary disease, and infections) and relatively fewer data from ethnic groups other than Caucasians (Hackmand and Anand 2003). However, when CRP is combined with traditional risk factors, it adds to the predictive value of CVD risk assessment.

E. Risk Factors for Osteoporosis

1. Assessment of Bone Mineral Density

Bone densitometry is the single best predictor of osteoporotic fracture risk and is used for assessing therapeutic interventions. Because of its predictive power, densitometric assessment is by far the most important information to obtain about fracture risk. Other independent predictors of fracture are readily known, such as age, history of previous fracture, and low body weight. Together, the risk profile of an individual can be accurately determined as long as the BMD is known (Miller et al. 1999). Several techniques are available for quantitative measurement of BMD (Table 2). The three most widely used techniques in a research setting are dual energy x-ray absorptiometry (DXA), quantitative computed tomography, and calcaneal ultrasonography. Among these, DXA is the most widely used technique and is considered the gold standard for measuring BMD. DXA is noninvasive, rapid, accurate, and safe. Although DXA can measure BMD at any skeletal site, the standard sites are the lumbar spine, the proximal femur, and the distal forearm. The high level of precision and low level of radiation exposure for this technique allows not only diagnosis, but also monitoring response to therapy (Brunader and Shelton 2002).

Table 2: Methods of Measuring Bone Mineral

Method	Central Skeleton	Whole Body	Peripheral Skeleton	Ionizing Radiation		Research Method	Clinical Method
				Low Dose	High Dose		
Single-energy photon absorptiometry[a]			√	√			
Single-energy x-ray absorptiometry			√	√		√	
Dual-energy photon absorptiometry[a]	√			√			
Dual-energy x-ray absorptiometry	√	√	√	√		√	√
Neutron activation analysis[a]		√			√	√	
Compton scattering		√			√	√	
Quantitative computed tomography (QCT)	√				√	√	√
Peripheral QCT		√	√	√		√	
Magnetic resonance imaging[b]	√	√				√	
Ultrasonography[b]		√	√			√	√

[a] Method no longer in use

[b] Nonionizing radiation

From Brunader and Shelton (2002)

Other techniques for measuring BMD have been developed. Quantitative computed tomography (QCT) measures lumbar spine and more recently has been adapted to measure peripheral sites as well (peripheral quantitative computed tomography; pQCT). The potential advantage of quantitative computed tomography over DXA is its ability to measure true volumetric density (grams/cm^3) compared with DXA, which provides an areal density (grams/cm^2). Moreover, quantitative computed tomography measures trabecular bone of the lumbar spine exclusively, devoid of the cortical envelope (Miller et al. 1999). This is of extreme importance because trabecular bone, due to its higher rate of turnover, is expected to show metabolic changes earlier (Brunader and Shelton 2002) than cortical bone. Trabecular bone is highly vascularized and provides the scaffolding for the ends of long bones. It is also responsible for hematopoiesis and mineral homeostasis and is the type of bone primarily lost with menopause (Nilas and Christiansen 1988). However, quantitative computed tomography, is expensive, not widely available, and exposes individuals to higher radiation levels. Fracture prediction by quantitative computed tomography is as good as but no better than that with DXA (Miller et al. 1999).

Peripheral quantitative computed tomography is a noninvasive method (Grampp et al. 1995) and provides accurate (Ebbesen et al. 1997) and precise (Sievanen et al. 1998) volumetric BMD, bone size, cortical geometry (Rauch et al. 1999), and apparent trabecular structure and connectivity (Takagi et al. 1995). High resolution pQCT measurements allow visualization of the trabecular bone microstructure. Using high resolution pQCT, osteoporotic changes are apparent on images of both the radius and tibia and structural parameters, such as trabecular number and separation, can be measured (Glüer et al. 1997). This is of importance, because in osteoporotic females, trabecular number is reduced, while in osteoporotic males, trabecular thickness is reduced (Mullender et al. 2005). For planar measurements like DXA, changes in density are substantially obscured by changes in bone size. Peripheral quantitative computed tomography measurements offer better methodology in such cases. Peripheral quantitative computed tomography also provides complete information on the distribution of bone mineral within the computed tomography slice image, and hence makes it possible to calculate biomechanically relevant parameters describing bone strength. Moreover, because of the distance from radiation sensitive organs, the

effective radiation exposure for pQCT measurements is very small (Glüer et al. 1997).

Determination of BMD alone explains about 60-80% of the variation in bone strength but provides no information on bone architecture. Bone ultrasonography measures the speed of sound as well as broadband ultrasonic attenuation of the site being measured. Ultrasonic attenuation is thought to be related to bone architecture and ultrasound speed to material bone properties such as density, although these theories are not universally accepted. The most common site of measurement is the calcaneus. However, no cut-off values for diagnosis of osteoporosis using ultrasound have been developed and the World Health Organization criteria for defining bone density are largely based on DXA (Brunader and Shelton 2002).

2. Established Risk Factors for Osteoporosis

Osteoporosis is a devastating disorder with significant physical, psychosocial, and financial consequences. The risks for osteoporosis, as reflected by low bone density, and the risks for fracture overlap but are not identical (National Institutes of Health, NIH Consensus Statement 2000). The established risk factors for osteoporosis are variables associated with osteoporosis and can be categorized as primary and secondary risk factors. Primary risk factors include aging, female gender, low peak adult bone mass, Caucasian or Asian race, thin and/or small frame, estrogen deficiency (menopause or ammenorhea), family history of osteoporosis, immobilization, and use of certain medications (corticosteroids and anticonvulsants). Secondary risk factors include cigarette smoking, excessive use of alcohol, lack of physical activity, dietary deficiencies of protein and vitamin K, dietary excesses of sodium, caffeine and protein, inadequate lifetime calcium intake, and poor vitamin D status. Heavy coffee consumption or caffeine intake has been thought to be a risk factor for low bone density, fracture risk, and osteoporosis by some researchers (Kiel et al. 1990) while other studies have contradicted this (Sakamoto et al. 2001; Lloyd et al. 1997). Cummings et al. (1995) assessed potential risk factors for hip fracture in 9,516 white women. They reported that risk was higher among women who had previous fractures of any type after the age of 50, were tall at the age of 25, rated their own health as fair or poor, had previous hyperthyroidism, had been treated with long-acting benzodiazepines or anticonvulsant drugs, ingested high amounts of caffeine, or spent four hours a day or less on their feet. Additionally, low calcaneal bone density was also an independent risk factor. The incidence

of hip fracture was 1.1 per 1000 woman-years among women with normal calcaneal bone density who had two or fewer risk factors, whereas this incidence increased to 27 per 1000 woman-years among those with bone density in the lowest tertile who had five or more risk factors. Adequate calcium and vitamin D intake are crucial to develop optimal peak bone density and to preserve bone throughout life. Supplementation of these two components in bioavailable forms may be necessary in individuals who do not achieve recommended intake from dietary sources (National Institutes of Health, NIH Consensus Statement 2000). Calcium intake has been shown to reduce the risk of fracture in several prospective cohort studies (Feskanich et al. 1997; Cumming et al. 1997). However, a meta-analysis of six prospectively studied cohorts did not show an increase in fracture risk with low calcium intake (Kanis et al. 2005). Regular exercise, especially resistance and high-impact activities, also contribute to development of high peak bone density and may reduce the risk of falls in older individuals (National Institutes of Health, NIH Consensus Statement 2000). Lifetime habitual physical activity prevents bone loss at weight bearing sites of the skeleton (Greendale et al. 1995; Korpelainen et al. 2006) because the skeleton responds to mechanical stress with a stimulation of osteoblast activity resulting in increased BMD (Korpelainen et al. 2006). In addition to the above risk factors, genetics account for a significant proportion of the variation in BMD. In a recent study in 570 Amish women, genetics accounted for 58-88% of the total variation in BMD in pre-menopausal women compared to 37-54% of the total variation in postmenopausal women (Brown et al. 2005).

3. Homocysteine and Bone

Osteoporosis is a multifactorial disease. In addition to the traditional risk factors for osteoporosis, Hcy has recently emerged as a novel risk factor. It has been hypothesized that the metabolism of Hcy is involved in osteoporosis. Homocystinuria, a rare autosomal recessive disease characterized by markedly elevated concentration of plasma Hcy, has several clinical manifestations involving the eyes, the vasculature, and the central nervous system. The presence of homocystinuria is associated with the early onset of osteoporosis. The underlying pathophysiological mechanism for the occurrence of early osteoporosis in patients who have homocystinuria is not completely understood. However, *in vivo* and *in vitro* studies support the concept that an Hcy-associated disturbance in collagen cross-linking

in bone is involved. The increased prevalence of osteoporosis among people with homocystinuria suggests that a high serum Hcy concentration may weaken bone (van Meurs et al. 2004).

a. Epidemiologic evidence for homocysteine and bone density/fracture risk

Numerous studies have demonstrated a link between increased plasma Hcy concentration and the risk of osteoporotic fracture. Results from 825 men and 1,174 women aged 59-91 years in the Framingham Study showed that subjects in the highest quartile of total Hcy had a greater risk of hip fracture than those in the lowest quartile (McLean et al. 2004). In addition, this risk was almost four times as high for men and 1.9 times as high for women. In 2,268 men aged 47-50 years and 3,070 women aged 71-75 years from the Hordaland Homocysteine Study cohort, plasma total Hcy was inversely related ($P<0.001$) to BMD among middle-aged and elderly women but not among men. This association remained significant after additional adjustments for plasma folate or intake of calcium and vitamin D. The multiple adjusted odds ratio for low BMD was 1.96 in those with high (≥15 μmol/L) total Hcy compared with low (<9 μmol/L) total Hcy, but this was not significant for men. Interestingly, there were no associations between plasma folate, vitamin B_{12}, or MTHFR polymorphisms and BMD (Gjesdal et al. 2006).

Two large prospective studies examined the relation between Hcy and risk of osteoporotic fracture in a population setting. van Meurs et al. (2004) studied 2,406 subjects from the Rotterdam Study and the Longitudinal Aging Study Amsterdam. The relative risk of fracture after adjusting for other variables was 1.4 for each SD increase in Hcy concentration. Subjects with a Hcy concentration above the cut-off value had a two-fold increase in fracture risk compared to subjects with lower values. These risk estimates were similar between men and women. Reporting similar results, Sato and coworkers (2005) examined the association between plasma Hcy and the risk of hip fracture in 433 stroke patients. Age-adjusted incidence rates per 1000 person-years for hip fracture increased linearly from 2.9 in the lowest quartile to 27.9 in the highest quartile of Hcy concentration. Multivariable-adjusted Cox proportional hazards regression analysis for quartiles of total Hcy showed that for each SD increase in total Hcy concentration, the risk of hip fracture increased by 35%.

A Turkish study in 79 postmenopausal women stated that Hcy concentration was significantly higher in osteoporotic postmenopausal women compared to their healthy counterparts (Yilmaz et al. 2006). Sato et al. (2005a) studied the risk of hip fracture in elderly women with Parkinson's disease. Results from this study revealed that the risk of hip fracture in women with Parkinson's disease was greatest in the highest quartile of Hcy and this risk was 2.4 times higher than the risk in the lowest quartile. It is known that L-3,4-dihydroxyphenylalanine that is used to treat Parkinson's disease, increases Hcy concentration (O'Suilleabhain et al. 2004); the increased risk of fracture might have been related to L-3,4-dihydroxyphenylalanine use. Additionally, these patients exhibit altered calcium and vitamin D metabolism (Sato et al. 2005b) which might have possibly increased fracture rate.

b. Purported mechanism for homocysteine in osteoporosis

McKusick in the mid 1960's first hypothesized that a disturbance in the metabolism of mucopolysaccharides could result in altered collagen synthesis due to a deficiency of sulfur donors (McKusick 1966). McKusick also speculated that Hcy interferes with collagen cross-linking, impairing bone integrity. One of the first studies to examine the McKusick hypothesis was published by Lubec et al. (1996) who studied collagen type I and type III synthesis in 10 homocystinuric patients. Although collagen type I and type III synthesis was not different between patients and controls, collagen type I cross-links expressed by serum carboxyterminal telopeptide of collagen type I were significantly lower in the patient group compared to the control group. This significant reduction of cross-links in the homocystinuric group did not correlate with serum Hcy or homocysteic acid concentrations. These results partly support the McKusick hypothesis since disturbed collagen cross-linking occurs in homocystinuria, but the bone manifestations are not necessarily due to deficient collagen synthesis. In apparent contrast to these results, Bode et al. (2000) found a positive correlation between serum carboxyterminal telopeptide of collagen type I and Hcy concentration in 109 patients undergoing a coronary bypass graft. Because of the low number of subjects in both studies and lack of further studies on carboxyterminal telopeptide of collagen type I concentration in homocystinuric patients, the results of these studies require corroboration.

In developing chicks supplemented with 0.6% dl-Hcy for the first eight weeks, hyperhomocysteinemic animals grew faster and had longer and wider tibiae at the end of eight weeks, despite normal indices of bone formation. Additionally, the bone carbonate to phosphate ratio was significantly decreased, demonstrating qualitative alterations in the cortical bone mineral composition (Massé et al. 2003). Herrmann et al. (2005) recently showed that high Hcy stimulates osteoclast activity *in vitro*. Interestingly, this activity did not increase in the presence of equimolar concentrations of the related thiols cysteine and glutathione, implicating Hcy in bone resorption.

To understand the mechanism by which hyperhomocysteinemia affects bone, Sakamoto et al. (2005) recently investigated the effects of Hcy on expression of osteocalcin and osteopontin, two non-collagenous extracellular bone proteins, in MC3T3-E1 preosteoblastic cells. Osteocalcin decreased by approximately 61% in cells treated with 500 μmol/L Hcy, while osteopontin increased from 134% to 209% of the control level. Hcy decreased osteocalcin mRNA but increased osteopontin mRNA without affecting cell viability (Sakamoto et al. 2005). On the contrary, a recent study by Herrmann et al. (2005a) in peri- and postmenopausal women showed that circulating osteocalcin was not associated with Hcy. Additionally, partial correlation analysis controlling for several variables did not reveal significant relation between Hcy and BMD of the hip (P=0.23) or lumbar spine (P=0.07). However, weak but significant associations between Hcy and urinary deoxypyridinoline cross-links, a marker of bone resorption (r=0.19, P=0.022), and serum calcium (r=0.17, P=0.045) were observed, suggesting a mechanistic link between Hcy and bone metabolism (Herrmann et al. 2005a).

Cell culture work in primary human bone marrow stromal cells and a human bone marrow stromal cell line showed that Hcy induced apoptosis via the reactive oxygen species-mediated mitochondrial pathway and nuclear factor-kappaB activation in human bone marrow stromal cells. Hence, Hcy may contribute to the development of osteoporosis by reducing bone formation. Hence, antioxidants may play a role in preventing bone loss in individuals with hyperhomocysteinemia (Kim et al. 2006). Osteoprotegerin is a member of the tumor necrosis factor superfamily and is involved in the regulation of bone metabolism and calcification of vascular tissue. A recent study by Vik et al. (2006) in 58 patients (40-60

years) with verified myocardial infarction and age- and sex- matched case controls showed that serum osteoprotegerin concentration was similar in cases and controls (P=0.92). However, there was a significant correlation between osteoprotegerin and Hcy in patients (r=0.30, P=0.02) and controls (r=0.35, P=0.007), suggesting a mechanistic link between bone and vascular tissue.

c. Factors affecting the relationship between homocysteine and bone

1) Methylenetetrahydrofolate reductase polymorphisms:

Polymorphism in the gene encoding MTHFR is probably the most extensively studied of the genetic factors influencing hyperhomocysteinemia. Miyao et al. (2000) have noted that this polymorphism is associated with reduced BMD in postmenopausal Japanese women with the TT genotype. Data from the Danish Osteoporosis Prevention Study revealed similar associations in early postmenopausal women. Subjects with the TT genotype had significantly lower BMD at the hip and lumbar spine and a two-fold higher fracture rate compared with their wild-type CC counterparts (Abrahamsen et al. 2003). In contrast, a study in early postmenopausal Scottish women showed that there was no significant association between femoral neck and lumbar spine BMD with the MTHFR genotype. However, dietary riboflavin, a vitamin upon which MTHFR is dependent, correlated positively with lumbar spine and femoral neck BMD in homozygotes for the MTHFR 'T' allele, indicting the possibility that riboflavin intake and MTHFR genotype might interact to regulate BMD (MacDonald et al. 2004).

