

**Cardiovascular disease risk factors in postmenopausal women:  
Excess iron is related to central adiposity but not to oxidative stress**

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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**DEDICATION**

I would like to dedicate this thesis to my parents, Kevin and Donna Deardorff, for their love and support throughout my college career. Their constant encouragement has allowed me to appreciate and value education and hard work. Thus, it is my goal to never stop learning and pursue challenges throughout my life with a positive attitude and desire for excellence.

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## EXCESS IRON IS RELATED TO CENTRAL ADIPOSITY BUT NOT TO OXIDATIVE STRESS IN POSTMENOPAUSAL WOMEN

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**LIST OF ABBREVIATIONS**

AGF ratio	androidal-to-gynoidal fat mass ratio
BMI	body mass index (kg/m <sup>2</sup> )
CVD	cardiovascular disease
DXA	dual-energy x-ray absorptiometry
GPX	glutathione peroxidase
HDL-C	high density lipoprotein cholesterol
8-OHdG	8-hydroxy-2'-deoxyguanosine
LDL-C	low density lipoprotein cholesterol
SF	serum ferritin
SOD	superoxide dismutase
ROS	reactive oxygen species
TC	total cholesterol
TG	triacylglycerides
TNF- $\alpha$	tumor necrosis factor-alpha
oxLDL	oxidized low density lipoprotein
PGF <sub>2<math>\alpha</math></sub>	urinary isoprostanes F <sub>2<math>\alpha</math></sub>
VLDL	very low density lipoprotein
WHR	waist-to-hip ratio

## ACKNOWLEDGEMENTS

I would like to thank Dr. Manju Reddy for allowing me to work in her lab and inviting me to be a graduate research assistant on this project. Her many hours of teaching and assistance have made this research experience unforgettable and very enjoyable. She has inspired me to continue my nutrition career with a researcher's mindset. I would like to thank my committee members, Drs. D. Lee Alekel and Kenneth Koehler, for their expertise and contribution to my thesis work. I would like to thank Dr. Alekel for the opportunity to work on a NIH-funded clinical trial which has prepared me for my future career goals. Dr. Koehler has been a valuable resource in regards to the statistical analysis. In addition, our statistician, Ulrike Genschel, should also be recognized for her many hours of analysis of the data.

The American Heart Association and the National Institutes of Health are appreciated for their financial support of the study. I would like to especially thank other researchers who were vital to the completion of our study including Jeanne Stewart, Dr. Kathy Hanson, and Laura Hanson for their contribution. My fellow graduate and undergraduate students who helped with subject testing, data collection, and sample analysis are very appreciated for their hard work and dedication. Lastly, I would like to thank the subjects for their participation in our study.

## GENERAL INTRODUCTION

### Thesis Organization

This thesis begins with a general introduction including objectives, hypotheses, specific aims, limitations, and the significance of the proposed study followed by a review of literature, a manuscript, general conclusions, and references.

### Objectives

- 1) The primary objective of this study is to determine the relationship between atherosclerotic cardiovascular disease (CVD) risk factors, particularly oxidative stress indices, central adiposity, and body iron stores in postmenopausal women.
- 2) The secondary objective of this study is to determine whether soy isoflavones would reduce the risk of atherosclerotic CVD in postmenopausal women.

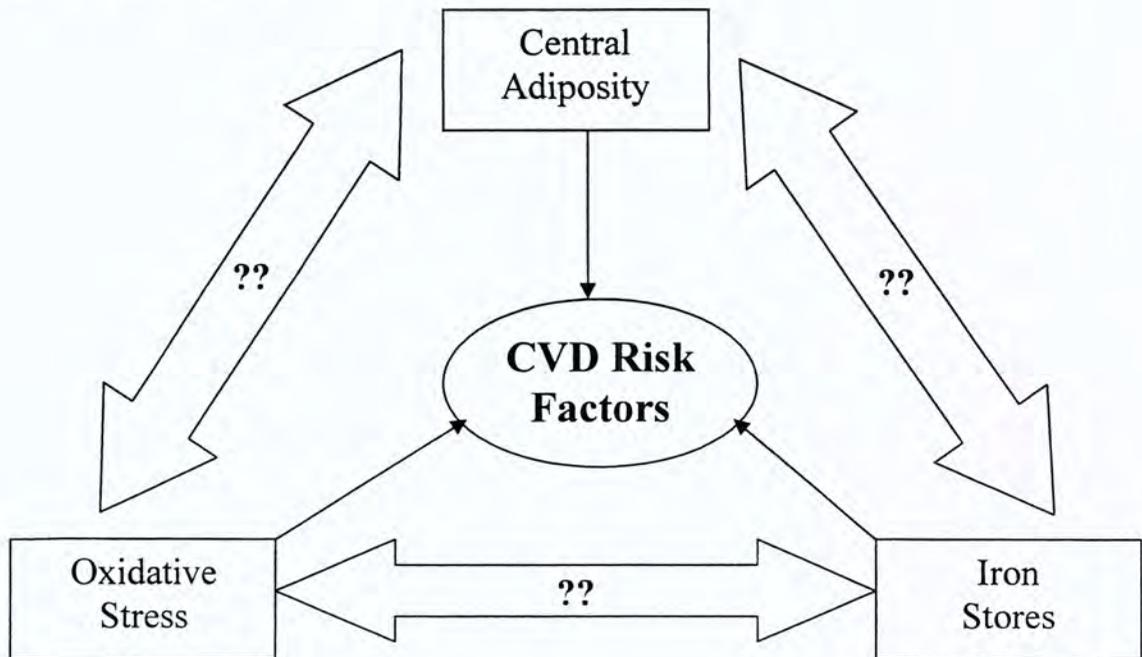
### Hypotheses

- 1) Oxidative stress and central adiposity is positively associated in postmenopausal women.
- 2) Excess body iron stores induce oxidative stress thereby increasing CVD risk.
- 3) Soy isoflavones will reduce oxidative stress by improving antioxidant status and reducing oxidative damage in postmenopausal women, thus reducing CVD risk. (These data will not be available until 2008).

### Specific Aims

- 1) To determine the relationship between oxidative stress, as measured by erythrocyte antioxidant enzymes [catalase (CAT), superoxide dismutase (SOD), and glutathione peroxidase (GPX)] and oxidative damage indices [oxidized LDL (oxLDL), urinary isoprostanes (PGF<sub>2α</sub>), protein carbonyls, and 8-hydroxy-2'-deoxyguanosine (8-OHdG)] with body fat in postmenopausal women, taking into account additional risk factors including dietary intake and other associated risk factors (Figure 1).
- 2) To determine the relationship of serum ferritin with oxidative stress and body fat, as measured by dual-energy x-ray absorptiometry (DXA) in postmenopausal women.

Figure 1. Conceptual framework of primary objective



- 3) To determine whether isoflavones reduce CVD risk as measured by oxidative stress indicators: antioxidant enzymes (CAT, SOD, and GPX) and oxidative damage indices (oxLDL,  $\text{PGF}_{2\alpha}$ , protein carbonyls, and 8-OHdG) in postmenopausal women.

### Limitations

There are several limitations to our study objectives. The primary objective is an observational study and is not causal. In addition, the women in our study are considered healthy and may not accurately represent the entire population. In addition, the correlations among CVD risk factors in these women may be stronger if the women were obese, diabetic, dyslipidemic, or had elevated iron stores.

The secondary objective cannot be completed until completion of the parent project in 2008. The laboratory analyses are complete; however, statistical analyses according to treatment will not be conducted until completion of the three-year clinical trial. The National Institutes of Health, the parent project's funding source, does not allow unblinding of data prior to trial completion.

**Significance of Study**

Women are at an increased risk of CVD after menopause due to a decrease in circulating estrogen concentrations, elevated iron stores, and increased abdominal fat accumulation. In addition, increased oxidative stress and decreased oxidative defenses after menopause contribute to a rise in CVD risk. Excess iron has been associated with increased oxidative damage, contributing to an increased risk of CVD.