Villadsen et al. (2005) more recently demonstrated that the TT genotype is significantly more common (14.3%) in Danish women with vertebral fractures than normal controls (8%). However, these women did not have a significantly lower BMD than their counterparts. Logistic regression analysis showed that vertebral fracture was associated with BMD, but only weakly (P=0.06) with MTHFR genotypes. In another study with 689 elderly Danish twins, the risk of fracture was 1.5 times higher in the TT genotype versus the CT group, and 1.5 times higher in the CT group versus the CC group. Interestingly, Hcy provided no significant contribution to fracture risk (Bathum et al. 2004). In a similar study in Danish postmenopausal women, logistic regression analyses showed that individuals homozygotic for the C-allele had a higher risk of lower forearm fracture (odds ratio=3.93,

P=0.02), hip fracture (odds ratio=6.99, P=0.02) and combined fractures (odds ratio=4.33, P=0.002) compared with those homozygotic for the T-allele (Jorgensen et al. 2002).

2) Folate: The inconsistencies in results from studies examining the MTHFR genotype could arise due to a possible gene-nutrient interaction. The MTHFR and associated enzymes together with a number of the B vitamins are required for the remethylation of Hcy to methionine (Cashman 2005). Folate, an important co-enzyme in the Hcy metabolic pathway, is emerging as a predictive factor for BMD. McLean and coworkers (2004a) determined whether folate status might modify an association between the C677T polymorphism and bone, possibly by influencing Hcy concentrations. Researchers found that compared with participants with at least one wild type allele (CC+CT group), the TT group had lower mean broadband ultrasound attenuation (P=0.06) and Ward's triangle BMD (P=0.08) within the lower plasma folate (< 4 ng/ml) group, but significantly higher hip BMD (P<0.05) within the higher plasma folate (≥ 4 ng/ml) group. The authors chose 4 ng/ml as a cut-off because this was considered below the normal level of 5 ng/ml and was close to the median value for the population. The mechanism underlying this association with bone is still unclear, but researchers hypothesize that the effects may be partially mediated by Hcy. However, it must be noted that determination of plasma folate in this study was assessed at least four years before bone phenotype measurements were made. Plasma folate concentration reflects recent dietary intake of folate rather than long term folate status. Moreover, the government mandated a nationwide fortification of enriched grains with folic acid, which was introduced during this four year period.

Cagnacci et al. (2003) demonstrated that serum folate but not Hcy or vitamin B_{12} was independently related to lumbar spine BMD in 161 postmenopausal women. Similarly, an Italian study assessed the risk of osteoporotic fractures in 978 elderly persons using data from the Conselice Study of Brain Aging. Following adjustment for serum folate and vitamin B_{12}, for each 1 SD increase in plasma Hcy, the odds ratio for fracture risk decreased from 1.4 to 1.2. Multivariate-adjusted risk of fracture doubled for persons in the lowest serum folate quartile compared to the higher quartiles. No independent association with BMD was found for vitamin B_{12} (Ravaglia et al. 2005). Conversely, recent work in healthy non-osteoporotic women showed that short-term folate supplementation (1 mg/d or 5 mg/d) for eight weeks

did not affect biochemical bone markers (Herrmann et al. 2006). An Iranian study found that BMD of femoral neck and lumbar spine were negatively correlated with Hcy but not MTHFR polymorphism. However, this association of Hcy with bone weakened when adjusted for circulating folate and vitamin B_{12} (Golbahar et al. 2004). Results of these studies are somewhat limited in that serum folate, an indicator of short-term folate status, rather than intracellular folate, a long-term indicator of folate status, was studied.

3) Vitamin B_{12}: Vitamin B_{12}, an important co-enzyme in Hcy metabolism, has also been implicated for its role in low BMD. Morris et al. (2005) studied the relation among intracellular folate, vitamin B_{12}, Hcy, and BMD in participants of NHANES III. Although serum vitamin B_{12} was related to BMD in a dose-response fashion, serum or intracellular folate was not associated with osteoporosis and BMD. Subjects with serum Hcy $\geq$ 20μmol/L had significantly lower BMD than those with serum values $\leq$ 20μmol/L. Similar results were published by Dhonukshe-Rutten et al. (2003) in a population of frail, elderly Dutch women. Women with osteoporosis had a significantly lower serum concentration of vitamin B_{12} than women with osteopenia or normal BMD. A more recent randomized double-blind placebo-controlled trial by Sato and co-workers (2005c) in 628 patients with residual hemiplegia at least one year following their first ischemic stroke showed that a Hcy lowering therapy with 5 mg folate and 1500 μg vitamin B_{12} reduced the hip fracture rate. The number of hip fractures per 1000 patient-years was 10 and 43, respectively, for the treatment and placebo groups. However, it is not clear from this study if improved folate and vitamin B_{12} status or decreased Hcy concentrations caused the decrease in fracture risk.

Results from data collected on adult participants in the Framingham Offspring Osteoporosis Study demonstrated that men and women with low vitamin B_{12} concentrations (<200 pg/ml; <148 pmol/L) had lower than average BMD compared with those above this cut-off (Tucker et al. 2005). A prospective study in 615 men and 652 women in the Longitudinal Aging Study Amsterdam showed that low vitamin B_{12} status, high Hcy concentration, or both was associated with a higher risk for fracture in men (relative risk=3.8) and in women (relative risk=2.8). This study supports the McKusick hypothesis that women with elevated Hcy also had high deoxypyridinoline /creatinine, suggesting that high Hcy

results in an altered bone matrix by interfering with the formation of collagen cross-links (Dhonukshe-Rutten et al 2005).

In one of the first studies relating vitamin B_{12} to bone, Carmel et al. (1988) suggested that osteoblast activity depends on vitamin B_{12} and that bone metabolism is affected by vitamin B_{12} deficiency. In several studies, vitamin B_{12} has been associated with osteoblast activity and bone formation (van Dommelen et al. 1964; Carmel et al. 1988; Kim et al. 1996). Anemia due to vitamin B_{12} deficiency is associated with reduced BMD of the lumbar spine (Eastell et al. 1992) and with vertebral fractures (Goerss et al. 1992). Whether this relationship is independent of Hcy is unclear. Stone and colleagues (2004) examined the relationship between low serum vitamin B_{12} and bone loss in 83 elderly women who participated in the Study of Osteoporotic Fractures. They reported that after adjusting for age, weight, and clinic site, women with vitamin B_{12} ≤280 pg/ml experienced an annual change of -1.6% in total hip BMD, compared with -0.2% in women with concentrations >280 pg/ml (P=0.003). Serum vitamin B_{12} was not associated with rate of change in calcaneal BMD in this cohort.

4. C-Reactive Protein and Bone

a. Epidemiologic evidence for immune modulators and bone

Although a few studies have demonstrated an association between CRP concentration and BMD or biochemical markers of bone turnover in several immune (Bakri Hassan et al. 1998) and inflammatory diseases (Haugeberg et al. 2006; Punzi et al. 2005), there is a paucity of information examining this relation in non-inflammatory conditions and in osteoporotic or postmenopausal subjects. In a multivariate linear regression model, mean CRP was found to be independently associated (P<0.001) with change in hand BMD during follow-up of patients with early undifferentiated rheumatoid arthritis (Haugeberg et al. 2006). Similarly, CRP concentration is higher in erosive osteoarthritis than in non-erosive osteoarthritis patients (Punzi et al. 2005). Osteoporotic patients with ankylosing spondylitis were also found to have significantly higher CRP (Lange et al. 2005). In one of the first studies relating CRP concentration to BMD in pre- and postmenopausal women, Koh and coworkers (2005) demonstrated that higher circulating CRP was associated with lower BMD. Compared with normal subjects, serum CRP was higher in osteopenic and osteoporotic

subjects (P<0.001), after adjustment for age, BMI, and menopausal status. These studies suggest a link between systemic inflammation as reflected by CRP and BMD.

b. Purported mechanisms for C-reactive protein in osteoporosis

1) IL-6 and bone: A plethora of information suggests that the immune system modifies bone resorption and probably bone formation through interactions involving T and B lymphocytes, dendritic cells, cytokines, and cell to cell interactions. The immune system plays a critical role in the bone remodeling cycle and this interaction between osteoclasts and osteoblasts is mediated primarily through the receptor activator of nuclear factor kappaB (RANK)/RANK ligand (RANKL)/osteoprotegerin system Various cytokines, including IL-6, IL-1, and TNF-α regulate both the immune response and the bone remodeling process (Clowes et al. 2005).

Since CRP is produced in the liver in response to IL-6, the relation between IL-6 and bone is important to consider. IL-6 is a key cytokine for osteoclastogenesis (Carlsten 2005). Ohsaki et al. (1992) demonstrated that giant tumor cells, which have many features of osteoclasts, synthesize and secrete IL-6, suggesting that IL-6 plays an autocrine/paracrine role in bone resorption. Adebanjo and coworkers (1998) showed that IL-6 receptor expression was present in single osteoclasts. Additionally, IL-6 reversed the inhibition of osteoclastic bone resorption induced by high extracellular Ca^{2+} (Adebanjo et al. 1998). IL-6 has been implicated as a facilitator of osteoclast precursor proliferation and a stimulator of bone resorption by mature osteoclasts (Pfeilschifter et al. 2002). In accordance with this, Tsangari and coworkers (2004) documented an increased expression of IL-6 mRNA in human trabecular bone of patients who had undergone a fragility fracture of the femoral neck, but not from those in the control group. These findings may be representative of the role IL-6 plays in the bone microenvironment, independent of circulating IL-6 concentration (Tsangari et al. 2004). de Hooge et al. (2005) demonstrated that IL-6 gene knockout male mice had lower BMD and developed more advanced osteoarthritis with aging. Recent work by O'Brien and colleagues (2005) showed that IL-6 gene expression *in vivo* did not change with an increase in parathyroid hormone and IL-6 was not required for the osteoclast formation and bone loss that accompanies continuous elevation of parathyroid hormone. On the contrary, work by Nakchbandi and others (2002) has shown that circulating concentration

of soluble IL-6 receptors correlate significantly ($P<0.01$) with rate of total femur bone loss in patients with hyperparathyroidism. In a study in Poland, postmenopausal women with type 1 diabetes had significantly lower BMD at the femoral neck and higher serum bioactive IL-6 concentration compared with the control group. However, using multiple regression analysis, no association was found between IL-6 and BMD in these women (Rachon et al. 2003).

2) Estrogen and IL-6: Menopause triggers changes in pro-inflammatory cytokines. Changes in cytokine activity have been purported as a common underlying mechanism for various diseases. It is now well known that estrogen deficiency enhances the responsiveness of cells toward some of these cytokines by up-regulating cytokine receptor number and cofactors, thus amplifying the increase in cytokine response (Pfeilschifter et al. 2002). Because bone-regulating cytokines, such as IL-1, TNF-α, and IL-6, synergize to stimulate their own and each other's synthesis, a small change in one cytokine in the bone microenvironment could dramatically alter the concentration of the others (Riggs 2000).

Estrogen withdrawal is associated with an increased potential of human bone marrow cells to release bone-resorbing cytokines, especially IL-6 (Bismar et al. 1995). The receptor for estrogen is known to suppress IL-6 gene expression through interaction with nuclear factor-kappaB in a hormone-dependent manner (Liu et al. 2005) and hence estrogen deficiency is associated with increased IL-6 concentration. Animal studies have shown that a loss of ovarian function up-regulates osteoclastogenesis *in vivo* in mice through an increase in IL-6 production in the microenvironment of the bone marrow (Jilka et al. 1992). *In vitro* evidence from cell culture work on murine bone marrow-derived stromal cell lines, normal human bone-derived cells, and nontransformed osteoblast cell lines from mice and rats have shown that 17β-estradiol decreases IL-6 mRNA concentration. This suggests, for the first time, a mechanistic paradigm by which estrogens might exert at least part of their antiresorptive influence on the skeleton (Girasole et al. 1992). Work on bone marrow cultures showed that estrogen loss causes an up-regulation of IL-6 by bone marrow cells and a similar phenomenon can be elicited *in vivo* by withdrawal of 17β-estradiol from primary cultures of bone cells (Passeri et al. 1993). Consistent with these observations, increased IL-6 concentration was detected in bone marrow supernatant from ovariectomized rats compared

to sham operated mice (Miyaura et al. 1995) and in serum of postmenopausal compared with premenopausal women (Lakatos et al. 1997). On the contrary, in an *in vivo* mouse model, Vargas et al. (1996) did not find an increase in IL-6 production with estrogen withdrawal. Work in postmenopausal women revealed that there were no significant correlations between serum IL-6 concentration and menopausal status, serum estradiol concentration, or markers of bone turnover (McKane et al. 1994). Also, there were no differences in IL-6 concentration in bone marrow aspirates of postmenopausal women receiving estrogen therapy or controls (Kassem et al. 1996). However, raloxifene, a selective estrogen receptor modulator, reduced bone loss and prevented vertebral fractures (Ettinger et al. 1999) by inhibiting IL-6 production by 25-45% ($P<0.001$) in human trabecular osteoblasts (Viereck et al. 2003). In an ovariectomized rat model of menopause, a standardized soy extract produced a decrease in serum IL-6 and showed a bone-sparing effect, which was partially attributed to the modulation of osteoclastogenesis induced by IL-6 (Gallo et al. 2005).

F. Hormone Therapy to Reduce Risk of Cardiovascular Disease and Osteoporosis

Hormone therapy for peri- and postmenopausal women prevents or decreases the discomfort associated with menopausal symptoms caused by diminished circulating estrogen. Hormone therapy is generally provided as a low dosage of one or more estrogens and typically combined with either progesterone or with its chemical analogue, progestin. The combination of estrogen and progesterone (combined hormone therapy) is usually prescribed for women with intact uteri. In women who have had a hysterectomy, an estrogen compound is usually given without any progesterone, a therapy referred to as unopposed estrogen therapy.

The routes of estrogen administration include oral (pill), transdermal (patch), creams, gels, topical emulsions, and vaginal ring. Several oral and transdermal estrogen formulations are indicated for treatment of both vasomotor symptoms and symptoms of vulvar and vaginal atrophy (Stefanick 2005). In contrast, all combination hormone therapies (estrogen plus progesterone) are indicated for treating both vasomotor and vulvar/vaginal symptoms associated with menopause, and progestins (synthetic progesterone) are indicated for

managing estrogen-induced hyperplasia (Stefanick 2005). Dosage varies, with estrogen taken daily and progesterone or progestin taken for about 10 days to two weeks every month (sequentially combined hormone therapy). Alternatively, a constant dosage of both hormones may be taken daily and is referred to as continuous combined hormone therapy.

Originally researchers believed that hormone therapy would protect against osteoporosis and CVD. However, several recent studies have provided conflicting data on the health effects of hormone therapy. For example, estradiol valerate and dionegest therapy for six months did not produce any change in plasma Hcy concentration (Pirimoglu et al. 2005), while oral doses of 17β-estradiol effectively reduced lipoprotein (a), a risk factor for CVD (Hemelaar et al. 2003). Recent research indicates that while hormone therapy may help reduce bone loss and fractures, there are substantial risks for breast cancer, thromboembolism and CVD (Writing Group for the Women's Health Initiative [WHI] Investigators 2002).

1. Cardiovascular Disease

Several large clinical trials have documented the effects of hormone therapy on CVD. Results from the Framingham Heart Study in the mid 80's (1985) with postmenopausal women (N=1,234) revealed that estrogen users had over a 50% elevated risk of cardiovascular morbidity ($P<0.01$) and more than a two-fold risk for cerebrovascular disease over an eight year period (Wilson et al. 1985). Interestingly, in the same year, the Nurses' Health Study with postmenopausal women (N=32,317) reported a 50% lower risk (adjusted relative risk=0.5; $P=0.007$) of coronary heart disease in estrogen users versus non users for an average 3.5 years of follow-up (Stampfer et al. 1985). The discrepancy between the results of these two highly respected prospective cohort studies was attributed to inclusion in the Framingham study of cardiovascular events other than myocardial infarction and coronary heart disease (e.g., angina pectoris, intermittent claudication, transient ischemic attack) and adjustment for HDL-C. At the time, this was considered possibly inappropriate because it was thought to be the most plausible mechanism of action for estrogen (Stefanick 2005). In 1995, the Postmenopausal Estrogen/Progestin Intervention (PEPI) clinical trial demonstrated favorable lipoprotein changes in women assigned to conjugated equine estrogen with or without a progestin. These results reinforced the belief that estrogens reduce coronary heart disease risk, but also showed that the addition of cyclic or daily medroxyprogesterone, a

synthetic form of progesterone, reduced the beneficial effect of estrogen on HDL-C (The Writing Group for the PEPI Trial 1995). The Heart and Estrogen/Progestin Replacement study with postmenopausal women (N=2,763) who had established coronary disease reported that treatment with oral conjugated equine estrogen plus medroxyprogesterone acetate (for an average of 4.1 years) did not reduce the overall rate of coronary heart disease events. Instead, hormone therapy increased the rate of thromboembolic events and gallbladder disease.

In 2002, after a mean of 5.2 years of follow-up, the data and safety monitoring board of the WHI trial recommended stopping the estrogen plus progestin arm due to increased risk of breast cancer. Final analyses revealed a hazard ratio of 1.24 for coronary heart disease (Writing Group for the WHI Investigators 2002) in women in the estrogen plus progestin arm, which was most apparent and significant in the first year (hazard ratio=1.81). These findings were consistent with the effects of conjugated equine estrogen plus medroxyprogesterone acetate, observed in women with coronary heart disease in the Heart and Estrogen/Progestin Replacement trial.

a. Homocysteine and hormone therapy

Since Hcy is an independent risk factor for CVD, several studies have focused on reducing Hcy through hormone therapy to ameliorate the risk of heart disease in postmenopausal women. Work by Boers and colleagues (1983) in the early 80's revealed that fasting plasma Hcy concentration was significantly higher in postmenopausal women and in men than in women of reproductive age. Premenopausal women have a unique efficiency of methionine handling (Boers et al. 1983), and thereby are protected against increases in Hcy typically seen after menopause. Menopause is therefore a critical time for Hcy metabolism, suggesting that the increased risk of CVD observed in many women may be partially due to an increase in Hcy concentration (De Leo et al. 2004). Several but not all studies have documented the effectiveness of hormone therapy in reducing plasma Hcy.

Findings from the NHANES III demonstrated the importance of estrogen in preventing increases in Hcy concentration. The mean serum total Hcy concentration of estrogen users greater than 55 years was significantly lower relative to non-estrogen users, independent of nutritional status or muscle mass (Morris et al. 2000). In a large prospective observational study in 438 Australian women, hormone therapy use was associated with

lower Hcy (Guthrie et al. 2005). In a prospective, randomized, placebo-controlled six month study in 107 healthy postmenopausal women, Hcy concentration decreased in all treatment arms: by 20% with unopposed conjugated equine estrogen, by 27% with conjugated equine estrogen plus medroxyprogesterone acetate, by 22% with conjugated equine estrogen plus nomegestrol acetate (a synthetic progestin) and by 34% with raloxifene. These decreases in Hcy were not statistically different between the groups (Gol et al. 2006) indicating a similar effect of estrogen, combined hormone therapy, and raloxifene. Similarly, Tutuncu et al. (2005) demonstrated no difference in the decrease in Hcy due to unopposed conjugated equine estrogen or conjugated equine estrogen combined with either of two doses of medroxyprogesterone acetate. Langer et al (2005) reported an Hcy lowering effect of hormone therapy in 608 women from the WHI cohort. Similarly, hormone therapy administered either as transdermal estrogen therapy or combined hormone therapy was effective in decreasing Hcy concentration in 120 postmenopausal women. These therapies decreased Hcy to premenopausal levels (Bednarek-Tupikowska et al. 2005).

Oral administration of 17β-estradiol alone or combined with progesterone decreased Hcy concentration by 13% and 10%, respectively, in a randomized, placebo-controlled, double-blind study in 25 postmenopausal women. In a multivariate analysis, the change in Hcy change was related to the change in albumin. The authors also observed that these changes were not accompanied by a change in the methionine-Hcy flux rate but hypothesized that a change in albumin metabolism plays an underlying mechanistic role in Hcy metabolism (Smolders et al. 2005). The initial concentration of Hcy may also be important. In a prospective, randomized study, sequential administration of a combination of estradiol and synthetic progesterone (norethisterone acetate), either orally or transdermally, did not cause a significant change in plasma Hcy in Finnish postmenopausal women with normal baseline Hcy concentration (Evio et al. 2000). In one of the earlier studies evaluating the effect of hormone therapy on Hcy concentration, van der Mooren and colleagues (1994) reported that in postmenopausal women with high Hcy, there was a 16.9% decrease in Hcy with a combination of continuous 17β-estradiol and cyclic progesterone therapy. Hormone therapy did not cause a significant change in women with low Hcy. However, the sample size for this study was extremely small (N=21). The results of these studies suggest that hormone

therapy effectively lowers Hcy only in women with high Hcy and is of little use in women with normal concentration.

Similar to the van der Mooren study (1994), administration of 17β-estradiol plus progesterone either orally or transdermally for a period of six months in 49 healthy postmenopausal women caused significant reductions in Hcy (Chiantera et al. 2003). Using similar treatments, Mijatovic et al. (1998) evaluated Hcy concentrations in postmenopausal women treated with 17β-estradiol and various doses of progesterone. The authors found a mean reduction of 13.5% in Hcy concentration without a dose-dependent effect of progesterone. This decrease was reached by three months and sustained thereafter indicating a new stable metabolic steady state. However, results from the Postmenopausal Estrogen/Progestin Intervention trial have shown that administration of both conjugated equine estrogens and combined hormone therapy decreased Hcy only through 36 months, indicating a transient effect of hormone therapy (Barnabei et al. 1999). Similar results were also reported by van der Mooren and coworkers (1997) in 39 healthy postmenopausal women. The authors reported a reduction of 12.3% in plasma Hcy during the first six months of therapy in women with higher Hcy at baseline. However, these lower values returned to baseline during the second half year of treatment. Conversely, work by Hsu et al. (2005) revealed that the beneficial effects of hormone therapy were noticeable even after three years of treatment. It is thus difficult to conclude from these data whether the effects of hormone therapy are transient or have a sustained effect on Hcy.

In apparent contrast to the above findings, D'Anna and colleagues (2005) found slight albeit not significant decreases in Hcy with six months of combined hormone therapy. Furthermore, Bruschi et al. (2004) found increases in plasma Hcy with oral or transdermal treatment with estradiol plus normegestrol acetate, a progesterone derivative, in 199 late postmenopausal women. In the oral treatment group, Hcy increased at 12 months by 22%; in the transdermal group, after a slight decrease at three months (1.5%), the increase at 12 months was 13%. In the same year, Lacut et al. (2004) reported similar results in 196 postmenopausal women who received either 17β-estradiol orally or transdermally, both combined with a daily intake of progesterone, or placebo over a period of six months.

Additionally, the authors also reported decreases in serum vitamin B_{12} with oral but not transdermal estrogen therapy.

Altogether, it appears that hormone therapy either in the form of estrogen or combined with progesterone decreases the Hcy concentration in early postmenopausal women. The route of administration does not seem to have an effect on Hcy concentration. Nonetheless, it needs to be ascertained if these beneficial effects are transient or long lasting. However, hormone therapy in late postmenopausal women seems to have an adverse rather than beneficial effect by increasing Hcy. Plausible mechanisms underlying these increases should be further researched.

b. C-reactive protein and hormone therapy

In women, CRP concentration correlates with age and menopause (Woodward et al. 2003), coincident with increased CVD risk. Numerous studies have demonstrated that estrogen and combined hormone therapy increase CRP in postmenopausal women. An Italian study indicated that either estrogen or estrogen-progestogen increased CRP, whereas estrogen decreased other inflammatory markers (Vitale et al. 2005). Results from the Estrogen Replacement in Atherosclerosis trial have shown that estrogen alone increased CRP by 40% at the end of 1 year of treatment (Lakoski et al. 2005). Data from a subset of postmenopausal women (n=221) from the Postmenopausal Estrogen/Progestin Intervention trial showed that all five treatment groups (3 different combined estrogen-progestin regimens, conjugated equine estrogen only, or placebo) resulted in increases at 12 months of estrone, sex hormone binding globulin, and CRP. Using an adjusted model to predict 12-month CRP change, progestin plus estrogen, but not estrogen alone, increased CRP through an inflammatory mechanism (Reuben et al. 2006). Recent data from postmenopausal women (N=346) showed that the CRP concentration is increased in women using hormone therapy and is significantly higher in women with cardiovascular events compared to those without events (Vitale et al. 2005a).

McKenzie and colleagues (2003) suggested that the typical increase in CRP concentration demonstrated with traditional hormone therapy is not evident with a low-dose continuous combined hormone therapy regimen in postmenopausal women with type 2 diabetes. This study reported a significant decrease in IL-6 (P=0.015) but no significant

alteration in CRP (P=0.62) concentration. Results from an Italian study conducted with postmenopausal women (N=389) with increased CVD risk have noted a discrepancy between increased plasma CRP and reduced plasma concentration of all other markers of inflammation. These results suggest that the increased CRP with oral hormone therapy may be related to metabolic hepatic activation and not to an acute-phase response (Silvestri et al. 2003). The route of administration also seems to impact CVD risk. For example, women using oral hormone therapy usually experience an increase in CRP (Hu et al. 2006; Bukowska et al. 2005; Eilertsen et al. 2005; Wakatsuki et al. 2004), while this increase is not typical in transdermal estrogen users (Sumino et al. 2005; Yilmazer et al. 2003; Kawano et al. 2003). Other studies have shown that transdermal hormone therapy does not have an effect on CRP (Lacut et al. 2003; Post et al. 2002; Vongpatanasin et al. 2003). In a subset of women (n=608) from the WHI Observational study, CRP was highest among women using unopposed conjugated equine estrogen. Among users of estrogen plus progestin, CRP concentration was higher in women with incident coronary events than healthy controls. Additionally, transdermal estrogen users had a lower CRP concentration (Langer et al. 2005). In a Turkish study, intranasal administration of 17β-estradiol in 29 healthy hysterectomized, postmenopausal women did not change CRP concentration after six months of treatment (Kiran et al. 2004). It is thought that these adverse effects of oral hormone therapy may be due in part to first-pass hepatic processing (Bassuk et al. 2004).

Contrary to the above mentioned findings, Kiran and Kiran (2006) illustrated that although conjugated equine estrogens plus medroxyprogesterone acetate caused a 29% increase in CRP concentration in postmenopausal women, these changes were not significant. In contrast, a prospective, randomized, placebo-controlled six month study in healthy postmenopausal women (N=107), CRP was not influenced by raloxifene, whereas conjugated equine estrogen or conjugated equine estrogen plus medroxyprogesterone acetate significantly increased CRP. On the other hand, another study showed that treatment with conjugated equine estrogen plus progesterone reduced serum CRP concentration (Gol et al. 2006).

Taken together, estrogen therapy is consistently associated with an elevation in CRP. On the other hand, progestin use appears to be beneficial as it seems to attenuate the effect of

oral estrogen on CRP. Transdermal and intranasal routes of administration do not seem to produce an elevation in CRP typically seen with oral use. Selective estrogen receptor modulators have various effects on CRP concentration.

Despite these different effects of hormone therapy on CRP and Hcy, there is no substantive evidence that a change in these markers results in a modification of CVD risk. In fact, the WHI has demonstrated that hormone use increases CVD risk (Writing Group for the WHI Investigators 2002), particularly in late postmenopausal women. Further studies are required to specifically investigate whether treatments that increase or decrease these markers in fact modulate the risk of cardiovascular events in women.

2. Osteoporosis

Accelerated bone loss during the perimenopausal years has been attributed to estrogen deficiency due to ovarian failure. This bone loss contributes to a 20-30% loss in trabecular bone and a 5-10% loss in cortical bone (Riggs et al. 1998). Estrogen alone or in combination with progestin prevents bone loss at the spine and hip (Komulainen et al. 1999) and reduces hip fracture rates (Cauley et al. 1995). In elderly men from the Framingham study, the adjusted hazard ratio for a hip fracture was highest in men in the lowest quartile of estradiol concentration compared to men in the highest quartile (3.1 versus 0.9). Testosterone had no effect on hip fracture rates, thereby strengthening the idea that estrogen is the major player involved in osteoporosis. However, in an analysis in which men were grouped by both estradiol and testosterone concentrations, men with both low estradiol and low testosterone concentrations had the greatest risk for hip fracture (adjusted hazard ratio=6.5) (Amin et al. 2006).

In a large prospective cohort of Danish female nurses (N=7,082) aged 50-69 years, women who used estrogen alone had a 40% reduced fracture risk compared to a 56% reduction in women using combined therapy. The authors also found no protective effect of hormone therapy in women who discontinued hormone therapy use, irrespective of duration or recency of discontinuation (Hundrup et al. 2004). In the Framingham study, the relative risk of hip fracture for estrogen users at any time was 0.65 after adjustment for age and weight. This risk declined to 0.34 in those women who had taken estrogen in the last two years, supporting the hypothesis that use of estrogen protects against bone loss associated

with menopause (Kiel et al. 1987). Data from the WHI, in which women (with an intact uterus) were randomly assigned to estrogen plus progestin or a placebo, showed that hormone therapy increased total hip BMD and further reduced the risk of fracture at the hip, vertebrae, and wrist (Cauley et al. 2003). Similarly, in women who underwent hysterectomy, the estrogen alone arm also demonstrated a reduced risk of hip fracture by 30%-39% (The WHI Steering Committee 2003).

Liu and Muse (2005) studied the efficacy of hormone therapy in preventing postmenopausal bone loss. In this two-year double-blind, placebo-controlled clinical trial, 132 menopausal women were randomized into one of six treatment groups: micronized progesterone, medroxyprogesterone acetate, norethindrone (a progestin derived from testosterone), micronized estradiol, micronized estradiol plus medroxyprogesterone acetate or placebo. Micronized estradiol or micronized estradiol plus medroxyprogesterone acetate treatment increased lumbar spine BMD by 2%-4% over two years. Spinal BMD showed a tendency toward decline with micronized progesterone, medroxyprogesterone acetate, and placebo treatments. Change from baseline for the norethindrone arm was not found. At the femoral neck, BMD did not change significantly for any treatment group. The authors concluded that estrogen remains the primary bone active agent in hormone therapy and progestins have significantly less activity.

In a 10 year study of the effect of hormone therapy on lumbar spine BMD and distal forearm bone mineral content (BMC), Eiken and coworkers (1996) found that lumbar spine BMD was significantly higher (14.5%; $P < 0.001$) in women who received hormone therapy compared to the placebo. However, forearm BMC decreased by 0.7% over the 10 year period in the hormone therapy treatment group compared with a reduction of 17.6% ($P<0.001$) in untreated women. In a randomized, double-blind, placebo-controlled multicenter trial (Women's Health, Osteoporosis, Progestin, Estrogen), more than 85% of women on low dose conjugated estrogen plus medroxyprogesterone acetate did not experience continued hip BMD loss at 12 and 24 months, in contrast to 30.6% of women on placebo at 12 months and 36.5% at 24 months (Lindsay et al. 2005). In an earlier study by the same group (Lindsay et al. 2002), data from healthy postmenopausal women (N=822) revealed that women using various doses of estrogen alone or combined estrogen plus progesterone had significant gains

($P<0.001$) in spine and hip BMD and total body BMC. Results from bone biopsies obtained from postmenopausal women have shown that high doses of estradiol increase mineralization in total (cortical and trabecular) iliac bone by $6.9 \pm 1.9\%$ ($8.6 \pm 2.1\%$ in cortical bone, $6.5 \pm 2.1\%$ in trabecular bone) (Boivin et al. 2005). An interesting study in postmenopausal women ($N=81$) showed that compared to placebo, medroxyprogesterone reduced the rate of loss in cortical areas of the skeleton, but not in the spine, which contains more trabecular bone. In contrast, estrogen reduced the rate of loss in both cortical and trabecular areas of the skeleton (Gallagher et al. 1991).

To summarize, hormone therapy has demonstrated clinical fracture risk reduction. However, the increased risk of CVD and stroke, which were not counterbalanced by the small reduction in hip fracture rate in the WHI trial and the Heart and Estrogen/Progestin Replacement study suggest that hormone therapy could possibly exert a favorable risk/benefit ratio. The use of hormone therapy is indicated for prevention of osteoporosis in postmenopausal women but not approved for sole use in the relief of vasomotor symptoms. Further research for safer alternatives to hormone therapy needs to continue.

G. Isoflavones as an Alternative Therapy to Reduce Risk of Cardiovascular Disease and Osteoporosis

Isoflavones belong to a class of diphenol compounds called phytoestrogens and occur naturally in many plants. However, the isoflavones (genistein, daidzein, and glycitein) derived from soy are relatively unique and are what we used in our study. Soy isoflavones exist either as polar β-glucosides, such as genistin, daidzin, and glycitin, or in the free form as aglycones that include genistein, daidzein, and glycitein. In nature these soy isoflavones, whether free or attached to a sugar moiety, occur in an approximate 5:4:1 ratio, respectively (Reinwald and Weaver 2006). Due to their structural similarity to 17β-estradiol, soy isoflavones have been widely studied for their estrogen-like effects and potential health benefits.