Soy foods contain compounds, such as isoflavones, which are thought to exert antioxidant effects and may protect women from heart disease. However, the protective mechanism by which isoflavones act is unclear. Isoflavones may provide protection from heart disease by improving antioxidant enzymes or by decreasing oxidative damage.

## REVIEW OF LITERATURE

### Cardiovascular Disease

Cardiovascular disease (CVD) is the major cause of illness and death in men and postmenopausal women, particularly in Western societies. About 13 million Americans have CVD, 1.5 million have a myocardial infarction each year, and approximately 450,000 die of CVD each year (American Heart Association 2000). Over the past couple of decades, there has been much interest and research on the etiology of CVD, including environmental, lifestyle, and genetic factors. However, it has been more recent that CVD has been defined as having an inflammatory etiology component. While high total cholesterol (TC) concentration contributes to increased CVD risk, cholesterol may also be involved in the pathogenesis of CVD via inflammatory mechanisms. Adhesion molecules, cytokines, and oxidative stress may also contribute to the inflammatory state of CVD (Meng 2006). Therefore, therapies directed at these markers may have potential beneficial effects in reducing and treating CVD.

#### **Risk Factors**

Many risk factors are associated with CVD; some are modifiable and others are nonmodifiable. CVD is a condition with multiple etiologies, and the modifiable risk factors are of particular importance in reducing CVD risk.

#### ***Non-modifiable***

There are nonmodifiable risk factors for CVD including aging, male gender, and a positive family history (Mosca et al. 2002). CVD has traditionally been considered a disease targeted towards middle-aged men; however, it has a high prevalence in women, particularly after menopause. The number of deaths from CVD in women after 65 years of age surpasses men by 11% (Lewis 2002), making it the leading cause of mortality. The connection between menopause and dyslipidemia may play a significant role in the increased incidence of CVD observed with increased age in women. Early menopause may place women at increased risk for CVD (van der Schouw et al. 1996; Hu et al. 1999). Physical changes, including increased fat accumulation and abdominal adiposity, coincident with menopause, have been considered part of the biological mechanism by which postmenopausal women experience an increase in CVD risk (Matthews et al. 1989).

Between the ages of 44 and 65, the percentage of women with serum TC above the normal range increases dramatically (Centers for Disease Control and Prevention 1999). In addition, a study with perimenopausal women showed significant increases in low density lipoprotein cholesterol (LDL-C) and triacylglycerides (TG) and a significant decrease in high density lipoprotein cholesterol (HDL-C) within six months of their last menstrual period (Jensen et al. 1990). Thus, postmenopausal women are at risk due to a more atherogenic lipid profile compared to their menstruating counterparts.

The dramatic rise in CVD risk in women after menopause can be attributed to the loss of endogenous estrogen. Estrogen is known to have a direct effect on the arterial wall; it can reverse the vasoconstrictive response of acetylcholine, suggesting the hormone is important for vascular smooth muscle response to endothelial-dependent vasodilator capacity (Herrington et al. 1994). Estrogen is also associated with a decreased concentration of plasma endothelin, a vasoconstrictor (Polderman et al. 1993). Thus, an increase in vascular resistance after menopause may be related to estrogen deficiency. The synergistic effect of an adverse lipid profile and vascular resistance after menopause may place women at increased risk of CVD.

Racial differences in cardiovascular mortality among women remain largely unexplained. African-American women may be at increased risk which may be attributable to impaired endothelial function (Loehr et al. 2004). Data from the Third National Health and Nutrition Examination Survey, 1988–1994, showed that after accounting for age and socioeconomic status, both black and Mexican-American women had a significantly higher prevalence of type II diabetes than white women (Sundquist et al. 2001). In addition, black women were significantly more likely to have abdominal obesity and hypertension and to be physically inactive than white women.

### ***Modifiable***

The major modifiable risk factors for heart disease in women include dyslipidemia, diabetes, hypertension, obesity, cigarette smoking, sedentary lifestyle, and poor nutrition (Lewis 2002; Mosca et al. 1997; Kip 2004). Additional atherosclerosis-associated risk factors that have more recently been identified include abnormal circulating concentrations of lipoprotein(a), homocysteine, C-reactive protein, serum amyloid A, intercellular adhesion

molecule-1, and interleukin-6. (Guyton et al. 1985; Ridker et al. 2000; Kang et al. 1986). The relationship between dyslipidemia and CVD will be discussed in more detail in the following section.

The influence of diabetes on CVD risk has a greater impact in women than in men (Lewis 2002). Diabetic women have a three- to seven-fold higher CVD risk than non-diabetic women, whereas the risk for diabetic men is two-to three-fold higher than non-diabetic men. A potential reason for the sex-based difference may be the negative impact diabetes has on both lipid markers and blood pressure in women (Manson et al. 1996).

Hypertension is also a modifiable risk factor; when it is reduced, one's risk of CVD also declines. A positive correlation between hypertension and age has been well documented, with a prevalence of 4% for young adults, increasing to 65% for those  $\geq 80$  years of age (Whelton et al. 1996). Similarly, the correlation of hypertension with increased risk of stroke and coronary artery disease has been established. Systolic blood pressure and mean arterial pressure were recently reported to be highly correlated to CVD-related death (van Trijp et al. 2005). A large prospective study including nearly 14,000 Dutch women, found a two- to three-fold increase in overall death rate from CVD in individuals with systolic blood pressure  $\geq 185$  mm Hg compared with those whose blood pressure was  $\leq 135$  mm Hg (van der Giezen et al. 1990).

Obesity has been shown to have a positive relationship with CVD risk in women (Rich-Edwards et al. 1995). Results from the Nurses' Health Study indicated that after adjustments for age and smoking, a mildly to moderately overweight woman has almost twice the risk of CVD compared with a lean woman, and that this risk increases to three-fold for a woman in the heaviest category (Manson et al. 1990). Some of this risk may be related to hypertension, diabetes, and hyperlipidemia, factors that are often associated with obesity. However, it has been reported that moderate weight gain may provide a protective effect among very lean postmenopausal women, evidenced by more than a threefold decrease in cardiovascular disease mortality risk. It is postulated that the protection might be due to an increase in adipose-tissue-derived estrogen production as a result of weight gain (Singh et al. 2001).

The damaging effects of cigarette smoking are well known and well documented. Among middle-aged women, greater than 60% of CVD events are attributable to tobacco exposure. The rates of smoking have decreased since the 1960's, but the decrease has been slower in women than in men (Rich-Edwards et al. 1995; van der Giezen et al. 1990).

Prevention of CVD through a "healthy lifestyle" appears to have a substantial impact on the incidence of CVD in middle-aged and older women. The Nurses' Health Study observed that the women who had a good diet, were not overweight, did not smoke, and exercised moderately or vigorously for 30 minutes each day, had an 80% lower incidence of cardiovascular events than the rest of the population (Stampfer et al. 2000). In addition, the study illustrated that improvements in diet and smoking cessation, respectively, accounted for a decrease in the incidence of CVD by 16% and 13%. A decrease in one's risk of CVD can be accomplished by lowering elevated blood pressure, which is often associated with weight reduction, dietary intervention, and reversal of a sedentary lifestyle. An estimated 60% of men and women in the United States engage in physical activity on a regular basis. An increase in physical activity in the general population could greatly affect the prevalence of CVD. The risk of CVD is estimated to be reduced by as much as 75% in American women who exercise regularly compared with those who lead a sedentary lifestyle (Rich-Edwards et al. 1995).

Depression has also been shown to be an independent predictor of CVD in women with no history of disease after adjusting for age, race, education, income, diabetes, hypertension, smoking, high TC requiring medication, body mass index, and physical activity (Wassertheil-Smoller et al. 2004). Also, the relationship between depression and CVD might be related to cytokines and will be discussed in more detail in the cytokine section of this thesis. Thus, it is important for women and their healthcare providers to recognize the symptoms of depression and effectively treat it in an effort to reduce CVD risk.

#### Dyslipidemia and other risk factors

Evidence indicates the severity of atherosclerotic disease of the carotid vessels is strongly associated with severity of dyslipidemia, especially increased LDL-C and low HDL-C concentrations (Wilson et al. 1997). Although elevated concentrations of LDL-C and TG, and decreased concentrations of HDL-C, are risk factors for CVD in both men and women,

the relative contribution to disease risk development of the lipid profile is sex-dependent. Decreased HDL-C and increased TG are independent predictors of CVD risk and stronger in women than men (Manolio et al. 1992). Likewise, low HDL-C, after adjusting for age, was found to be the most significant predictor of death from CVD in women (Jacobs et al. 1990). The Framingham Study, which evaluated HDL-C concentration in women, determined that those with  $<35$  mg/dL had a risk of CVD that was eight times greater than women with  $\text{HDL-C} \geq 65$  mg/dL (Gordon et al. 1977). Improving the serum lipoprotein profile results in a decreased risk of cerebral vascular events, such as stroke (Crouse et al. 1997). A meta-analysis estimated that for every 1% reduction in TC, there is a 2.5% decrease in the incidence of coronary heart disease (Holme 1990).

Dietary intake, especially source and type of fatty acids, has been well studied in relation to reducing CVD risk (Hu et al. 2001). Intake of saturated and trans fatty acids is associated with an adverse lipid profile, while intakes of mono- and polyunsaturated fatty acids promotes a heart-healthy lipid profile. In addition, a high intake of trans fatty acids adversely affects endothelial function (Lopez-Garcia et al. 2005) and has been associated with biomarkers of systemic inflammation (Mozaffarian et al. 2004).

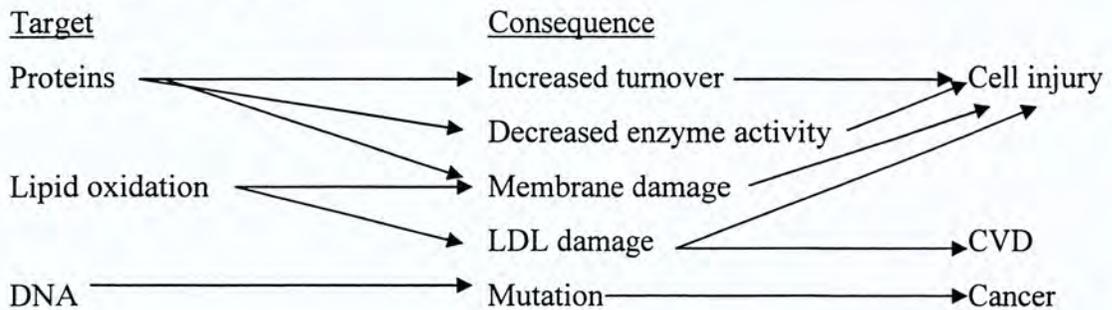
Not only is an undesirable lipid profile important in atherosclerotic CVD risk, but damage to LDL particles may also be an important factor. The oxidation of LDL plays a central role in the pathogenesis of atherosclerosis. The oxidation of LDL initiates a cascade of events including: accelerated platelet aggregation; injury to arterial endothelial cells; and production of cytokines, adhesion factors, growth factors, and other substances that facilitate development of foam cells and fatty streaks, eventually leading to atherosclerosis. Atherosclerosis is characterized by a thickening of the arterial wall due to smooth muscle cell proliferation, lipid deposits, and fibrosis. Subsequent rupture of the lipid-containing plaques results in thrombosis and leads to myocardial infarction and stroke.

### Oxidative Stress

Oxidative stress is an imbalance between the production of reactive species and the body's natural protective mechanisms to cope with these reactive compounds and prevent adverse effects (Mayne 2003). Oxidative stress can arise from many sources including cellular respiration, exercise, and the normal aging process. Oxidative stress has been

suggested to be involved in the aging process etiology of diseases including cancer, CVD, and cataracts (Institute of Medicine 2000). An illustration of free radical damage to various targets is shown in Figure 2.

Figure 2. Free Radical Damage

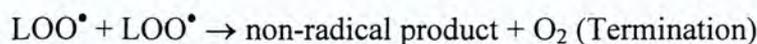
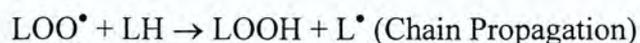
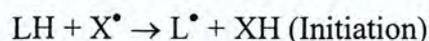


Most oxygen is used in cellular metabolism which mainly occurs in the mitochondria and is related to energy metabolism. Cellular enzymes and controlled metabolic processes normally keep oxidative damage to a minimum. However, at times of increased oxidative stress, including high metabolic demands and external factors such as sunlight, smoking, and pollution, body defense mechanisms may not be adequate and oxidative damage may occur due to the generation of free radicals. Free radicals are atoms or molecules with unpaired electrons, such as superoxide anion, peroxy radical, and hydroxyl radical. Many radicals are highly reactive and can either donate an electron to or accept an electron from other molecules. Thus, these molecules are generally short-lived and may react with macromolecules such as proteins, lipids, and nucleic acids (Young and Woodside 2001). Other reactive molecules, such as molecular oxygen, singlet oxygen, and hydrogen peroxide, are not free radicals per se, but are capable of initiating oxidative reactions and subsequently generating free-radical species. Together, free radicals and reactive oxygen molecules are called reactive oxygen species (ROS). Excessive production of ROS may be related to a variety of neurodegenerative and other chronic diseases. An accumulation of ROS may lead to increased activation of polymorphonuclear leukocytes during infection or be influenced by the pro-oxidant effect of tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ), produced by activated macrophages (Das et al. 1990).

ROS can attack cellular membrane lipids, nucleic acids, proteins, and enzymes resulting in cellular damage and cellular degeneration. Specifically, ROS attack double bonds in polyunsaturated fatty acids, inducing lipid peroxidation (shown in Figure 3), causing cellular oxidative damage. For example, oxLDL has been shown to be involved with the pathogenesis of CVD, while oxidatively modified DNA may play a role in carcinogenesis (Poulsen et al. 1998). In addition, cataracts are a result of oxidation of proteins, which aggregate and accumulate in the lens of the eye (Taylor 1993).

The following reactions illustrate the steps implicated in lipid peroxidation, consisting of three steps: initiation, propagation, and termination.

Figure 3. Lipid Peroxidation (Esterbauer 1995)



Lipid peroxidation is the process whereby free radicals accept electrons from the lipids in cell membranes, resulting in cell damage. Initiation is the step whereby a fatty acid radical is produced. The fatty acid radical is not a stable molecule so it reacts readily with molecular oxygen, thereby creating a peroxy-fatty acid radical, which is also unstable. It may then react with another free fatty acid, producing a different fatty acid radical and a hydrogen peroxide. Free radicals react by producing radicals, causing chain propagation. LDL resistance to oxidation may depend upon adequate dietary antioxidant status, including  $\alpha$ -tocopherol,  $\beta$ -carotene, and selenium.

Various markers of oxidative damage in lipids can be measured in vivo including: oxidized LDL, LDL resistance to oxidation, breath hydrocarbons, thiobarbituric acid-reactive substances, and  $\text{F}_2$  isoprostanes (Mayne 2003).  $\text{F}_2$  isoprostanes are produced from free-radical-induced peroxidation of arachidonic acid, formed in phospholipids, cleaved, and released into circulation before excretion in the urine as free isoprostanes (Roberts and

Morrow 1997). Urine is better than serum to quantify isoprostanes because some factors are shown to affect serum concentrations. For example, aspirin suppresses the most abundant F<sub>2</sub> isoprostane, 8-isoprostaglandin F<sub>2α</sub>, in the serum but not in urine (Reilly et al. 1996). Also, urine may be stored for a longer period of time than serum without isoprostanes degrading.