1. Cardiovascular Disease

In July 2002, results from the WHI trial drew much attention, when it provided strong evidence for an increased risk of CVD with the use of combined estrogen plus progestogen in

postmenopausal women. These unexpected results triggered an intense ongoing discussion about the role of hormone therapy in reducing CVD risk or bone loss associated with menopause. In a large population-based random sample of German women (N=8,380), aged 45-65 years, the percentage of current hormone therapy users dropped by 16%. Among current hormone therapy users, the percentage of combined conjugated estrogen/progestogen users decreased by 41 % (P=0.008) after the publications of the WHI trial and the Heart and Estrogen/Progestin Replacement Study II (Clanget et al. 2005). Although several studies have documented the efficacy of postmenopausal hormone therapy, results from WHI trial have underscored the continuing need for research into new treatments for postmenopausal women.

a. Homocysteine and isoflavones

Data on the effect of isoflavones on Hcy have been relatively few and inconsistent. In a recent multi-center double-blind, crossover intervention trial in postmenopausal women (N=89), daily consumption of a fruit cereal bar containing 50 mg soy isoflavones for eight weeks had no effect on plasma Hcy (Reimann et al. 2006). A prospective, randomized, placebo-controlled trial in hypercholesterolemic men (N=89) showed that a soy based dietary supplement (containing 30 g soy protein, 9 g cotyledon fiber, and 100 mg isoflavones) verses a placebo (30 g casein) for 24 weeks did not affect Hcy. However, in a sub group, the active arm experienced a reduction in LDL-C and total cholesterol (Hermansen et al. 2005). Results from a Finland study demonstrated that daily intake of 41 g of soy protein in the form a yoghurt had no effect on plasma Hcy in a population of men (18-75 years) and postmenopausal women (45-70 years). However, significant decreases in total cholesterol, LDL-C, non-HDL-C, and for total cholesterol/HDL-C ratio were observed (Puska et al. 2004). In an observational study in premenopausal Japanese women (N=201), soy product intake (soy protein 9.0 g/d; soy isoflavones 30 mg/d) assessed by a dietary questionnaire was inversely associated with serum Hcy, but this relation attained significance (P=0.04) only when age and total energy intake were controlled (Nagata et al. 2003). Interesting results from a randomized crossover trial by Jenkins et al. (2002) revealed that consumption of low- (10 mg/d) but not high-isoflavone foods (73 ± 3 mg/d) for one month decreased Hcy concentration. However, when the authors pooled data from low- and high-isoflavone phases,

there was a significant decrease in plasma Hcy (P=0.044) during the soy phase but not the control phase.

Evaluating the molecular mechanism underlying the protective effect of isoflavones, Fuchs et al. (2006) found that both an extract of soy protein and a combination of two isoflavones, daidzein and genistein, in a concentration similar to the native soy extract, inhibited Hcy-induced apoptosis of endothelial cells to the same extent. However, the soy protein extract and the isoflavones affected different cellular target proteins. In an earlier study by the same group, Fuchs et al. (2005) showed that genistein reversed Hcy (25 μmol/L)-induced change in proteins involved in metabolism, detoxification, and gene regulation. Additonally, genistein inhibited Hcy-mediated apoptotic cell death as indicated by inhibition of DNA fragmentation and chromatin condensation.

Isoflavones from sources other than soy have been researched as well. In a 12 week intervention in 25 menopausal women suffering from severe hot flushes and night sweats, consumption of a combination of isoflavones from kudzu and red clover decreased hot flushes by 46% and there was a modest but not significant improvement in Hcy (Lukaczer et al. 2005). Additonally, the ratio of 2-hydroxyestrone-to-16 alpha-hydroxyestrone, a proposed marker of breast cancer risk, also improved significantly.

b. C-reactive protein and isoflavones

Because of the structural and functional similarity that isoflavones share with estrogen, it has been hypothesized that isoflavone consumption might exert a cardioprotective effect. The reduction in circulating inflammatory marker concentrations by estrogen may be one of the mechanisms by which premenopausal women are protected against CVD (Hall et al. 2005). In a double-blind, placebo controlled study in postmenopausal women (N=117) consuming soy-derived isoflavones (50 mg/d) for eight weeks, isoflavones lowered elevated (>1 mg/L) CRP (Hall et al. 2005). In contrast, Nikander et al. (2003) found that isoflavone (114 mg/d) intake had no effect on CRP in postmenopausal women (N=56) with a history of breast cancer. Likewise, Jenkins et al. (2002) reported no significant effects of a high- (73 mg/d) or low-isoflavone soy-based diet for one month on CRP in postmenopausal women. Similarly, D'Anna et al. (2005) determined that genistein (54 mg/d) supplements consumed by healthy postmenopausal

women (N=90) for six months had no effect on CRP. Consistent with these findings, neither dietary soy isoflavones nor conjugated equine estrogen had any effect on CRP determined at the end of a three-year study in ovariectomized monkeys consuming a moderately atherogenic diet (Register et al. 2005).

Hilpert and coauthors (2005) showed that a diet containing 25 g/d soy protein (+ 90 mg/d isoflavones) did not produce a change in CRP compared to a control diet in moderately hypercholesterolemic men (N=18) and postmenopausal women (N=14) receiving hormone therapy. Similar work by Teede et al. (2004) in postmenopausal women and by McVeigh et al. (2006) in healthy young men found no effect of soy protein isolate with different doses of isoflavones on circulating CRP concentration. Additionally, Ryan-Borchers and colleagues (2006) also found that neither soy milk with isoflavones nor isoflavones provided as a supplement for 16 weeks had any effect on CRP in healthy postmenopausal women. However, there were significant changes in markers of oxidative stress, suggesting a protective effect of isoflavones.

2. Osteoporosis and Isoflavones

In the post era after the publication of the Heart and Estrogen/Progestin Replacement Study and WHI trials, soy isoflavones have increasingly been studied as a safe alternative to hormone therapy for bone loss. The low rate of hip fracture in Asian women has been hypothesized to be due to the purported effect of soy isoflavones on bone (Ho et al. 2003). However, the lower rate of hip fracture is likely due to the shorter hip axis length and shorter stature of Asian women (Chin et al. 1997). Studies conducted in postmenopausal women have examined biochemical markers of bone turnover and change in bone loss via changes in BMD and BMC at the lumbar spine and hip.

In one of the first randomized, double-blind clinical trials on the effect of isoflavones on bone, Alekel and coworkers (2000) demonstrated (N=69) that 40 g/d soy protein attenuated bone loss from the lumbar spine of perimenopausal women, whereas the whey protein control group had significant loss. Regression analysis showed that the active arm had a positive effect on change in BMD (5.6%; P=0.023) and BMC (10.1%; P=0.0032). Contrast coding using analyses of covariance with BMD or BMC as the outcome showed that isoflavones, not soy protein, exerted this favorable effect. In a randomized, double-blind,

placebo-controlled trial by Lydeking-Olsen et al. (2004) in postmenopausal women (N=107), significant lumbar spine bone loss occurred in the control group ($P<0.01$) and in the group that consumed isoflavone-rich soy milk and applied progesterone cream ($P<0.01$ for BMD and $P<0.05$ for BMC). The only beneficial effect of the soy only treatment was that it prevented bone loss in the lumbar spine. No changes were seen in the femoral neck BMC and BMD or in markers of bone formation and resorption among different treatments. Potter et al. (1998) studied hypercholesterolemic, postmenopausal women (N=66) in which they reported a significant ($P<0.05$) increase in the lumbar spine BMC and BMD at the end of 24 weeks in the 90 mg soy isoflavone group. Again, there was no change in the proximal femur (hip) region. Chen et al. (2003) examined the effect of two doses of soy isoflavones (40 mg and 80 mg) compared to a placebo in postmenopausal Chinese women (N=203). After one year, researchers found an increase in BMC at the total hip and trochanter in the 80 mg isoflavone group ($P<0.05$). Further analysis revealed that the positive effect of soy isoflavones was observed only among women with lower (median or less) initial baseline BMC. In contrast, Kreijkamp-Kaspers et al. (2004) found no effect of 99 mg of soy isoflavones on BMD of spine or hip in older (60-75 years) postmenopausal women (N=175). Arjmandi et al (2005) examined the effect of an active treatment containing 25 g soy protein with 60 mg of soy isoflavones compared to a control. There was a decrease (albeit not significant) in whole body and lumbar spine BMC and BMD in both soy and control groups. These researchers reported an increase in serum markers of bone formation in both treatments (casein control vs. soy, respectively): bone-specific alkaline phosphatase activity (28% vs. 26%), insulin-like growth factor-I (13% vs. 26%), and osteocalcin (95% vs. 103%). However, there was no change in either group in urinary deoxypyridinoline, a marker of bone resorption.

Several studies have examined changes in markers of bone formation and resorption. In an Iranian study, postmenopausal women were supplemented with 35 g of soy protein containing 98.3 mg isoflavones daily for 12 weeks. They found an increase in total serum alkaline phosphatase, a non-specific and insensitive marker of bone formation, and a decrease in urinary deoxypyridinoline, a marker of bone resorption (Roudsari et al. 2005). In other similar studies using 40 mg of isoflavones, there were either no significant differences (Dalais et al. 2003) or no effect on markers of bone resorption (Mori et al. 2004). In a

randomized, placebo-controlled crossover clinical trial, Harkness et al. (2004) supplemented postmenopausal women (N=19) with 110 mg/day isoflavones for a period of six months. They found a 37% decrease in urinary concentrations of type 1 collagen alpha1-chain helical peptide, a marker of bone resorption, and a significant ($P<0.05$) increase in mean spine BMD.

A few observational studies have examined the relationship between intake of soy foods and BMD or fracture risk. Kritz-Silverstein and Goodman-Gruen (2002) examined the relationship between dietary isoflavone intake and bone density at the lumbar spine and hip and markers of bone turnover in postmenopausal women (N=208). They reported that after adjustment for covariates, there was a trend ($P=0.07$) whereby women with the highest level of isoflavone consumption had greater bone density at the spine. In another study, Somekawa et al. (2001) found that after adjustment for years since menopause and weight, lumbar spine BMD was significantly higher in the highest quartile (50– ≥65 mg/day) compared with lowest intake quartile (<35 mg/day) of isoflavone intake. Another study in early mid-life (30-40 years) Hong Kong Chinese women (N=132) observed a significantly higher spinal BMD in women in the fourth verses first soy isoflavone intake quartile after adjusting for covariates (Ho et al. 2001). Multiple linear regression analysis showed that soy isoflavones, together with known covariates, accounted for 24% of the variance in spinal BMD. Likewise, baseline data analysis from the Study of Women's Health Across the Nation, a US community-based cohort study in 42-52 year old women (Greendale et al. 2002), revealed that Japanese premenopausal women in the highest tertile of genistein intake had 7.7% and 12%, respectively, greater spine and femoral neck BMD compared to women in the lowest tertile. Among Chinese women, no association was found between genistein intake and BMD, probably because their median intakes (3511 μg/day) were nearly half that of the Japanese women (7151 μg/day). Zhang and colleagues (2005) examined the relationship between usual soy food consumption and fracture incidence in 24,403 postmenopausal women who had no history of fracture or cancer in the Shanghai Women's Health Study. After adjustment for age, major risk factors for osteoporosis, socioeconomic status, and other dietary factors, the relative risk of fracture ranged from 0.63 to 1.00 ($P<0.001$ for trend) in the highest to lowest quintiles respectively of soy protein intake, with a more pronounced

inverse association among women in early menopause. Similar results were also found for isoflavones.

Taken together, results of these studies suggest that isoflavones may prevent bone loss from the lumbar spine in postmenopausal women, who may otherwise be expected to lose 2-3% of bone yearly. Such attenuation of bone loss, particularly if started during the early postmenopausal years and continued through menopause, could translate into a decreased risk for osteoporosis. Since a bone-remodeling cycle ranges from 30-80 weeks (Heaney 1994), a long-term clinical trial is needed to determine whether soy isoflavones will prevent bone loss and possibly favor bone formation.

H. SUMMARY

In summary, CVD and osteoporosis are major public health problems that frequently coexist and share a common mechanistic link. Hcy and CRP are emerging as novel risk factors for both CVD and osteoporosis in mid-life women. These novel risk factors also have common mechanisms for their effect on atherosclerotic CVD. These include: 1) endothelial cell damage and dysfunction, 2) monocyte activation and recruitment, and 3) vasodilation. However, Hcy and CRP are thought to affect osteoporotic risk by different mechanisms that are not yet completely understood. Additionally, there have been no studies examining the relationship between Hcy and CRP and trabecular bone, the type of bone that is preferentially lost with menopause. Our research team is currently investigating the relationship between total Hcy, CRP, cortical, and trabecular bone in healthy postmenopausal women.

Hormone therapy has a favorable effect on Hcy but tends to increase CRP concentration in mid-life women. Alternatives for hormone therapy that reduce CVD and osteoporotic risk but do not impose further risk, as is associated with hormone therapy, are currently being investigated. There have been a paucity of population-based data on the effects of these alternative forms of therapy on postmenopausal women. Soy protein, particularly in combination with isoflavones, has been documented to produce an improved lipid profile. Isoflavone supplementation has been shown to have a modest bone-sparing effect but there have been few well-designed studies of sufficient duration to reach definitive

conclusions. More research is needed to determine whether isoflavones preserve or perhaps increase BMD in mid-life women.

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Relationship of Trabecular and Cortical Bone to Circulating Total Homocysteine and C-Reactive Protein in Postmenopausal Women

A paper to be submitted to the Journal of Clinical Densitometry

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ABSTRACT

Background: Homocysteine (Hcy) and C-reactive protein (CRP) are established risk factors for atherosclerotic cardiovascular disease and are emerging as novel risk factors for osteoporosis.

Objectives: The primary purpose of this study was to determine whether total Hcy and CRP concentrations are associated with trabecular and/or cortical bone mineral content (BMC) or bone mineral density (BMD) in postmenopausal women. The secondary objective was to determine the body composition and nutritional status indices (dietary and circulating) and key biologic factors related to total Hcy and CRP. The tertiary purpose of this study was to examine changes in total Hcy and CRP over one year.

Methods: We enrolled healthy postmenopausal women (N=242) as part of a randomized, double-blind, placebo-controlled multi-center clinical trial designed to examine the effect of two doses of isoflavones extracted from soybeans on bone loss over three years in early postmenopausal women. We assessed volumetric BMD at the distal tibia and femur (1/3 site) using peripheral quantitative computed tomography (pQCT) in a subset of women (N=184 for distal tibia; N=237 for 1/3 femur site). Total proximal femur and lumbar spine (L1-L4) BMC and BMD, as well as overall body composition, were assessed via dual-energy x-ray absorptiometry. We assessed plasma total Hcy, serum CRP, intracellular folate, serum folate, and vitamin B_{12}.

Results: Total Hcy and CRP did not contribute to the variability in trabecular BMC of the distal tibia or cortical BMC of the 1/3 femur site using pQCT. Approximately 22% of the variability in trabecular BMC was accounted for by weight, hemoglobin, serum uric acid, and

blood glucose. Study site, weight, and age accounted for about 14% of the variability in cortical BMC. The overall variability (19%; $p \leq 0.0001$) in total Hcy was accounted for by serum vitamin B_{12} and creatinine; the overall variability (28%; $p \leq 0.0001$) in CRP was accounted for by serum iron, overall percent body fat, serum uric acid, triglycerides, and white blood cell count. Total Hcy and CRP increased, while serum vitamin B_{12}, serum folate, and intracellular folate decreased over a one year period.

Conclusion: Total Hcy and CRP were not related to trabecular or cortical bone, but this may be because these women were healthy and non-osteoporotic. Since hemoglobin was a significant contributor to trabecular BMC, while iron was a significant contributor to CRP, it is possible that inflammation may mediate the relationship between iron and trabecular BMC.

Key Words: Homocysteine, C-reactive protein, pQCT, trabecular bone, cortical bone, osteoporosis

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INTRODUCTION

Osteoporosis and atherosclerotic cardiovascular disease (CVD) are major public health problems that often coexist and account for significant morbidity and mortality in postmenopausal women. The postmenopausal period typically occupies one-third of a woman's life, with more than 40 million women in the United States now in the postmenopausal phase (Hargrove and Eisenberg 1995). Homocysteine (Hcy) is emerging as a novel risk factor for osteoporosis in mid-life women, with the increase in osteoporosis among those with homocystinuria suggesting that a high serum Hcy concentration may weaken bone (van Meurs et al. 2004). Typically, Hcy increases with age (Ganji and Kafai 2003) and is higher in post- than premenopausal women (Hak et al. 2000). Additionally, estrogen

deficiency during menopause is associated with increased release of bone-resorbing cytokines, particularly interleukin-6 (IL-6) by bone marrow cells (Bismar et al. 1995). Atherogenesis and osteoporosis consist of an inflammatory component, thus providing the biological plausibility for the potential use of CRP, a proinflammatory risk factor for CVD, as a marker of both atherogenesis and osteoporotic risk.