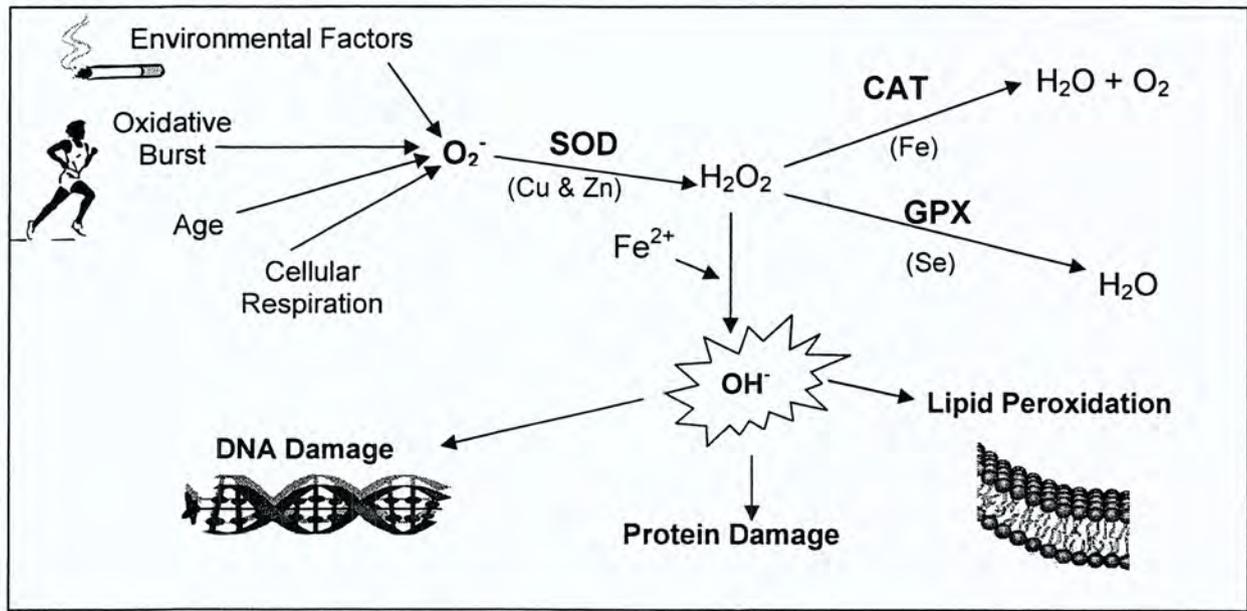
Elevated markers of protein oxidation have also been shown to be associated with diseases such as: Alzheimer's disease, Parkinson's disease, muscular dystrophy, amyotrophic lateral sclerosis, and rheumatoid arthritis (Stadtman et al. 1998). Oxidatively modified proteins are not repaired and must be removed by proteolytic degradation (Levine et al. 1990). A decrease in efficiency of proteolysis causes an increase in the cellular content of oxidatively modified proteins. The concentration of these modified molecules can be quantified by measurement of the protein carbonyl content. Accumulation of modified proteins disrupts cellular function either by loss of catalytic and structural integrity or by interruption of regulatory pathways (Stadtman and Levine, 2000). In addition to cataractogenesis (Garland 1990), exercise-induced oxidative stress in muscle may be related to elevated protein oxidation (Witt et al. 1992).

Biomarkers of DNA oxidation include 8-hydroxy-2'-deoxyguanosine (8-OHdG), antibodies to oxidized DNA, and the Comet assay which detects DNA strand breaks. Of the three available methods, 8-OHdG is the most commonly used marker for DNA damage. This method is based on a common oxidative event in DNA, oxidation of the C-8 of guanine, which ultimately results in a mutagenic lesion that produces a G-to-T transversion mutation (Mayne 2003).

The antioxidant defense system is composed of enzymatic and nonenzymatic substances, or dietary antioxidants. An antioxidant is any substance that significantly delays or inhibits the oxidation of a substrate (Halliwell and Gutteridge 1995). Thus, the physiological role of antioxidants is to prevent damage to cellular components arising as a consequence of chemical reactions involving free radicals. Oxidative damage may be prevented by a normal antioxidant defense system that scavenges ROS. The body's antioxidant defenses depend upon 1) the integrity of the enzymatic system which requires an adequate intake of trace minerals such as iron, selenium, copper, zinc, and manganese and 2) adequate concentrations of vitamins A, C, E, and β-carotene in the cytoplasm and lipid

membrane of the cells that are derived from dietary sources (Allard et al. 1998). Many dietary constituents may scavenge free radicals and have direct antioxidant activity, such as flavonoids, or indirect antioxidant activity, such as zinc and selenium, trace minerals that are necessary for antioxidant enzymes.

Figure 4. Oxidative Stress



Intracellular antioxidant enzymes include superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GPX). Erythrocyte SOD catalyzes the dismutation of superoxide anions to hydrogen peroxide and oxygen, while CAT catalyzes the conversion of hydrogen peroxide to water and oxygen. Additionally, GPX has direct radical-scavenging activity and may represent glutathione activity in other tissues (Meister et al. 1979), and appears to be sensitive to body selenium stores (Cohen et al. 1985). Figure 4 illustrates oxidative status, including antioxidant enzymes and free radical damage.

Antioxidant effects are of interest because of their ability to protect against oxidative damage, which is linked to the development of atherosclerotic CVD and cancer. For instance, oxidative DNA damage is high in women who have first-degree relatives with breast cancer (Djuric et al. 1998) and in patients with invasive ductal carcinoma (Malin et al 1993). Breast cancer patients were also shown to have increased ROS production and lipid

peroxidation (Ray et al. 2000; Kumaraguruparan et al. 2002). Interestingly, studies in both animals and humans have shown a potentially important antioxidant role for both endogenous and exogenous estrogens (Yagi 1997; Nathan and Chaudhuri 1998). This observation supports the hypothesis that postmenopausal women are at increased risk for oxidative stress due to loss of endogenous estrogen production. In American women, age, markers of glucose metabolism, insulin resistance, and postmenopausal status have been associated with increased oxidative stress and reduced antioxidant potential (Trevisan et al. 2001). While it is generally accepted that insulin resistance may play a role in contributing to oxidative stress, it has been suggested that oxidative stress may result in insulin resistance (Laville et al. 1995). In addition, endothelial dysfunction and increased vascular oxidative stress predict the risk of cardiovascular events in patients with coronary heart disease (Heitzer et al. 2001). It has also been shown that obesity increases with oxidative stress which may trigger the development of insulin resistance (Urakawa et al. 2003). Adipose tissue is highly metabolic and abdominal fat is involved in the production of oxidative stress. A large waist circumference is associated with a high concentration of oxLDL independently of BMI in both men and women (Weinbrenner et al. 2006).

Dietary antioxidants protect the body against oxidative stress either by directly reacting with or quenching free radicals directly, or by indirectly decreasing the damage caused by the free radicals.  $\beta$ -carotene is a fat-soluble carotenoid and antioxidant that concentrates in lipoprotein fractions, such as HDL and LDL, and accumulates in atherosclerotic plaques (Stahl and Sies 1997). High serum  $\beta$ -carotene concentration has been shown to protect endothelial tissue from damage by decreasing cholesterol uptake into those cells and may inhibit the formation of atherosclerotic lesions (Buring and Hennekens 1997; Vogel et al. 1997). An increase in lipid peroxidation has also been associated with lower plasma concentrations of antioxidants such as vitamin C,  $\alpha$ -tocopherol,  $\beta$ -carotene, and selenium (Allard et al. 1998).

Lycopene is a carotenoid present in tomato products and has been shown to have the highest antioxidant capacity of the carotenoids by quenching singlet oxygen and trapping peroxy radicals (Di Mascio et al. 1989; Miller et al. 1996). It has also been reported that high consumption of tomato products improves plasma antioxidant status, lowers lipid

peroxidation (Parfitt et al. 1994), and improves the antioxidant defense of LDL against attack by singlet oxygen (Oshima et al. 1996). Furthermore, the consumption of tomato products has also been shown to reduce the susceptibility of lymphocyte DNA to oxidative damage (Riso et al. 1999). More recently, the combination of the carotenoids, lutein,  $\beta$ -carotene, and lycopene, at a dose achievable through the diet (12 mg total or 4 mg of each), has been shown to exert protection against oxidative DNA damage (Zhao et al. 2006).

It is well known that a higher intake of omega-3 fatty acids from fish or plant sources lower the risk of CVD (Hu and Willet 2002). Omega-3 fatty acids reduce CVD risk by reducing TG, decreasing platelet aggregability, and exerting antiarrhythmic effects (Conner 2000). In normotriglyceridemic and hypertriglyceridemic individuals fish oil supplementation has been shown to stimulate omega-3 fatty acid incorporation into erythrocyte membranes (Mabile et al. 2001). Likewise,  $\alpha$ -lipoic acid has been shown to be protective against oxidative stress-induced endothelial function (Park et al. 2004).  $\alpha$ -Lipoic acid is a thiol antioxidant with direct free-radical scavenging potential (Packer et al. 1995, 1997) and exhibits beneficial effects on vascular and endothelial function (Evans et al. 2002; Visioli et al. 2002).

### Iron Status

Iron is an essential nutrient needed by humans for normal redox reactions that occur under tightly controlled conditions. As a part of the protein hemoglobin, iron is essential to transport oxygen and carbon dioxide to and from tissues. Iron is needed in the electron transport system to provide energy to the cell. In addition, iron is necessary for cell proliferation, DNA synthesis, brain, and immune function (Corti et al. 1997). On average, one third of total body iron is bound to the storage protein ferritin primarily found in the liver, spleen, and bone marrow. The other two thirds of body iron facilitates metabolic or enzymatic function, and is transported by transferrin. Iron-containing enzymes catalyze the transfer of electrons and oxidation-reduction reactions (Swanson 2003). Hence, it is the biological property of iron to exist in two oxidation states [(ferric ( $\text{Fe}^{3+}$ ) and ferrous ( $\text{Fe}^{2+}$ )], which may pose a threat when not properly modulated by iron-binding proteins or antioxidants.

During adulthood, iron stores gradually increase in men; in women, iron stores begin to increase after menopause as a result of the cessation of menstruation. Data from the Third National Health and Nutrition Examination Survey report the mean serum ferritin concentration in premenopausal women, postmenopausal women, and men of 33.6, 93.4, and 139.9  $\mu\text{g/l}$ , respectively. Serum ferritin has been shown to be higher in postmenopausal women compared to premenopausal women, both in blood donors (43.4 vs. 23.1  $\mu\text{g/L}$ ,  $P<0.001$ ) and in nondonors (71.7 vs. 32.8  $\mu\text{g/L}$ ,  $P<0.001$ ) (Berge et al. 1994). Since iron stores, TC, and LDL-C increase with age, it is not surprising that iron stores and blood lipids are positively associated. Serum ferritin has been shown to be positively associated with TC and LDL-C, after adjusting for age (Berge et al. 1994). Whether there is any relationship between iron and estrogen or hormone therapy remains unclear. There is evidence that women using hormone therapy have better iron status parameters and lipid profiles than women not on hormone therapy (Penckofer et al. 2000). It has also been shown that postmenopausal women have higher TC, LDL-C, and TG compared to premenopausal women with similar diet, body weight, and body fat distribution (Masse et al. 2004).

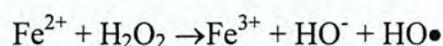
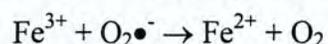
Epidemiological studies suggest a positive relationship between body iron stores and risk for CVD (Salonen et al. 1992; Danesh et al. 1999). However, some reports suggest that there is not enough evidence to link iron status consistently with CVD in epidemiological studies (Corti et al. 1997). Since aspirin is associated with reduced risk of myocardial infarction and lowered iron stores, this suggests a role for iron in CVD. The Framingham Heart Study illustrated aspirin of serum ferritin lowering effect of aspirin, which may be related to findings from epidemiologic studies regarding associations between elevated serum ferritin and heart disease risk (Fleming et al. 2001). Furthermore, in the presence of other risk factors, serum ferritin may adversely affect ischemic heart disease risk in the elderly (Klipstein-Grobusch et al. 1999).

A decrease in body iron stores has been shown to be cardioprotective due to a reduction of iron availability which impairs nitric oxide action, thereby improving endothelial function in patients with CVD (Duffy et al. 2001). Transition metals, such as iron and copper, catalyze free radical oxidation of lipids and proteins. Iron may contribute to atherosclerosis by promoting oxidative modification of LDL (or oxLDL), a key step that

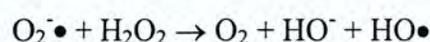
leads to scarring and inflammation of arteries. In vitro, free iron participates in the Fenton and Haber-Weiss reactions, reducing hydrogen peroxide to the harmful hydroxyl radical, as shown in the Figure 5.

Figure 5. Fenton and Haber-Weiss Reactions

Fenton reaction (Emerit et al. 2001)



Haber-Weiss reaction (Haber and Weiss, 1934)



Unbound iron catalyzes the reaction of the superoxide radical and hydrogen peroxide to form the hydroxyl radical. The highly reactive hydroxyl radical is capable of binding a hydrogen atom from polyunsaturated fatty acids, which initiates lipid peroxidation. In addition, once lipid hydroperoxides accumulate, free iron may directly initiate additional lipid peroxidation. However, in healthy individuals, iron is tightly bound to transferrin in circulation. Thus, only a small amount of catalytic iron is typically found in the body under normal physiological conditions (Reddy and Clark 2004). Any released iron is immediately chelated in cells by low molecular weight compounds, such as citrate or adenosine diphosphate (Emerit et al. 2001).

The hypothesis that transition metals accumulate in human arteries remains unconfirmed. Elevated concentrations of iron and copper were shown in the intima of atherosclerotic lesions compared to healthy controls, which supports the hypothesis that iron may accumulate and contribute to atherogenesis. Women, with and without carotid plaques, had average serum ferritin of 90.8 and 67.3  $\mu\text{g/L}$  ( $P < 0.001$ ), respectively, whereas men, with and without plaques, had average serum ferritin of 163 and 147  $\mu\text{g/L}$  ( $P < 0.31$ ), respectively. In the same study, a dose-response relationship between serum ferritin and carotid atherosclerosis was implicated in that a higher ferritin concentration was positively associated with a greater odds ratio for carotid plaque prevalence in both men and women (Wolff et al. 2004).

The brain may be especially vulnerable to oxidative stress and neurological damage due to high amounts of polyunsaturated fatty acids in membrane lipids, iron, and low concentrations of antioxidant enzymes. A case-cohort study of stroke patients matched with a random sample from the European Prospective Investigation Into Cancer and Nutrition measured serum ferritin, serum iron, and transferrin saturation (van der et al. 2005). Serum ferritin concentration ranged from 4.6 to 1158.0  $\mu\text{g/L}$ , and the overall median concentration was 103.0  $\mu\text{g/L}$ . Of the 1,132 women in the study, 1.9% had serum ferritin concentration  $<15 \mu\text{g/L}$  and 4.3% had  $>300 \mu\text{g/L}$ . The study showed that the highest tertile of serum ferritin concentration was associated with an increased risk of ischemic stroke compared to the lowest tertile. A mechanism explaining the observation between high ferritin and stroke could be attributed to the catalytic properties of iron in the production of hydroxyl radicals. In a different study, zinc antagonized the effect of iron in atherosclerotic lesions when free radicals were produced by iron, suggesting zinc and iron have opposite roles (Minqin et al. 2003). In the Iowa Women's Health Study, researchers found that a higher intake of heme iron may be harmful, while a high intake of zinc may be beneficial in relation to CVD mortality in the presence of a trigger, such as alcohol disturbed homeostasis (Lee et al. 2005).