At the time these data were analyzed, no studies were published to examine the effect of total Hcy and CRP on trabecular and cortical bone separately. This is of primary importance as trabecular bone reflects metabolically active bone, which is preferentially lost during menopause and thus is of interest in assessing early osteoporotic risk (Takagi et al. 1995). Peripheral quantitative computed tomography (pQCT) offers a novel, noninvasive (Grampp et al. 1995), accurate (Ebbesen et al. 1997), and precise (Sievanen et al. 1998) method to assess volumetric BMD, bone size, cortical geometry (Rauch et al. 1999), and apparent trabecular structure and connectivity (Takagi et al. 1995). In addition, determination of volumetric (three dimensional) BMD via pQCT provides a more accurate assessment of trabecular bone than areal (two dimensional) BMD via dual energy x-ray absorptiometry (DXA). The purpose of this analysis was to investigate the relationship of Hcy and CRP to trabecular and cortical bone in a sample of healthy postmenopausal women. We hypothesized that Hcy would be inversely associated with trabecular bone, whereas CRP would be inversely related to either trabecular or cortical bone as assessed by pQCT. We determined the body composition and nutritional status indices (dietary and circulating) and key biologic factors related to total Hcy and CRP. We also assessed change in CRP, total Hcy, serum vitamin B_{12}, serum folate, and intracellular folate over the course of one year in these postmenopausal women.

MATERIALS AND METHODS

Research Design

We enrolled healthy postmenopausal women (45.8-65.0 years of age) as part of a randomized, double-blind, placebo-controlled multi-center (Iowa State University [ISU], Ames, IA and University of California at Davis [UC-Davis], Davis, CA) clinical trial. This parent study (**S**oy **I**soflavones for **R**educing **B**one **L**oss; SIRBL) is designed to examine the

effect of two doses of isoflavones extracted from soybeans on bone loss during the course of three years in 242 at-risk early postmenopausal (e.g., less than 10 years since their last menses) women (Elders et al. 1989). Eligible participants (non-osteoporotic, without diseases or conditions, not taking hormones or medications) have been randomly assigned to one of three treatment groups: isoflavone-rich soy extract with 80 mg of isoflavones, with 120 mg of isoflavones (aglycone form), or placebo without isoflavones. We have collected and are in the process of collecting data from women in the laboratory at baseline, six, 12, 24, and 36 months. As part of an ancillary project, this report examines total Hcy and CRP at baseline and at six and 12 months. We are also examining the relationship between total Hcy and CRP and bone (trabecular and cortical) using pQCT.

Subject Screening, Selection, and Characteristics

We recruited subjects throughout the state of Iowa and the Sacramento region in California through direct mailing lists, stories in local newspapers, local/regional radio advertisements, a story on a local television channel, community announcements, cooperative extension nutrition and health field specialists, and mailings/flyers at local school systems, medical centers/clinics, grocery stores, university campus, public libraries, local women's groups, and local businesses. We screened women who responded (N=5,255) to outreach materials initially via a telephone questionnaire to identify healthy women who went through a natural menopause (cessation of menses nine months to ten years), were ≤ 65 years of age, non smokers, and had a body mass index (BMI, kg/m^2) ranging from 18.5 through 29.9 (inclusive). We excluded women with currently diagnosed or previous history of bone disease, renal disease, urinary stones, cancer, malignancy or tumor of any kind, cardiovascular disease, diabetes mellitus, respiratory disease, parathyroid disease, thyroid or liver disease, and/or those women who had a first-degree relative with breast cancer. Additionally, we also excluded women currently using chronic medications, such as cholesterol-lowering or anti-hypertensive medications, or women who refused to cease taking herbal therapies and/or nutritional/dietary supplements. We also excluded vegans and high alcohol consumers (>7 servings per week). Use of oral hormone or estrogen therapy, selective estrogen receptor modulators, or other hormones within the last 12 months, use of estrogen or progestogen creams or calcitonin within the last six months, use of antibiotics

within the last three months, and/or any previous use of bisphosphonates were grounds for exclusion. We also excluded subjects with excessive vasomotor symptoms from the study. Because the purpose of the parent study is to examine the effect of isoflavones on bone, subjects were required to consume one of three treatments daily for three years, discontinue use of non-study nutritional supplements, and avoid consuming isoflavone-rich foods.

Women who met the initial criteria via telephone (N=677) attended a pre-baseline appointment at the testing center to determine additional entry criteria. We measured height and weight to confirm BMI status. We used DXA to determine BMD of the lumbar spine and left proximal femur. Women with evidence of osteopenia or osteoporosis based on lumbar spine and/or proximal femur BMD (using >1.5 SD below the young adult mean as cut-off) and women with evidence of previous or existing spinal fractures were excluded. We also excluded women with spine and/or femur BMD >1.0 SD above the mean. If a woman qualified based on her BMD, our phlebotomist drew blood for a chemistry profile. We excluded women if their fasted blood values indicated diabetes mellitus (fasting blood glucose $\geq$126 mg/dl), abnormal renal, liver and/or thyroid function, or abnormal lipid profile (low density lipoprotein-cholesterol >160 mg/dl; triacylglycerol >200 mg/dl). To be qualified for the study, each woman needed to undergo a transvaginal ultrasound at area clinics (ISU: McFarland Clinic, Ames IA; UC-Davis: Mather VA, Mather, CA). Each woman was required to have an endometrial thickness $\leq$ 5.0 mm to qualify. However, women with an endometrial thickness between 5.0 and 6.0 mm had to undergo an endometrial biopsy that proved normal to be considered eligible. We enrolled subjects who met all entry criteria (N=242) in the parent study (**Figure 1**).

The study protocol, consent form, and subject-related materials were approved by ISU Human Subjects Review Committee (Institutional Review Board; IRB ID# 02-199) and the UC-Davis IRB (ID# 200210884-2). Approvals for the DXA and pQCT procedures were obtained from each institution's IRB and appropriate safety boards. We obtained informed consent from all women at the start of pre-baseline screening.

Data Collection

At the pre-baseline visit, trained interviewers administered three questionnaires: nutrition history, health and medical history, and reproductive history. Each subject filled out

a historical physical activity questionnaire at home; this questionnaire was reviewed by interviewers during the baseline visit for completeness. Estrogen exposure (in years) was calculated for each subject by subtracting her age at menarche from her age at her last menstrual cycle. At the baseline testing appointment, we assessed dietary intake using a semi-quantitative food frequency questionnaire from Block Dietary Data Systems (Berkeley, CA) and soy food intake using a soy food questionnaire (Kirk et al. 1999). A trained anthropometrist at each geographic site measured standing and sitting heights (Ayrton stadiometer, Model S100; Ayrton Corp., Prior Lake, MN); weight (abco Health-o-meter; Health-o-meter Inc., Bridgeview, IL); waist, hip, and abdominal circumferences; sagittal diameter (Holtain-Kahn abdominal caliper; Holtain Ltd., Crosswell, Crymych Dyfed, U.K.); and skeletal widths/breadths at the shoulder (biacromial), hip (biiliac), thigh (bitrochanteric) (Harpenden skeletal anthropometer; Holtain Ltd., Crosswell, Crymych, Dyfed, UK), and elbow (Gneupel Swiss made SERITEX; Rutherford, New Jersey) using anthropometers. We also measured resting heart rate and blood pressure using an automatic digital sphygmomanometer (HEM-907; Omron Healthcare, Inc., Vernon Hills, IL) after the subject sat quietly for at least 20 minutes. We also assessed balance and propensity to fall (using a one-foot stance balance test) and torso, quadriceps, and grip strength using two dynamometers (back strength [torso and quadriceps]: TKK 5002 Back-A; Takei Scientific Instruments Co., Ltd, Japan; grip strength [0 to 100 kg]: Lafayette Instrument Co., Lafayette, IN). Each woman collected a 24-hour urine sample the day before her testing appointment. Urine samples were thoroughly mixed and aliquots were frozen at -20°C for further analyses. Phlebotomists collected fasted (9 hours) blood samples between 7:00 and 8:00 a.m. We separated serum and plasma from whole blood by centrifuging for 15 minutes (4°C) at 1000 x g and stored aliquots at -80°C until analyses.

Bone and Body Composition Measurements

We assessed volumetric BMD at the distal tibia and femur (1/3 site) using pQCT (XCT 3000; STRATEC Medizintechnik, Pforzheim, Germany, Division of Orthometrix; White Plains, NY) in a subset of women (ISU=122, UC-Davis=62 for distal tibia; ISU=122, UC-Davis=115 for femur 1/3 site). Total proximal femur and lumbar spine (L1-L4) bone mineral content (BMC) and BMD were assessed via a Delphi W DXA (Hologic, Inc;

Bedford, MA) instrument. In addition, whole body DXA scans provided total body composition.

Matching instruments at each site and daily calibration ensured that the pQCT and DXA instruments provided comparable results. One operator at each site performed all pQCT and DXA scans. Cross-training for pQCT and DXA scanning between sites has ensured comparable quality control. Laboratory personnel at each site were trained by the manufacturers' technicians and received further training on pQCT software analysis (Bone Diagnostic, Inc.; Fort Atkinson, WI). A research assistant at UC-Davis performed all pQCT scan analyses following guidelines provided by Bone Diagnostic, Inc. The ISU DXA operator performed all DXA scan analyses following Hologic guidelines for BMC, BMD, and overall body composition using software version 12.3:7. The procedures were approved by the State Department of Public Health in both Iowa and California. The ISU laboratory within-subject (n=10 per skeletal site) *in vivo* precision error (coefficient of variation) for pQCT and DXA measurements is presented in **Table 1**.

Laboratory Measurements

Total Hcy concentration was determined using a high performance liquid chromatography (HPLC) method adapted from Araki and Sako (1987) and Ubbink et al. (1991). The total Hcy in plasma consists of free Hcy (i.e., reduced plus oxidized Hcy in the non-protein fraction of plasma) and protein-bound Hcy (Araki and Sako 1987). The thiol compounds in heparinized plasma, which were reduced or liberated from plasma proteins with tri-n-butylphosphine (Sigma, St. Louis, MO), were derivatized with a thiol-specific fluorogenic reagent, ammonium 7-flourobenzo-2-oxa-1,3-diazole-4-sulphonate (Sigma, St. Louis, MO). *N*-Acetylcysteine (1 mM) was added to the plasma samples prior to derivatization as an internal standard. All derivatized samples were filtered (ISO-DISC PTFE-4-2, 4 mm x 0.2 μm; SUPLECO, Bellefonte, PA) into vials and frozen at -20°C until use. Derivatized samples (100 μl) were analyzed on HPLC (Beckman Coulter System Gold 126 Solvent Module and Beckman Coulter System Gold 508 Autosampler; Fullerton, CA) using a reverse LC-18 column (SUPELCOSIL™; 25cm x 4.6mm, 5μm; SUPLECO, Bellefonte, PA), protected by an LC-18 guard column (SUPELCOSIL™ LC-18 Supelguard® Cartridge; SUPLECO, Bellefonte, PA). The following solvents were used: (A) acetonitrile

(B) 0.1 M potassium phosphate (monobasic) buffer (pH 1.75 adjusted with o-phosphoric acid) in HPLC water. The buffers were filtered through a MAGNA, Nylon, Supported Plain, 0.22 micron, 47 mm filter just prior to use. The final pH of the 0.1 M potassium phosphate (monobasic) buffer after filtering was 2.1. The initial starting conditions were 96% solvent B and 4% solvent A at a flow rate of 1.0 ml/min. Samples were introduced with an injection valve fitted with a 100 µl sample loop. At 14.10 minutes, a one minute gradient from 96% to 84% for solvent B was applied and allowed to pump for 10 minutes. At 25.10 minutes, B was increased to 96% over five minutes and pumped for a 10 minute requilibration period before the next sample was injected. The fluorescence intensities were measured with excitation at 385 nm and emission at 515 nm, using a JASCO FP-1520 fluorescence detector. At the end of each sequence, we performed a wash for 160 minutes using 100% HPLC grade water at 1.0 ml/min. At the end of 160 minutes, we changed the pumps to 50% water and 50% acetonitrile for one hour at a flow rate of 1.0 ml/min. At the end of 220 minutes, initial conditions were restored with a five minute gradient back to 96% solvent B and 4% solvent A and allowed the system to requilibrate. The intraassay variability was 3.8% and the interassay variability was 6.3%.

CRP concentration was determined in serum using a high sensitivity sandwich enzyme-linked immunosorbent assay kit (ALPCO Diagnostics; Salem, NH). Intracellular folate, serum folate, and serum vitamin B_{12} were measured using a radioactive immunoassay kit (MP Biomedicals; Irvine, CA). We used manufacturer-provided and in-house quality controls. The intraassay variability for CRP, intracellular folate, serum folate, and serum vitamin B_{12} are 3.5%, 3.2%, 3.4%, and 3.4%, respectively. The interassay variability for CRP, intracellular folate, serum folate, and serum vitamin B_{12} are 6.0%, 11.8%, 10.1% and 10.1%, respectively. Blood samples were analyzed by a certified clinical laboratory (LabCorp; Kansas City, Kansas at the ISU site and the UC-Davis Medical Center Laboratory; Sacramento, CA at UC-Davis site) for a complete blood count (CBC) with differential, general chemistry panel (ChemScreen), and thyroid screen (thyroid stimulating hormone (TSH) with reflex to free thyroxine (T_4) if TSH was abnormal).

Subject Follow-up

For the parent project, women underwent serial testing through three years of treatment. However, we are reporting data only through the first year in this ancillary project. Since the parent project follows an intent-to-treat model, our current analyses included all eligible women at baseline, regardless of whether or not they were compliant with study protocol. This study includes three women who did not take treatment tablets, but returned for testing visits as part of the intent-to-treat model: one was diagnosed with breast cancer (ISU), one experienced a severe gastrointestinal adverse event (UC-Davis), and one chose to consume soy products rather than treatment tablets (UC-Davis). In addition, 13 women were lost to follow-up: two at ISU after baseline, seven at UC-Davis after baseline, and four at UC-Davis after six months. Thus, the sample size for this study varied across time points: N=242 at baseline, N=233 at six month, N=229 at 12 month (**Figure 1**).

Power Analysis and Statistical Analyses

Power analysis for this study is based on the NIAMS-funded project (#RO1 AR046922-01 A2), reflecting a random effects repeated measures (Laird and Ware 1982) regression model of change in lumbar spine BMD via DXA as the outcome variable. Our sample size is fixed at 184 for this project. Statistical analyses were performed using SAS (version 9.1; Cary, NC) with results considered statistically significant at $p \leq 0.05$. Descriptive statistics included means for normally distributed data (age, years since menopause, estrogen exposure, body size, body composition, and bone data) and medians for data that were not normally distributed (total Hcy, serum CRP, and dietary intake). We excluded CRP values >10 mg/L for all inferential statistics, based on recommendations from the Centers for Disease Control and the American Heart Association (Pearson et al. 2003). To examine the relationship between total Hcy and CRP versus bone, we used Spearman's rho correlation analyses. In all regression models, study site was included as an obligatory variable to account for potential site differences. In modeling the outcomes of interest, we removed variables that exhibited multicollinearity as indicated by the variance inflation factor. We performed multiple regression analyses to assess the combined contribution of factors related to volumetric BMC and BMD (weight, age, blood glucose, hemoglobin, serum uric acid, plasma total Hcy, serum CRP, and total calcium [dietary plus supplemental] intake). Classes

of variables in modeling the outcomes of baseline trabecular and cortical BMC and BMD included all covariates that were biologically plausible and/or significantly related to bone in the Spearman's rho correlation analysis. We have presented the best models for trabecular and cortical BMC because these models were better than the BMD models. Given that total Hcy and CRP are important CVD risk factors that also possibly contribute to osteoporotic risk, we examined contributing factors to total Hcy and CRP. The following variables were initially included in the model: serum vitamin B_{12}, serum or intracellular folate, serum creatinine, serum uric acid, and specific dietary factors (vitamin B_6; carbohydrate [because grains are fortified with folate]; magnesium, phosphorus, dietary or total calcium, and vitamin C [as indicators of an overall adequate diet]; retinol; and riboflavin). In modeling CRP, we initially included the following variables: age, whole body percent fat, waist circumference, white blood cell count, serum iron or total iron binding capacity, serum uric acid, and total cholesterol/HDL-C ratio. Similar to the BMC models, we included all variables that were biologically plausible and/or significant in the Spearman's rho correlation analysis for total Hcy and CRP. To determine whether change over the course of time (baseline to one year) was significantly different from zero for CRP and total Hcy (as well as intracellular folate, serum folate, and vitamin B_{12}), we performed a matched pair analysis. Since these circulating variables were not normally distributed, we performed a Wilcoxon signed-rank test. It is important to note that the matched pair analysis does not take treatment into account; these results are presented merely as a basis for discussion.

RESULTS

Subject Characteristics

The baseline characteristics of women are described in **Table 2** and **Table 3**. At the start of this study, women were 65 or younger and were within 10 years of menopause. The majority of women were Caucasian at each site. The vast majority of the subjects reported that they were not vegetarians, with only four being vegetarian (three pesco, one lacto-ovo). More than half of the subjects reported a family history of cardiovascular disease, while one third reported a family history of osteoporosis and/or a personal history of bone fracture. The

most common form of hormone therapy use was combined estrogen plus progestogen therapy, with a wide range of duration in general for any formula.