A recent study using mice confirmed in vivo results showing an increase in vascular deposition accompanying superoxide release with a high-fat and iron-supplemented diet (Yao et al. 2005). Fatty acids might mediate iron intracellular translocation and subsequent oxidative injury. An in vitro study showed that palmitic acid facilitated iron translocation into cells and mitochondria through a transferrin receptor-independent mechanism, resulting in intracellular iron overload and subsequent ROS overproduction and lipid oxidation. Results from the Bruneck Study, after adjusting for major vascular risk factors, revealed that serum ferritin was one of the strongest indicators of carotid artery disease in both men and women. In addition, the predictive significance of ferritin was found to be synergistic with hypercholesterolemia (Kiechl et al. 1994). Therefore, the relationship between body iron stores and blood lipids seems to be interrelated with regards to increased CVD risk.

However, some studies disagree with the hypothesis that excess body iron is associated with CVD risk. Eichner et al. (1998) found no relationship between increased iron stores in atherosclerosis patients. Likewise, in a rat model, serum iron was found to have no

effect on serum lipids or blood pressure; however, rats have distinct differences in lipid and plasma lipoprotein metabolism (no hepatic lipase or a protein similar to apolipoprotein-AII) compared to humans (Turbino-Ribeiro et al. 2003). Iron supplementation did not increase the susceptibility of LDL to oxidative modification in women with low iron status (Binkoski et al. 2004). In addition, three controlled feeding studies found no relationship between iron status and LDL oxidative susceptibility (Derstine et al. 2003). Lastly, Sempos et al. (2001) found no evidence that elevated iron stores is directly related to CVD. Therefore, in a healthy population, iron excess is not a major concern; however, in persons with hyperlipidemia, elevated oxidative stress may place individuals at greater risk for CVD (Reddy and Clark, 2004).

There has been less research on the relationship between body iron stores and body fat distribution, despite the association of each factor with CVD risk. In Mexican-American men, serum ferritin concentration was negatively associated with waist-to-hip ratio (WHR) and other indices of body fat distribution and obesity (Gillum 2001). It is important to note that waist and hip circumferences assess different aspects of body composition and fat distribution and may have independent and opposite effects on CVD risk factors. For example, a narrow waist and large hips may both be protective against CVD, hence the WHR is a crude index for determining disease risk (Seidell et al. 2001). In addition, there may be ethnic or cultural differences in the association between iron stores and body fat distribution. Chambers et al. (2006) found that serum iron was inversely correlated with BMI, waist circumference, and fat mass among Hispanic women but not among African-American, white, or Asian women.

Serum ferritin concentration was also related to the degree of insulin resistance in Chinese women, but not in men, after adjusting for age, BMI, and menopausal status (Sheu et al. 2003). More recently, a study reported a direct association between serum ferritin and visceral fat area and subcutaneous fat area using computed tomography. These results suggest that serum ferritin may be a useful indicator of systemic fat content and degree of insulin resistance (Iwasaki et al. 2005). A possible mechanism explaining this relationship could be that increased iron stores in the liver may induce liver-mediated insulin resistance and reduce the ability of insulin to suppress hepatic glucose production (Fernandez-Real et

al. 1998). Ferritin is associated with decreased insulin sensitivity and increased fasting plasma insulin and glucose, abnormalities which may lead to increased adiposity (Gillum 2001). Lastly, iron stores in muscle might enhance the oxidation of free fatty acids, which in turn might interfere with glucose disposal. Elevated free fatty acids can damage pancreatic beta cells and cause insulin resistance (Tuomainen et al. 1997). Nonetheless, the direct association of serum ferritin to central adiposity warrants further investigation.

### Homocysteine

Homocysteine (Hcy) is a sulfur-containing amino acid that is an intermediary product in methionine metabolism. Deficiencies in vitamin cofactors in the Hcy pathway, including folate, pyridoxine, and cobalamin may also lead to hyperhomocysteinemia. Individuals with increased plasma Hcy are likely to have inadequate concentrations of one or more of these vitamins (Selhub et al. 1993, Robinson et al. 1995). In patients with an inborn error of Hcy metabolism (homocysteinuria), due to a deficiency of an enzyme involved in the Hcy pathway, lowering the elevated circulating Hcy concentrations greatly reduces CVD risk (Wilcken and Wilcken 1997; 1998).

Hyperhomocysteinemia has evolved from a rare condition predisposing individuals to atherothrombosis to a common disorder of atherosclerosis in the general population. The pathogenesis of atherothrombotic vascular disease may be closely associated with hyperhomocysteinemia in relation to oxidative stress, vitamin deficiencies, and endothelial dysfunction (Loscalzo 1996). Increased circulating Hcy is a contributing factor to the development of premature atherosclerosis and an independent risk factor for stroke and myocardial infarction (Stampher et al. 1992). Moderate hyperhomocysteinemia, defined as plasma concentrations of Hcy > 15-16  $\mu\text{mol/L}$ , has been identified in 20-40% of patients with atherosclerotic vascular disease (Duell and Malinow 1998).

In healthy, postmenopausal US women, increased concentration of Hcy is correlated with increased risk of future CVD (Ridker et al. 1999). Estradiol has been shown to have a beneficial effect on preserving the endothelial integrity and function in a condition of Hcy-induced oxidative stress (Dimitrova et al. 2002). In a prospective cohort, the Hordaland Homocysteine Study of almost 5000 Norwegian men and women, plasma Hcy was a strong predictor of both cardiovascular and noncardiovascular mortality in individuals 65-72 year of

age (Vollset et al. 2001). This study supports results from elderly Framingham subjects (Bostom et al. 1999) and from a study of Jerusalem men and women (Kark et al. 1999). Plasma Hcy concentration was significantly correlated with the proinflammatory cytokines interleukin-1 receptor antagonist and interleukin-6 (IL-6) in Italians over 65 years of age (Gori et al. 2005), contributing to the association between Hcy and atherosclerosis. In addition, the following factors were significant independent correlates with Hcy: a sedentary state; intakes of vitamin B-6, B-12, and folic acid; serum folate; and serum  $\alpha$ -tocopherol. Graham et al. (1997) detected hyperhomocysteinemia in 42% of patients with cerebrovascular disease, 28% with peripheral vascular disease, and 30% with coronary vascular disease. In addition, with an increase of 5  $\mu\text{mol/L}$  in Hcy concentration, the relative risk of CVD has been estimated to increase by approximately 60% for men and 80% for women (Graham et al. 1997). Interestingly, women may be able to control their Hcy concentration by decreasing coffee and alcohol consumption, whereas men should focus on increasing physical activity, dietary fiber, and dietary folate (Mennen et al. 2002), indicating a difference in Hcy metabolism between the two sexes.

Studies have shown that elevated Hcy concentration is associated with premature vascular disorders, including myocardial infarction and cerebral and vascular diseases (Malinow et al. 1993; Clarke et al. 1991). A meta-analysis showed that increasing Hcy concentrations has a graded effect on CVD risk, meaning the greater the Hcy concentration, the greater risk of CVD will result (Boushey et al. 1995). Jang et al. (2001) revealed that male CVD patients had greater visceral fat accumulation, higher insulin and Hcy concentrations, and lower insulin-like growth factor I, superoxide dismutase, and  $\beta$ -carotene concentrations than healthy men matched for age and BMI. In addition, CVD patients with diabetes had the greatest visceral fat and Hcy concentration and the lowest HDL-C and carotenoid concentrations.

It is proposed that Hcy may have direct cytotoxic effects, producing free radicals that may cause endothelial damage and dysfunction (Currie et al. 1996). High circulating Hcy concentration may promote oxidative damage because the sulfhydryl group of Hcy acts catalytically with ferric or cupric ions to generate hydrogen peroxide, oxygen, and homocysteinyl radicals (Olszewski and McCully 1993). Thus, a high Hcy concentration has

been associated with low antioxidant capacity (Buczynski et al. 1993). Oxidative stress is increased by Hcy via stimulating superoxide production (McDowell and Lang 2000). This relationship was supported by a study (Jang et al. 2001) where patients with or without diabetes had lower superoxide dismutase activity than did control subjects.

Folic acid, or folate, is a micronutrient found in green leafy vegetables, some animal products, and fortified enriched grain products in the United States to prevent neural tube defects in newborns. Folate is needed to provide one-carbon units for more than 100 biochemical processes, including Hcy metabolism and deoxynucleotide synthesis. Increasing evidence shows that folate may contribute to CVD prevention via reducing plasma Hcy concentration by remethylating Hcy to methionine (Haynes 1999). Chait et al. (1999) demonstrated that a prepared meal plan providing >100% of the recommended dietary allowances (RDA) for 23 micronutrients, including folate, resulted in increased intakes and serum concentrations of folate and vitamin B-12 and were associated with reduced serum Hcy concentration in individuals at increased CVD risk. The current RDA of 180 µg/d may not be sufficient to maintain a low plasma Hcy concentration in some postmenopausal women (Jacob et al. 1998). Increased lipid peroxidation during folate deficiency may be a consequence of increased Hcy concentration. Folic acid may provide a beneficial effect on the vascular endothelium by reducing plasma Hcy or by reducing oxidative stress (Wilmink et al. 2000). An increase in oxidative stress may play a role in reducing endothelial nitric oxide activity in hyperhomocysteinemic patients (Faraci, 2003). However, a study has shown that folic acid administration improved endothelial function without any effects on Hcy concentration (Verhaar et al. 1998). In addition, folic acid was shown to have antioxidant properties and direct scavenging effects in vitro and may directly improve nitric-oxide production by enhancing enzymatic activity of nitric-oxide synthase (Stroes et al. 2000).

### Cytokines

Recent evidence has suggested that CVD is an inflammatory disease mediated by systemic inflammatory markers. The precise triggering of the inflammatory response is unknown. However, activated neutrophils, lymphocytes, monocytes, and increased

proinflammatory cytokines, such as IL-6, CRP, and TNF- $\alpha$  are involved in the pathophysiology of CVD (Meng et al. 2006).

IL-6 may play an important regulatory role in the development of atherosclerosis by metabolic, endothelial, and coagulation mechanisms. IL-6 increases glucose uptake, increases the release of adhesion molecules by the endothelium, increases the hepatic release of fibrinogen, and exerts procoagulant effects on platelets (Yudkin et al. 1999). Cytokines have also been associated with components of insulin resistance syndrome, including insulin sensitivity, high TG and low HDL-C concentrations, and elevated blood pressure (Dan et al. 1995). In addition, IL-6 and TNF- $\alpha$  inhibit lipoprotein lipase and stimulate lipolysis. Endothelial expression of other chemokines and adhesion molecules, induced by IL-6, may affect TG metabolism by altering endothelial generation of nitric oxide following elevated non-esterified fatty acid concentration (Samad et al. 1996). Lastly, a procoagulant state may be attributed to the effect of IL-6 on platelets, fibrinogen concentration, and coagulation, and to the effect of TNF- $\alpha$  on plasminogen activator inhibitor by hepatocytes, endothelial cells, and adipose tissue (Mohamed-Ali et al. 1997). Therefore, IL-6 plays a role in the progression and etiology of CVD.

In a study of healthy men and women, the synthesis and circulating concentration of IL-6 increase with adiposity. It is estimated that one third of circulating IL-6 originates from the adipocyte. In vitro data suggest a greater contribution of IL-6 and TNF- $\alpha$  from visceral fat than from subcutaneous fat (Mohamed-Ali V et al. 1997), which supports the idea that adipose tissue around the organs is more atherogenic than fat directly under the skin. Hence, obesity can be regarded as a low-grade inflammatory condition in which IL-6 is synthesized from adipose tissue.

Interestingly, it has also been well-documented that psychological factors, such as stress, can elevate IL-6 concentration influenced of catecholamines. The hypothalamo-pituitary-adrenal axis may be stimulated by IL-6, thereby increasing corticotrophin releasing hormone, adrenocorticotrophic hormone, and cortisol. Patients with melancholic depression patients were shown to have elevated IL-6 along with other CVD risk factors including: central adiposity, increased blood pressure, insulin resistance, hypertriglyceridemia, and low LDL-C (Chrousos and Gold 1998). Psychosocial stress may elevate IL-6 concentration,

thereby stimulating the hypothalamo-pituitary-adrenal axis, resulting in increased abdominal fat storage, insulin resistance, and dyslipidemia (Brunner et al. 1997).

Inflammation has also been reported to be increased in type 2 diabetics. Women with type 2 diabetes and CVD have a significantly greater IL-6 concentration compared to age-matched controls, suggesting an additive effect of the two diseases on inflammation (Tuttle et al. 2004). IL-6 elevation was shown to be proportional to insulin resistance and blood glucose (Bastard et al. 2000). When obese patients were placed on energy-restricted diets for three weeks, IL-6 significantly decreased and IL-6 and TNF- $\alpha$  were shown to be positively correlated with BMI. Hence, IL-6 may reflect amount of adipose tissue and degree of insulin resistance. In a study with 108 postmenopausal women, the women with a combination of high visceral adipose tissue and high insulin resistance were characterized as having a greater deterioration in metabolic risk profile than women with one of the two conditions (Piche et al. 2005).

The relationship between cytokines and hormone status has been studied in postmenopausal women who are at increased risk of CVD. Postmenopausal women on hormone therapy were shown to have elevated CRP, while no relation was found between IL-6 and CRP (Lakoski and Herrington 2005). However, Vitale et al. (2005) found plasma IL-6 to be a stronger predictor of adverse cardiovascular events than CRP and that CRP was associated with an adverse event only when IL-6 was also elevated. Thus, menopause and estrogen status have a variety of effects on inflammatory markers and hormone therapy may modulate the relationship between obesity and inflammatory cytokine production. The Women's Health Initiative study found a significant relationship between CRP, but not IL-6, and use of hormone therapy (Langer et al. 2005). This evidence suggests CRP is influenced by factors other than IL-6, possibly a component of hormone therapy. Further studies are needed to determine the relationship between estrogen status, cytokines, CVD risk in postmenopausal women.

## **Prevention**

### *Hormone and Estrogen Therapies*

Although CVD has often been regarded as a disease impacting men, after menopause the risk is more similar in both genders. Premenopausal women are relatively protected from

CVD compared to postmenopausal women attributed to the female sex hormone, estrogen. The difference in mortality related to CVD between pre- and postmenopausal women suggests that premenopausal women have endothelial protective factors that are lost after menopause. The site of estrogen action is not only reproductive tissues, but also the bone, liver, brain, and vasculature. Both genomic, mediated by estrogen receptor alpha and beta, and nongenomic mechanisms, through nitric oxide, do not explain the effects of estrogen on preserving the vasculature under conditions of oxidative stress (Dimitrova et al. 2002). In vitro and in vivo studies show that estrogen prevents vascular injury in conditions of induced hyperhomocysteinemia and decreases Hcy in animals on a hyperhomocysteinemic diet (Dimitrova et al. 2001a and Dimitrova et al. 2001b). In addition, estrogen was found to have antioxidant properties by enhancing glutathione, leading to a reduction in hydrogen peroxide concentration (caused by high Hcy), resulting in reduced endothelial damage (Dimitrova et al 2001). Hence, estradiol may have a beneficial effect on preserving the endothelial integrity and function in conditions of Hcy-induced oxidative stress.