Approximately half of the women had a BMI <25.0 kg/m^2. Although the mean BMI was comparable between the two sites, there was greater variability in BMI at UC-Davis. This was because at UC-Davis two women were below the exclusion criteria of 18.5 and eight women were above the exclusion criteria of 29.9. Consequently, body composition measures, particularly those reflecting the fat compartment, were more variable at the UC-Davis than at the ISU site. Integral bone (cortical and trabecular) measurements assessed via DXA and volumetric bone measurements (distal tibia reflecting trabecular bone and 1/3 femur site reflecting cortical bone) assessed via pQCT are shown in are presented in **Table 4**. In examining the DXA data and the pQCT data, it appears that the UC-Davis BMC data have greater variability than the ISU data, whereas this greater variability is no longer apparent for the BMD data at parallel bone sites. Thus, once bone area was taken into account, the variability differences in the BMD data are no longer apparent. All bone measures were normally distributed and thus mean values are reported. We reported median values on circulating analytes as they were not normally distributed (**Table 5**). Approximately 48% of the subjects had normal CRP (<1 mg/L), 36% had moderately elevated CRP (1-3 mg/L), and 16% had high CRP (>3 mg/L) values. We conducted intracellular folate analysis on 230 women (seven samples at ISU were incorrectly processed; one sample at UC-Davis was missing; two at ISU and two at UC-Davis were missing hematocrit values). The median dietary intake for carbohydrate, protein, riboflavin, vitamin B_6, and vitamin B_{12} approximated the dietary reference intakes (DRIs) for each of these nutrients. However, this was not the case for fiber, vitamin A, vitamin E, folic acid, magnesium, or calcium. The median total calcium (dietary and supplemental) intake at baseline was 1185 mg daily (**Table 6**) approximating the calcium DRI (1200 mg/day) for postmenopausal women. At both sites combined, lifetime calcium intake from dairy products decreased consistently from childhood, when milk intake was the highest, to mature adulthood, with this decline being particularly pronounced for the UC-Davis women. The percentage of women who took calcium supplements was 61.5% at ISU and 67.5% at UC-Davis.

Correlation Analyses

Correlation coefficients between bone measurements versus CRP and total Hcy are shown in **Table 7**. The highest positive correlation (r=0.192, p=0.003) was observed between CRP and total proximal femur BMD assessed via DXA. In addition, CRP was also correlated with distal tibia trabecular BMD (r=0.178, p=0.017) assessed via pQCT. Although our women did not report any chronic diseases, approximately 32% (n=77) had CRP values >1.8 mg/L. In a subsample analysis including women with elevated CRP (>1.8 mg/L), Spearman rho's correlation analysis revealed that CRP was significantly and negatively correlated with spine BMC (r= -0.227; $p \leq 0.05$). We found no relationship between total Hcy and bone measurements in these healthy postmenopausal women.

Regression Analyses

Since trabecular bone, cortical bone, total Hcy, and CRP were our primary outcomes of interest, we performed further analyses to examine factors contributing to their variability. Since the BMC was better than the BMD model for both trabecular and cortical bone, we have presented only the BMC regression models. Factors contributing to the variability of trabecular and cortical BMC are presented in **Table 8**. We initially included the following variables in the model: age, blood glucose, hemoglobin, serum uric acid, weight, serum CRP, and total calcium. After variable elimination was completed, multiple regression analyses revealed that 22% of the variability in trabecular BMC (F=9.59, $p \leq 0.0001$) was accounted for by hemoglobin, uric acid, weight, and fasting blood glucose. Although site did not reach significance, we kept site in the model to account for potential site differences. Serum uric acid (p=0.0027) contributed negatively to trabecular BMC. In contrast, baseline body weight ($p \leq 0.0001$) and hemoglobin (p=0.0006) contributed positively to trabecular BMC. Variables that remained in the model (except site) were significant at $p<0.10$. We used the same variables for the cortical BMC at 1/3 femur site that were initially included in the trabecular BMC model. Site, age, and weight emerged as significant positive contributors to cortical BMC and together explained 14% of the variability in cortical bone (F=12.54, $p \leq 0.0001$). Site accounted for the greatest proportion of the variability (9%), while age and weight contributed a smaller percentage.

In the final total Hcy model (**Table 9**), approximately 19% of the variability (F=17.72, $p \leq 0.0001$) was accounted for by serum vitamin B_{12} (12%) and creatinine (6%). Serum vitamin B_{12} ($p \leq 0.0001$) contributed negatively to total Hcy concentration. In the final CRP model (**Table 9**), whole body percent fat (5%), uric acid (3%), and triglycerides (3%) were the most significant and positive contributors to circulating CRP concentration. Additionally, white blood cell count (2%) was positively and serum iron (2%) was negatively related to CRP.

Longitudinal Analyses

The mean change in serum CRP, plasma total Hcy, intracellular folate, serum folate, and serum vitamin B_{12} during the course of one year is shown in **Figure 2**. Wilcoxon signed-rank test revealed that CRP decreased significantly from baseline to six months (-0.2 mg/L; p=0.05) but increased overall from baseline to one year (0.3 mg/L; p=0.0035). On the other hand, total Hcy increased significantly at the end of six months but did not change significantly thereafter. Similarly, serum folate, intracellular folate, and vitamin B_{12} decreased significantly by six months but no change was observed after six months.

DISCUSSION

Menopause contributes to significant risk in developing osteoporosis (Scheiber and Rebar 1999) and atherosclerotic CVD (Welty 2001) in postmenopausal women. Many risk factors including elevated Hcy (Hak et al. 2000) and CRP emerge after menopause. To our knowledge, this is the only published study examining the relationship between total Hcy and CRP versus trabecular and cortical bone in healthy postmenopausal women. A couple of studies have related high total Hcy to a low BMD (Gjesdal et al. 2006; McLean et al. 2004). However, no studies have examined the relationship between total Hcy and trabecular or cortical bone separately.

Contrary to previous findings (Gjesdal et al. 2006; McLean et al. 2004), we did not demonstrate a relationship between circulating total Hcy and integral bone. Although we hypothesized that total Hcy would be inversely associated with trabecular bone, correlation analysis did not reveal any association between total Hcy and trabecular bone (r=-0.041, p=0.58). However, the vast majority of women in our study had normal total Hcy, whereas

previous epidemiological studies have demonstrated an increased risk of osteoporotic fracture only in subjects with high Hcy (>15 μmol/L). For example, in the Hordaland Homocysteine Study (Gjesdal et al. 2006), the multiple adjusted odds ratio for low BMD was 1.96 in those with high (≥15 μmol/L) compared with low (<9 μmol/L) Hcy. Similarly, the Framingham Study (McLean et al. 2004) showed that subjects in the highest quartile (18.6±6.4 μmol/L) had a greater risk of hip fracture than those in the lowest quartile (7.6±1.0 μmol/L) of total Hcy. Plasma total Hcy concentration was slightly higher (7.6 ± 2.1 μmol/L) in our study than that recently reported for women (6.8 ± 0.1 μmol/L) from the 2001-2002 NHANES data (Ganji and Kafai 2006), but was comparable to the lowest quartile of total Hcy for women (7.6±1.0 μmol/L) in the Framingham Study (Gjesdal et al. 2006). Only four women in our study had high (≥15 μmol/L) plasma total Hcy, possibly explaining why we did not detect a relationship between Hcy and bone in these healthy postmenopausal women.

Nonetheless, we identified a number of known and unconventional factors that contributed to the variability in trabecular BMC. Hemoglobin emerged as a strong contributor to trabecular but not cortical BMC. This may be because trabecular bone is more highly vascularized, is well innervated, and is responsible for hematopoiesis and mineral homeostasis. Subjects with a higher hemoglobin concentration may have better oxygen delivery to all tissues, including bone, thereby increasing trabecular BMC. Reporting similar results, Cesari et al. (2005) concluded that hemoglobin was significantly (positive direction) associated with total bone density (at the calf; p=0.03), as well as both trabecular (p=0.02) and cortical (p=0.03) bone density, using pQCT in older women. Interestingly, African-American sickle cell disease (characterized by low hemoglobin concentrations) patients had 13% lower integral BMD of the forearm, but showed no differences in trabecular bone (Nelson et al. 2003). Yet, the lumbar spine BMD, which is about 66% trabecular bone, was low in most of the young adult sickle cell patients in another study (Miller et al. 2006). One may speculate that a low hemoglobin concentration due to iron deficiency anemia may impact bone by reducing trabecular BMC. To illustrate, the lumbar vertebrae of female rats either on an iron restricted (12 mg Fe/kg/day) but calcium adequate (5.2 g Ca/kg diet) diet (Parelman et al. 2006) or an iron deficient diet (< 8 mg Fe.kg/day; Medeiros et al. 2004) had a lower trabecular number and greater trabecular separation using micro-computed

tomography. It is important to note that hemoglobin is fairly stable in postmenopausal women who no longer have monthly blood losses. Perhaps this is one explanation why we noted the strong association of hemoglobin with trabecular bone in these women. An explanation for the role of iron in bone formation is that iron is a cofactor for prolyl and lysyl hydroxylases, enzymes that catalyze an ascorbate-dependent hydroxylation of prolyl and lysyl residues. These steps are essential to collagen crosslinking in bone by lysyl oxidase (Tuderman et al. 1977). Serum ferritin, a marker of iron stores, is a better indicator than hemoglobin of iron status and may also have shown this relationship to bone. We measured serum ferritin only in the ISU women, but plan to measure serum ferritin in the UC-Davis women as well. Ferritin will allow us to distinguish between iron status and inflammation in these women and perhaps will be related to trabecular bone. The negative relationship between circulating uric acid and trabecular BMC may parallel the relation between low BMD and elevated uric acid excretion, typical in calcium renal stone formers (Pietschmann et al. 1992). Thus, the relationship between uric acid and trabecular BMC may illustrate the importance of kidney function in bone health. Pertinent to our results, Ensrud et al. (1997) revealed that compared with heavier women, thin women had a nearly two-fold increase in the age-adjusted risk (relative risk=1.93) of hip fracture. Additionally, the Study of Osteoporotic fractures in older women and the Osteoporotic Fractures in Men Study demonstrated higher rates of hip bone loss among women (Ensrud et al. 2003) and men (Ensrud et al. 2006) undergoing weight loss. A very low body weight may exacerbate the already hypo-estrogenic state of postmenopausal women, contributing to an even greater imbalance between bone resorption and bone formation. This imbalance maybe reversed with weight gain and as a more optimal weight is maintained, this may be associated with increases in BMC and BMD (Bolton et al. 2005).

Although a few studies have demonstrated an association between CRP concentration and BMD or biochemical markers of bone turnover in immune (Bakri Hassan et al. 1998) and inflammatory (Haugeberg et al. 2006; Punzi et al. 2005) disease states, there is a paucity of information examining this relation in non-inflammatory conditions or in healthy postmenopausal subjects. Contrary to our hypothesis, we found no association between CRP and volumetric cortical bone at the 1/3 femur site, but it was significantly and positively

associated with total proximal femur BMD and distal tibia trabecular BMD. However, once others factors were taken into account, CRP was no longer related to trabecular BMD; yet, we identified factors contributing to volumetric cortical bone. A potential drawback in multi-center clinical trials is that measurement differences between study sites may emerge. Nonetheless, pQCT operators were trained similarly and we did not detect a significant study site effect for the distal tibia. However, for the 1/3 femur site, after controlling for study site (9%), age and weight together accounted for about 7% of the variability in cortical BMC. It was rather surprising that weight was the strongest contributor to trabecular BMC, but not to cortical BMC via pQCT. Perhaps this was due to study site accounting for a significant proportion of the variability in cortical BMC, thereby diminishing the effect of weight. It is important to note that UC-Davis had a greater proportion of women at the upper end of the weight spectrum, as well as a greater proportion of older women farther from last menses than ISU. Perhaps the importance of study site for cortical bone may be related to the higher weight and age of women at UC-Davis. Yet, study site had a very low variance inflation factor. Sigurdsson et al. (2006) noted that with every 10 year increase in age among elderly women there is a 0.8% decrease in cortical BMD. The Framingham Osteoporosis Study (Hannan et al. 2000) and the Rotterdam Study (Burger et al. 1998) reported an annual decrease of 0.9% and 0.6%, respectively, in femoral neck BMD, which is about 75% cortical bone.

The median CRP concentration (1.0 mg/L) in our study was similar (1.0 mg/L) to that reported by Koh et al (2005) in Korean women, but was slightly lower than the concentration (1.5 mg/L) in apparently healthy American women (Rifai and Ridker 2003). Our results showing a positive correlation between total proximal femur BMD and CRP do not agree with Koh et al. (2005), who demonstrated that higher circulating CRP was associated with lower femoral neck BMD in postmenopausal women. However, women from their study were late postmenopausal (7.7±5.7 years) compared to our women who were early postmenopausal (3.5±2.0 years). More importantly, in osteopenic and osteoporotic women, CRP was significantly greater ($p \leq 0.014$) compared with healthy postmenopausal women. Since we only recruited women whose BMD was within the normal range, it is possible that we were unable to detect a relationship between CRP and bone because our women were

more uniform with respect to BMD. To further explore this relationship, we used the 1.8 mg/L cut-off value because this value was associated with osteopenia and/or osteoporosis in the Korean study (Koh et al. 2005). When we examined our women with CRP greater than 1.8 mg/L, we found that lumbar spine BMC was significantly and negatively associated with CRP ($r= -0.227$; $p \leq 0.05$). High CRP (>1.8 mg/L) thus seems to be a risk factor not only for atherosclerotic CVD, but also for increased risk of osteoporosis.

Our finding that total Hcy increased over a period of one year could be due to several possibilities. As this is an ancillary study to an NIAMS/NIH funded project, we did not separate out the effect of treatment in this group of women. Since two-third of the subjects are on active treatment (80 or 120 mg isoflavones), it may be that: 1) total Hcy increased with age or time since menopause, 2) treatment altered total Hcy, or 3) total Hcy increased as time since subjects ceased taking all forms of supplements at baseline increased. However, the increase in Hcy is parallel to the significant decreases in serum vitamin B_{12}, serum folate, and intracellular folate values. Interestingly, the increase in CRP at the end of one year is similar to the increase in CRP seen with hormone therapy (Lakoski et al. 2005, Reuben et al. 2006). Since two-thirds of the women are on active treatment (isoflavones), it is possible that these compounds are exerting an estrogen-like effect by increasing CRP. It is also possible that CRP increased with age or time since menopause. We will not be able to determine what these changes are due to until we are able to unblind the trial at the end of treatment.

Since total Hcy and CRP are risk factors for atherosclerotic CVD and perhaps osteoporosis, we examined the factors contributing to circulating total Hcy and CRP. In our analyses we only included women with a CRP value <10.0 mg/L. According to the Centers for Disease Control and American Heart Association guidelines (Pearson et al. 2003), CRP >10.0 mg/L should be discarded and repeated in two weeks to allow acute inflammation to subside before retesting (Bassuk et al. 2004). Since we analyzed our samples in batch at the end of year one, we could not obtain another blood sample for those women with elevated CRP. Instead, we excluded nine CRP data points in the inferential statistics. Our finding that whole body percent fat, but not waist circumference as an indicator of central adiposity, was a strong predictor of CRP and has been shown in other studies (Gomez-Ambrosi et al. 2006; Manns et al. 2003; Saito et al. 2003). However, Iwasaki et al. (2006) demonstrated that both

visceral (r=0.55; $p \leq 0.0001$) and subcutaneous (r=0.40; $p \leq 0.0001$) fat were independently correlated to CRP in non-diabetic men and women. Thorand et al. (2006) concluded that adiposity was strongly associated with low-grade systemic inflammation and was especially strong in women as reflected by CRP. More recently, researchers have demonstrated that human adipocytes also produce CRP after stimulation with inflammatory cytokines IL-1 beta and IL-6 and by the specific adipocytokine, resistin, thereby suggesting a link between obesity and inflammation (Calabro et al. 2005).

Similar to our findings, Lee et al. (2006) demonstrated that low serum iron was associated with a pro-inflammatory state and thus an elevated CRP concentration. Hepcidin, a key regulator of serum iron during inflammation, is thought to bind to its receptor, protein ferroporotin, which serves as a transmembrane iron channel enabling iron efflux from cells. The hepcidin-ferroportin complex is then degraded in lysosomes and iron is not released from cells (mainly enterocytes, macrophages, and hepatocytes), thus leading to a decrease in iron absorption and a decrease in serum iron (Vyoral and Petrak 2005). This possibly explains the low serum iron concentration typically seen in inflammatory conditions. Analogous to previous findings (Yanagawa et al. 2006), we found that with increasing CRP, there was an increase in triglycerides. Esteve et al. (2005) explained that changes in lipid metabolism due to inflammation are aimed at protecting the host from acute injury and are mediated by cytokines. The activation of the inflammatory cascade induces a decrease in HDL-cholesterol, which stimulates compensatory changes, such as synthesis and accumulation of phospholipid-rich VLDL. These particles then bind to bacterial products and other toxic substances, resulting in hypertriglyceridemia. The final consequence is an increased accumulation of intracellular cholesterol. Additionally, CRP also influences the response to a low fat or high MUFA diet. Desroches et al. (2006) demonstrated that in subjects with high CRP, a low fat diet increased triglycerides. On the other hand, in subjects with low CRP, a high MUFA diet decreased triglycerides. Our finding of a positive relationship between serum uric acid and CRP has been reported in previous studies in healthy subjects (Dohi et al. 2006) and in chronic kidney disease patients (Caravaca et al. 2005). Uric acid was found to be independently and positively correlated with CRP in healthy subjects (Patel et al. 2006), but unlike our study, it failed to contribute to the

variability in CRP. The positive relation between white blood cell count and CRP has been reported in the past (Byrne et al. 2004) and likely reflects that some of our subjects may have had an infection or some degree of inflammation at baseline. Typically during inflammation, there is an increase in the white blood cell count, as a result of the bone marrow reacting to inflammation or infection (Abramson and Melton 2000).

Given that vitamin B_{12} is a co-enzyme with methionine synthase in the remethylation pathway of Hcy (Selhub 1999), it was not surprising that serum vitamin B_{12} contributed inversely to baseline total Hcy. Similar to our findings, other studies have also noted that creatinine is a predictor of total Hcy in patients on hemodialysis (Refsum et al. 2006) and in the general population (Righetti et al. 2006). Hcy is metabolized by the kidney and serum creatinine is a marker of renal function. Recent research has shown that total Hcy concentration is positively correlated with creatinine in the early stage of chronic renal failure (Cetin et al. 2006) and in elderly hospatilized patients (Rodriguez et al. 2006), but not in healthy populations (Taskin et al. 2006). Thus, it was surprising that serum creatinine emerged as a contributor to Hcy in these healthy women.

In conclusion, total Hcy and CRP were not related to trabecular or cortical bone, but this may be because these women were healthy and non-osteoporotic. Nonetheless, it is of prime importance to examine the type of bone that is lost in hyperhomocysteinemic subjects. Since hemoglobin was a significant contributor to trabecular BMC, while iron was a significant contributor to CRP, it is possible that inflammation may mediate the relationship between iron status (reflected by low serum iron and/or hemoglobin concentration) and low trabecular BMC.

ACKNOWLEDGEMENTS

We thank Ann Perera, MRL Metabolomics, for her help in HPLC setup. We also thank our phlebotomists, Cindy Kruckenberg, Marilyn Chrusciel, and Shirley Nelson. We also thank the subjects who volunteered for this study.

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Table 1: Precision Error (%) for Peripheral Quantitative Computed Tomography (pQCT) and Dual Energy X-ray Absorptiometry (DXA)

	pQCT		DXA		
	Distal Tibia	**Femur (1/3 Site)**	**Total Hip**	**Lumbar Spine**	**Whole Body**
BMD[a]	1.24	0.40	0.66	1.13	0.80
BMC[b]	2.98	0.79	1.56	1.21	0.76

[a] Bone Mineral Density

[b] Bone Mineral Content

Table 2: Characteristics of Subjects at Baseline

	ISU (n=122)	UC-Davis (n=120)	ISU+UC-Davis (N=242)
	Mean ± SD [min - max]		
Age (years)	54.0 ± 3.0 [46.1 - 60.2]	54.7 ± 3.5 [45.8 - 65.0]	54.3 ± 3.3 [45.8 - 65.0]
Years since menopause	3.4 ± 1.9 [1.1 - 8.0]	3.7 ± 2.1 [0.8 – 10.1]	3.5 ± 2.0 [0.8 - 10.1]
Estrogen exposure (years)	37.4 ± 3.3 [27.0 - 44.0]	38.2 ± 3.4 [28.0 - 48.0]	37.8 ± 3.4 [27.0 - 48.0]
Race	**Number of subjects (%)**		
Caucasian	120 (98.4)	84 (86.7)	224 (92.6)
African American	1 (0.8)	2 (1.7)	3 (1.2)
Native American	0	7 (0.8)	7 (0.4)
Asian	0	3 (2.5)	3 (1.2)
More than one race	1 (0.8)	6 (5)	7 (2.9)
Unknown	0	2 (1.7)	2 (0.8)
Not reported	0	2 (1.7)	2 (0.8)
Family history of cardiovascular disease	78 (63.9)	57 (47.5)	135 (55.8)
Family history of osteoporosis	34 (27.9)	40 (33.3)	74 (30.6)
Personal history of bone fracture	47 (38.5)	39 (32.5)	86 (35.5)

Table 2 (continued): **Characteristics of Subjects at Baseline**

	ISU (n=122)	UC-Davis (n=120)	ISU+UC-Davis (N=242)
Past hormone therapy use[a]	**Number of subjects (%)**		
Estrogen+progestogen	55 (45.1)	43 (35.8)	98 (40.5)
Estrogen	8 (6.6)	19 (15.8)	27 (11.2)
Progestogen/progesterone	11 (9.0)	15 (12.5)	26 (10.7)
Past hormone therapy use[b]**(mos)**	**Median [min - max]**		
Estrogen+progestogen	12 [0.3 - 120]	12 [0.3 - 114]	12 [0.3 - 120]
Estrogen	15.5 [1 - 60]	12 [1 - 132]	12 [1 - 132]
Progestogen/progesterone	6 [1 - 36]	6 [1 - 24]	6 [1 - 36]

[a] Number and percentage of women who used hormone therapy.

[b] Distributions for these variables were not normal and thus median [min - max] values are reported.

Table 3: Body Size and Body Composition at Baseline

Measurement	ISU (n=122)	UC-Davis (n=120)	ISU+UC-Davis (N=242)
	Mean ± SD [min - max]		
Height (cm)	165.3 ± 6.0	164.2 ± 6.7	164.7 ± 6.4
	[150.6 - 178.4]	[146.3 - 182.2]	[146.3 - 182.2]
Weight (kg)	68.0 ± 8.7	67.4 ± 10.0	67.7 ± 9.3
	[47.8 - 89.2]	[43.7 - 94.5]	[43.7 - 94.5]
BMI (kg/m^2)	24.9 ± 2.7	25.0 ± 3.4	25.0 ± 3.1
	[18.9 - 29.9]	[17.8 - 32.7]	[17.8 - 32.7]
Waist circumference (cm)	76.7 ± 7.2	79.2 ± 8.5	77.9 ± 8.0
	[62.8 - 98.6]	[59.1 - 100.6]	[59.1 - 100.6]
Hip circumference (cm)	102.0 ± 6.5	98.5 ± 6.7	100.3 ± 6.8
	[87.1 - 117.2]	[80.9 - 118.2]	[80.9 - 118.2]
Waist-to-hip ratio	0.8 ± 0.1	0.8 ± 0.1	0.8 ± 0.1
	[0.6 - 0.9]	[0.6 - 0.9]	[0.6 - 0.9]
Lean mass[a] (kg)	4.3 ± 0.4	4.3 ± 0.5	4.3 ± 0.5
	[3.4 - 5.5]	[3.0 - 5.6]	[3.0 - 5.6]
Fat mass[a] (kg)	2.3 ± 0.6	2.3 ± 0.7	2.3 ± 0.6
	[0.8 - 3.7]	[0.8 - 4.8]	[0.8 - 4.8]
Percent body fat[a] (%)	34.7 ± 5.1	34.3 ± 6.2	34.5 ± 5.7
	[18.1 - 44.6]	[18.4 - 56.0]	[18.1 - 56.0]

[a] Assessed via Dual Energy X-Ray Absorptiometry.

Table 4: Baseline Bone Measurements

Measurement	ISU (n=122)	UC-Davis (n=120)	ISU+UC-Davis (N=242)
		Mean ± SD [min - max]	
Total proximal femur bone mineral content	31.06 ± 3.31	31.89 ± 4.05	31.47 ± 3.71
(BMC) (g)	[22.30 - 42.15]	[22.89 - 43.28]	[22.30 - 43.28]
Total proximal femur bone mineral density	0.907 ± 0.076	0.913 ± 0.073	0.910 ± 0.075
(BMD) (g/ cm^2)	[0.744 - 1.048]	[0.765 - 1.075]	[0.744 - 1.075]
Lumbar spine BMC (g)	59.68 ± 6.52	57.80 ± 8.43	58.75 ± 7.57
	[45.29 - 74.10]	[40.44 - 83.50]	[40.44 - 83.50]
Lumbar spine BMD (g/cm^2)	1.003 ± 0.075	0.984 ± 0.079	0.994 ± 0.077
	[0.871 - 1.181]	[0.874 - 1.201]	[0.871 - 1.201]
Trabecular Bone	**n=122**	**n=62**	**n=184**
Distal tibia BMC (g)	183.58 ± 23.75	179.87 ± 30.86	182.33 ± 26.33
	[120.69 - 236.56]	[95.36 - 256.26]	[95.36 - 256.26]

Table 4 (continued)**: Baseline Bone Measurements**

Measurement	ISU	UC-Davis	ISU+UC-Davis
		Mean ± SD [min - max]	
Distal tibia BMD (g/cm^2)	234.389 ± 30.285	234.697 ± 31.139	234.492 ± 30.490
	[157.900 - 332.200]	[151.900 - 296.100]	[151.900 - 332.200]
Cortical Bone	**n=122**	**n=115**	**n=237**
Femur (1/3 site) BMC (g)	320.42 ± 30.73	339.40 ± 36.25	329.63 ± 34.77
	[249.47 - 406.81]	[264.83 - 432.78]	[249.47 - 432.78]
Femur (1/3 site) BMD (g/cm^2)	1110.919 ± 22.055	1124.063 ± 24.989	1117.297 ± 24.380
	[1048.200 - 1156.400]	[1033.900 - 1181.400]	[1033.900 - 1181.400]

Table 5: Circulating Analytes at Baseline: C-Reactive Protein (CRP), Total Homocysteine (Hcy), Folate, and Vitamin B_{12}

Circulating Variable[a]	Median [min – max]	Normal Range Reported from Literature
Serum CRP (mg/L)	1.0 [0.0001 - 29.9]	1.5[c]
Plasma total Hcy (μmol/L)	7.3 [4.2 - 17.6]	6.8 ± 0.1[d]
Intracellular folate[b] (ng/mL)	538.4 [249.3 – 2128.3]	277.1 ± 4.6[d]
Serum folate (ng/mL)	13.3 [4.2 – 59.4]	12.9 ± 0.2[d]
Serum vitamin B_{12} (pg/mL)	492.7 [59.7 – 1567.4]	628.2[e] [535.5 – 720.7]

[a] Distributions for these variables were not normal and thus median [min, max] values are reported.

[b] Values reported on 234 women as seven samples were incorrectly processed, one sample was missing, and two at ISU and two at UC-Davis were missing hematocrit values.

[c] Median value reported by Bassuk et al. (2004) for American women not on hormone therapy

[d] Values reported are geometric means ± SE from NHANES 2001-2002 data for women (Ganji and Kafai 2006).

[e] Value reported is mean [min - max] NHANES III data for women (Ganji and Kafai 2003).

Table 6: Dietary Intake Based on Semi-Quantitative Food Frequency Questionnaire

Nutrient[a]	ISU (n=122)	UC-Davis (n=120)	ISU+UC-Davis (N=242)
	Median [min-max]		
Energy (kcal/day)	1588 [423 - 4564]	1422 [424 - 3633]	1547 [423 - 4564]
Carbohydrate (g/day)	195 [33 - 476]	162 [27 - 421]	176 [27 - 476]
Protein (g/day)	66 [20 - 168]	58 [15 - 143]	61 [15 - 168]
Total fat (g/day)	67 [17 - 247]	62 [22 - 164]	65 [17 - 247]
Saturated fat (g/day)	20 [5 - 65]	18 [5 - 53]	19 [5 - 65]
Monounsaturated fatty acids (g/day)	25 [6 - 97]	24 [7 - 65]	25 [6 - 97]
Polyunsaturated fatty acids (g/day)	16 [3 - 70]	16 [5 - 44]	16 [3 - 70]
Alcohol[b,c] (g/week)	28.1 [0.7 - 302.4] [n=82]	53.3 [2.0 - 242.4] [n=100]	43.0 [0.6 - 302.4] [n=182]
Fiber (g/day)	17 [4 - 50]	16 [6 - 42]	16 [4 - 50]

Table 6 (continued): **Dietary Intake Based on Semi-Quantitative Food Frequency Questionnaire**

Nutrient[a]	ISU (n=122)	UC-Davis (n=120)	ISU+UC-Davis (N=242)
		Median [min-max]	
Caffeine[b,d] (g/day)	224 [0.3-842] [n=114]	217 [0.2-1392] [n=114]	219 [0.2-1392] [n=228]
Vitamin A (IU)	430 [85 - 1590]	306 [60 - 970]	371 [60 - 1590]
Vitamin E (α-TE)	9.2 [1.9 - 37.5]	10.0 [3.2 - 26.7]	9.5 [1.9 - 37.5]
Riboflavin (mg/day)	1.6 [0.4 - 3.9]	1.4 [0.3 - 3.2]	1.5 [0.3 – 3.9]
Vitamin B_6 (mg/day)	1.7 [0.4 - 4.6]	1.5 [0.4 - 3.5]	1.5 [0.4 – 4.6]
Folic acid (μg/day)	322 [78 - 1013]	326 [112 - 918]	324 [78 - 1013]
Vitamin B_{12} (μg/day)	3.8 [1.0 - 10.4]	2.8 [0.4 - 8.3]	3.2 [0.4 - 10.4]
Magnesium	290 [63 - 734]	269 [68 - 664]	283 [63 - 734]

Table 6 (continued): **Dietary Intake Based on Semi-Quantitative Food Frequency Questionnaire**

Nutrient[a]	ISU (n=122)	UC-Davis (n=120)	ISU+UC-Davis (N=242)
Calcium		**Median [min - max]**	
Dietary intake (mg/day)	762	634	686
	[152 - 2446]	[154 - 1856]	[152 - 2446]
Supplemental intake (mg/day)	323	286	323
	[0 - 1130]	[0 - 1130]	[0 – 1130]
Lifetime calcium intake from dairy products[b] (mg/day)			
Childhood (5 – 11 years)	910	638	870
	[0 - 2964]	[0 - 2607]	[0 – 2964]
Adolescence (12 - 18 years)	899	632	702
	[0 - 4118]	[0 - 2281]	[0 – 4118]
Young adult (19 - 34 years)	641	398	561
	[0 - 2989]	[0 - 2234]	[0 – 2989]
Midlife adult (35 – 49 years)	586	337	447
	[0 - 2409]	[0 - 2234]	[0 – 2409]
Mature adult[e] (≥ 50 years)	606	282	403
	[0 - 1886]	[0 - 1645]	[0 – 1886]

[a] Distributions for these variables were not normal and thus median [min - max] values are reported.

[b] Reported from nutrition history questionnaire.

[c] Amount of alcohol is based on women who reported intake and does not include abstainers.

[d] Amount of caffeine is based on women who reported intake and does not include abstainers.

[e] Number of women who were ≥ 50 years of age at baseline at ISU=108, at UC-Davis=105, and combined=213.

Table 7: Correlation Analysis: C-Reactive Protein (CRP) and Total Homocysteine (Hcy) versus Bone Measures

	CRP (mg/L)		Total Hcy (µmol/L)	
	r	*p*	r	*P*
Dual Energy X-Ray Absorptiometry	n=233[a]		n=242	
Total proximal femur BMC (g)	0.067	0.31	0.078	0.23
Total proximal femur BMD (g/cm^2)	0.192	0.003**	0.002	0.98
Lumbar spine BMC (g)	-0.065	0.32	0.051	0.43
Lumbar spine BMD (g/cm^2)	0.127	0.053	0.043	0.51
Peripheral Quantitative Computed Tomography				
Distal tibia (trabecular) BMC (g)	-0.055[b]	0.47	-0.041	0.58
Distal tibia (trabecular) BMD (g/cm^2)	0.178[b]	0.017*	-0.06	0.42
Femur (1/3 site; cortical) BMC (g)	-0.120[c]	0.07	0.099	0.13
Femur (1/3 site; cortical) BMD (g/cm^2)	-0.092[c]	0.17	0.036	0.58

[a] N=228 for CRP as nine data points with a CRP value >10mg/L were excluded.

[b] N=178 for correlation between CRP and distal tibia (trabecular BMC and BMD) as 64 data points were not available either due to improper data acquisition of trabecular bone measurements or CRP value >10mg/L.

[c] N=228 for correlation between CRP and 1/3 femur (cortical BMC and BMD) as five 1/3 femur measurements were not available because UC-Davis institutional approval was not yet secured for scanning these women and nine CRP data points with value >10mg/L were excluded.

*$p<0.05$

**$p<0.01$

Table 8: Regression Analysis: Contributors to Volumetric Bone Mineral Content

Trabecular Bone Mineral Content at the Distal Tibia[a]

Overall Model R^2=21.6% (Adj R^2=19.4%) [F=9.59; df=(5,174)] $p \leq 0.0001$

Parameter	Parameter Estimate	Percentage Variance[b]	Prob > |t|[c]	Variance Inflation Factor[d]
Intercept	77.0460		0.016	
Study site	1.8739	0.001	0.62	1.23
Weight	0.2028	12.37	≤0.0001	1.04
Hemoglobin	6.7740	5.54	0.0006	1.16
Serum uric acid	-5.3385	4.17	0.0027	1.19
Serum glucose	-0.4056	1.48	0.071	1.21

Cortical Bone Mineral Content at the Femur (1/3 site)[e]

Overall Model R^2=13.9% (Adj R^2=12.8%) [F=12.54; df=(3,233)] $p \leq 0.0001$

Parameter	Parameter Estimate	Percentage Variance[b]	Prob > |t|[c]	Variance Inflation Factor[d]
Intercept	378.8962		≤0.0001	
Study site	20.5374	8.65	≤0.0001	1.01
Weight	0.6947	3.48	0.0024	1.01
Age	-1.9596	3.33	0.0030	1.00

Additional variables included in the trabecular bone mineral content model: age, total Hcy, CRP, total calcium, and dietary calcium. Additional variables included in the cortical bone mineral content model: blood glucose, hemoglobin, serum uric acid, total Hcy, CRP, total calcium, and dietary calcium.

[a] N=62 for the trabecular bone mineral content model as 58 measurements were not available due to improper data acquisition.

[b] Squared semi-partial Type II correlation coefficient; accounts for shared variance among variables.

[c] Variables (except site) left in the model are significant at $p \leq 0.10$ level.

[d] Measures inflation in the variances of parameter estimates due to multicollinearities among regressors.

[e] N=237 for cortical bone mineral content model as five measurements were not available because UC-Davis institutional approval was not yet secured for scanning these women.

Table 9: Regression Analysis: Contributors to Cardiovascular Disease Risk Factors

Total Homocysteine

Overall Model R^2=18.6% (Adj R^2=17.2%) [F=17.72; df=(3,238)] $p \leq 0.0001$

Parameter	Parameter Estimate	Percentage Variance[a]	Prob > |t|[b]	Variance Inflation Factor[c]
Intercept	6.0234		≤0.0001	
Study site	0.2962	0.01	0.23	1.00
Serum vitamin B_{12}	-0.0032	11.81	≤0.0001	1.00
Serum creatinine	3.9617	5.84	≤0.0001	1.00

Serum C-Reactive Protein[d]

Overall Model R^2=27.9% (Adj R^2=25.9%) [F=14.24; df=(6,221)] $p \leq 0.0001$

Parameter	Parameter Estimate	Percentage Variance[a]	Prob > |t|[b]	Variance Inflation Factor[c]
Intercept	-3796.1226		≤0.0001	
Study site	-5.4312	0.00	0.98	1.12
Whole body percent fat	77.5817	4.93	≤0.0001	1.18
Serum uric acid	311.8754	2.96	0.0029	1.22
Serum triglycerides	8.3998	2.86	0.0034	1.23
White blood cell count	270.6307	1.93	0.016	1.19
Serum iron	-8.4490	1.79	0.019	1.03

Additional variables included in total Hcy model: Serum or intracellular folate, serum uric acid, dietary factors important for Hcy metabolism. Additional variables included in CRP model: Age, waist circumference, TIBC, total cholesterol/HDL-C ratio.

[a] Squared semi-partial Type II correlation coefficient; accounts for shared variance among variables.

[b] Variables (except study site) left in the model are significant at $p \leq 0.10$ level.

[c] Measures inflation in the variances of parameter estimates due to multicollinearities among regressors.

[d] N=228 for C-reactive protein model as five (2 at ISU and 3 at UC-Davis) complete blood counts were missing.

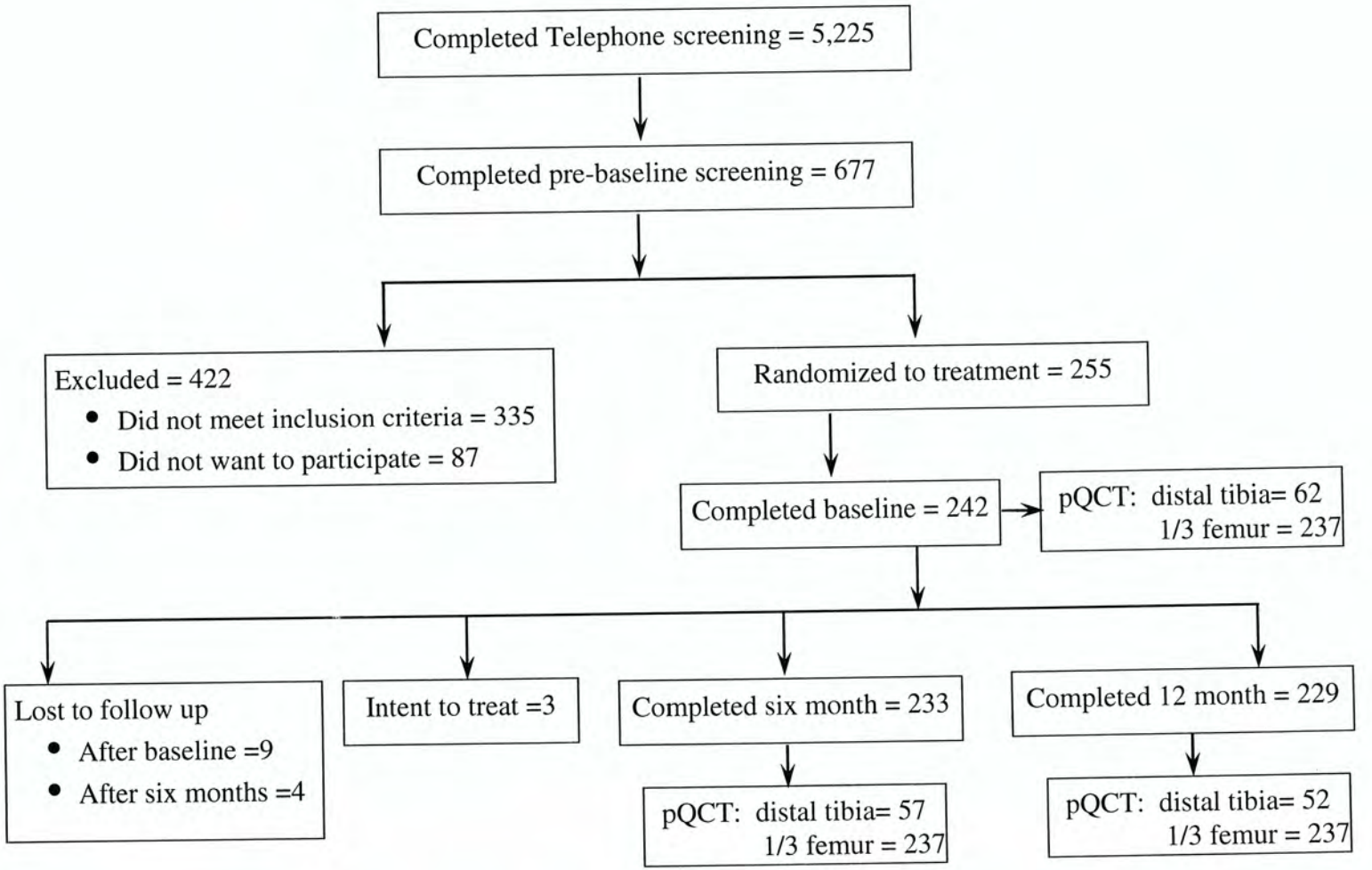
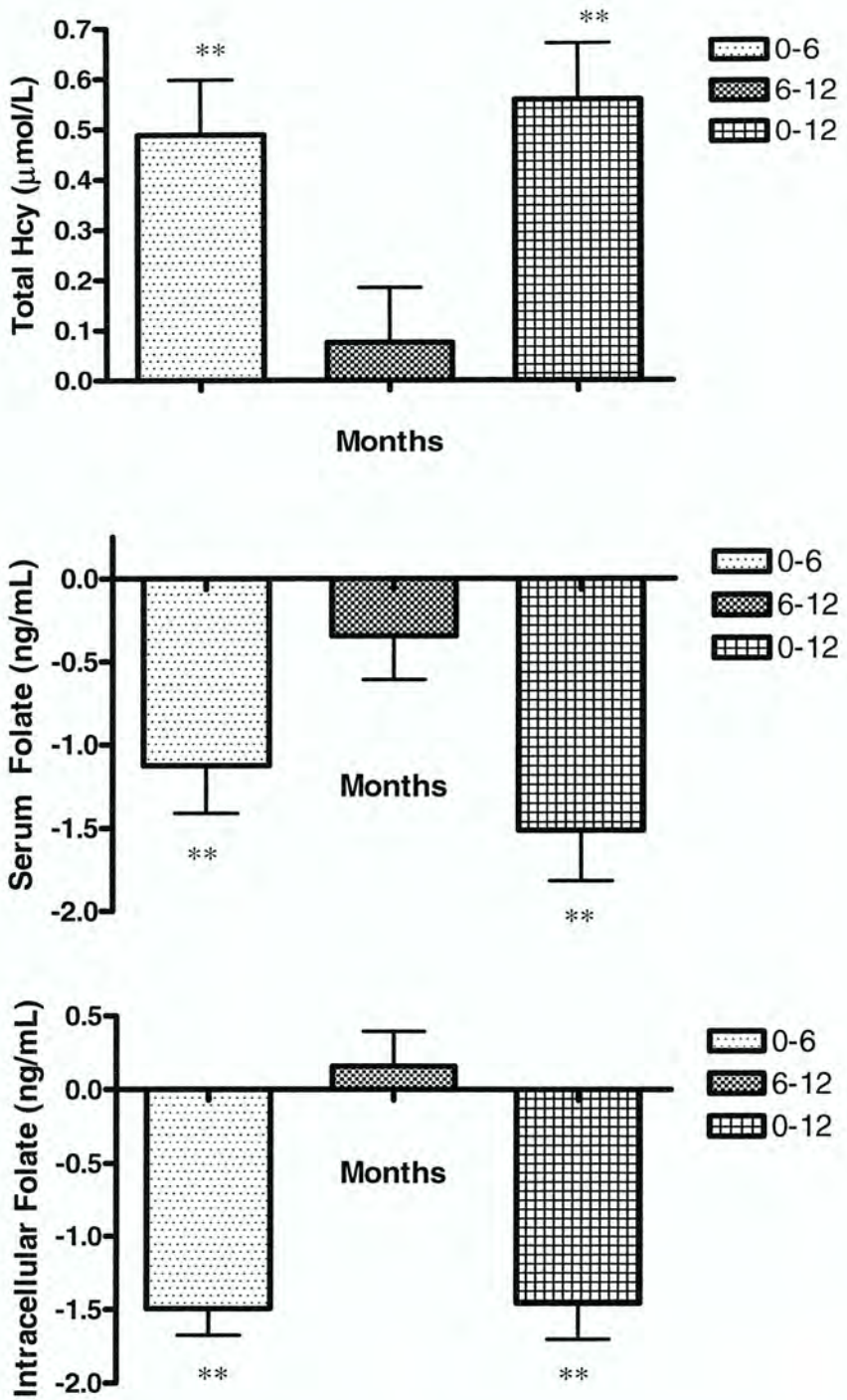
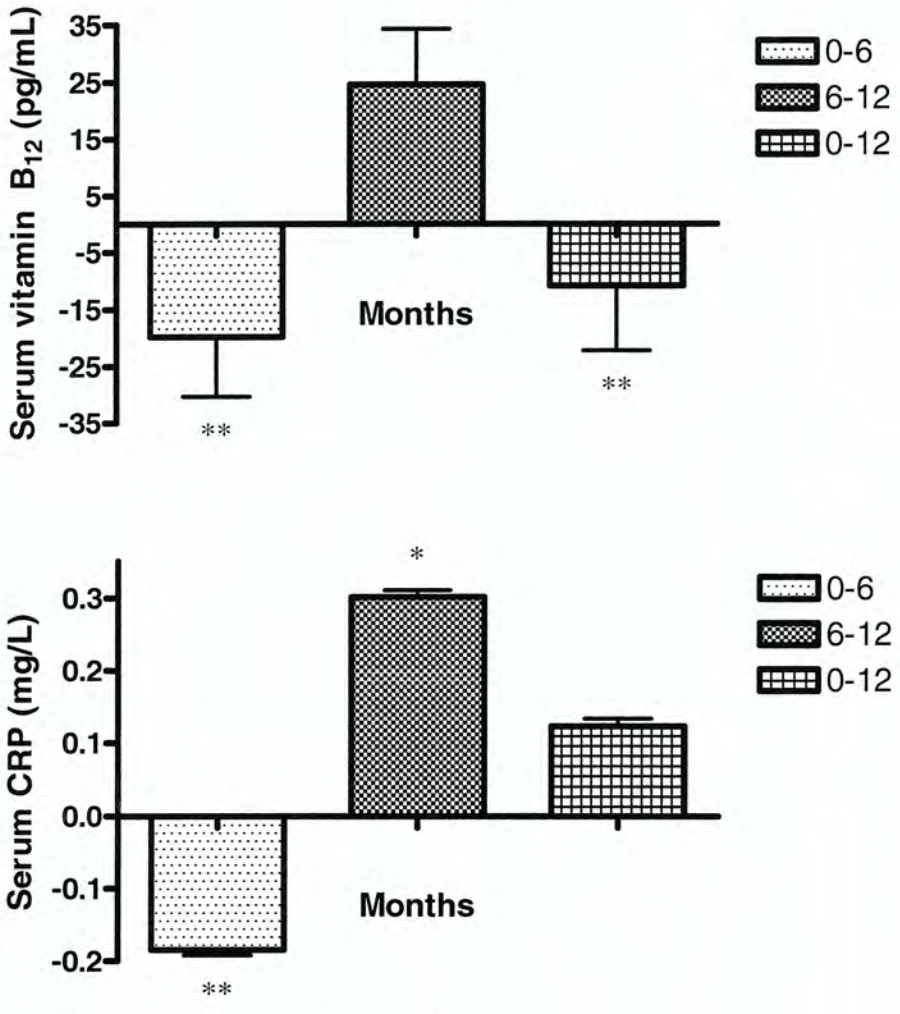


Figure 1: Subject enrollment and follow-up flow chart



*p<0.05, **p<0.01 using Wilcoxon signed rank test

Figure 2: Change in circulating analytes in postmenopausal women from baseline through one year.



*p<0.05, **p<0.01 using Wilcoxon signed rank test

Figure 2 (continued): **Change in circulating analytes in postmenopausal women from baseline through one year.**

GENERAL CONCLUSIONS

In summary, the results from our primary objective of this study suggest that Hcy and CRP are not related to trabecular or cortical bone in healthy, non-osteoporotic postmenopausal women. Since a vast majority of these women did not have elevated Hcy, we were not able to determine whether or not hyperhomocysteinemia was related to trabecular bone. Future studies should be conducted to establish which type of bone is primarily lost with hyperhomocysteinemia. In women with elevated CRP (>1.8 mg/L), lumbar spine BMD was inversely related to CRP, but this relationship needs to be further explored in epidemiological and intervention studies. The results from the secondary objective determined that weight, hemoglobin, and serum uric acid contributed to about 22% of the variability in trabecular BMC. In contrast, study site, weight, and age were the significant contributors to cortical BMC. Serum vitamin B_{12} and serum creatinine contributed to the variability (~19%) in total Hcy, indicating that renal function is important in Hcy metabolism. Serum iron contributed inversely to CRP, but whole body percent fat, serum uric acid, triglycerides, and white blood cell count were positively related to circulating CRP. Hepcidin, a key regulator of serum iron during inflammation, is thought to disable iron efflux from cells, thereby making iron less available for invading microorganisms. This may explain the inverse association between serum iron concentration and CRP. Since hemoglobin was a significant contributor to trabecular BMC, while iron was a significant contributor to CRP, it is possible that inflammation may mediate the relationship between low iron status (reflected by low serum iron and/or hemoglobin concentration) and low trabecular BMC. Results from the third objective demonstrate that total Hcy and CRP significantly increased at the end of one year, while serum folate and vitamin B_{12} decreased significantly. Future data analyses will be conducted to determine the effect of two doses of soy isoflavones (80 and 120 mg) on circulating total Hcy and CRP in relation to change in trabecular and cortical bone and in relation to CVD risk.