Due to a loss of estrogen in the postmenopausal years, some women consider taking hormone therapy to improve their lipid profile, risk of osteoporosis, and memory, mood, and hot flashes. However, there have been reports that the risks of hormone therapy outweigh the benefits and can lead to fatal cardiovascular events and breast and uterine cancers (Writing Group for the Women's Health Initiative Investigators 2002, Manson et al. 2003). In the Women's Health Initiative Study, estrogen alone was shown to have no effect on coronary heart disease risk but did increase the risk for stroke and deep vein thrombosis. Investigators recommend that estrogen alone should not be used to prevent chronic disease and should only be used at the smallest effective dose for the shortest amount of time to treat menopausal symptoms, especially for women less than 60 years of age (Writing Group for the Women's Health Initiative Investigators 2004). Whether hormone therapy protects women from CVD via reducing Hcy, iron stores, and oxidative damage needs further investigation.

### *Physical Activity*

It is estimated that 66% of Americans are overweight and 33% are considered clinically obese, defined as having a BMI  $\geq 30$  kg/m<sup>2</sup> (Flegal et al. 2002). Thus, it is crucial

for men and especially postmenopausal women to focus attention on a healthy diet and exercise to help combat CVD risk. Epidemiologic evidence clearly shows that physical activity is an important modifiable risk factor for CVD (Hoyert et al. 2001; Pate et al. 1995) and consistently reduces morbidity and mortality from CVD (Kohl 2001; Wannamethee and Shaper 2001). A meta-analysis of 30 studies using female subjects quantified the relationship of physical activity in healthy women to CVD outcomes and assessed the effect of minimal amounts of physical activity to reduce CVD risk. The conclusion was encouraging; inactive women could benefit slightly by increasing their physical activity level, to walking one hour per week (Oguma et al. 2004). Excess body weight and physical inactivity are associated with higher TG, TC, and LDL-C, and lower HDL-C (Brown et al. 2000). One reason for higher HDL-C in physically active individuals is due to the increased formation of HDL-C from apolipoprotein A-I and cellular lipids (Olchawa et al. 2004). Physical activity is effective in lowering TGs in particular and TC in general. In addition, physical training increases insulin sensitivity (Hardin et al. 1995) and lowers blood pressure (Blair et al. 1989). Therefore, physical activity is crucial to prevent chronic disease by maintaining a healthy weight and by maintaining normal concentrations of circulating lipids. Physical activity also improves immune response and overall optimal health.

In contrast, aerobic exercise increases oxygen consumption and free radical formation (Brownell and Bachorik 1982), thereby consuming circulating antioxidants (Davies et al. 1982) and increasing the rate of lipid peroxidation and oxidation of LDL particles (Berg et al. 1994; Sanchez-Quesada et al. 1995). In addition, relatively intense aerobic training decreases circulating antioxidant concentrations and impairs endothelial function in forearm vessels (Bergholm et al. 1999). However, a recent study reported that a physically active lifestyle was related to a lower CVD risk and lower oxidative stress (Phil et al. 2003).

### *Dietary Approaches*

Many approaches regarding dietary intake have been investigated to determine which components provide protection against CVD. Since the antioxidant effect of phytochemicals may be additive or synergistic, it is important to consume whole foods, such as fruits and vegetables, rather than pills or extracts. The benefit of a diet rich in fruits and vegetables is attributed to the complex mixture of phytochemicals in whole foods compared to individual

supplements (Liu 2003). This theory is supported by a small study of men and women given gazpacho, a Mediterranean vegetable soup rich in vitamin C, who exhibited a reduction in oxidative stress and biomarkers of inflammation (Sanchez-Moreno et al. 2004). Likewise, fruit and vegetable consumption was shown to increase glutathione peroxidase activity and resistance of plasma lipoproteins to oxidation more efficiently than supplemental vitamins and minerals (Dragsted et al. 2004). Thus, increasing vegetable consumption may greatly improve longevity and prevent oxidative stress-induced diseases.

Dietary sources rich in flavonoids, including apples, onions, red wine, chocolate, red fruits, citrus fruits, and tea, have been shown to be cardioprotective. Elderly women who consumed strawberries, spinach, or red wine, rich in antioxidant phenolic compounds, showed an increase in their antioxidant capacity (Cao et al 1998). In addition, French women who consume high amounts of flavonoid-rich foods are at less risk for CVD and have lower systolic blood pressure than women who consume low amounts of these foods (Mennen et al. 2004).

The dietary supplementation of lycopene (39.2-75.0 mg/day), a carotenoid available in tomato products, was shown to significantly reduce serum lipid peroxidation and LDL oxidation, yet provided no effect on serum TC or LDL-C (Agarwal and Rao 1998). Likewise, the inhibition of lipid peroxidation by polyphenolic antioxidants derived from products, such as tea and chocolate has also been documented (Gelejkjnse et al. 1999; Hodgson et al. 2000). However, in one study the regular ingestion of green or black tea did not alter the urinary excretion of F<sub>2</sub>-isoprostanes, a marker of in vivo lipid peroxidation, in subjects with mildly elevated blood pressure or serum TC concentration (Hodgson et al. 2002). Interestingly, cocoa products, rich in flavonoids, were shown to protect LDL from oxidation, but had no effect on F<sub>2</sub>-isoprostanes or other markers of inflammation (Mathur et al. 2002). Additionally, Wan et al. (2001) found that cocoa powder and dark chocolate may favorably affect CVD risk factors by reducing LDL oxidative susceptibility, increasing total antioxidant capacity and HDL-C concentration, but not adversely affect prostaglandins.

In addition to a diet rich in antioxidants, moderate alcohol intake has also been shown to provide protection against CVD. In a randomized crossover study investigating the effect of moderate alcohol consumption on CVD risk along with a controlled diet reported a

protective effect on serum lipids and lipoproteins. Consumption of 15-30 grams (or 1-2 drinks/day) of alcohol per day by postmenopausal women improved plasma LDL-C and HDL-C and apolipoprotein A-I and B concentrations (Baer et al. 2002). Procyanidins, the antioxidant predominant in wine, have been shown to be effective in preventing lipid oxidation of foods while in the digestive tract, thus preventing the rise in postprandial hydroperoxides (Ursini et al. 1998). In addition, several studies have shown an increase in plasma antioxidant capacity following wine intake (Maxwell et al. 1994; Whitehead et al. 1995). Studies with rabbits fed a high cholesterol diet have shown that grapeseed procyanidins are strongly protective in reducing plasma lipid peroxides and inhibit lipid-laden foam-cell deposition (Ursini and Sevanian, 2002). Furthermore, a study with pre- and postmenopausal women given lyophilized grape powder, rich in a variety of polyphenols, favorably altered lipoprotein markers, oxidative stress, and inflammatory markers (Zern et al. 2005). Interestingly, ingestion of dietary polyphenols, including wine, cocoa, or tea, along with a meal rich in oxidizable lipids, improves endothelial dysfunction, lowers the susceptibility of LDL lipids to oxidation, and may act as modulatory signaling molecules (Sies et al. 2005).

The consumption of plant stanol or sterol esters incorporated into food products, such as margarine and yogurt, is also an effective and safe method of lowering LDL-C (Thompson and Grundy 2005). Nonesterified, nonhydrogenated plant sterols from soybean oil, when incorporated to a low-fat milk product, has shown potential to substantially reduce LDL-C in mildly hypercholesterolemic individuals (Thomsen et al. 2004). This is possibly due to the ability of plant sterols to inhibit cholesterol absorption by competing for esterification in the enterocyte of the small intestine.

Another dietary modification is the inclusion of lignans found in flaxseed that have been shown to lower LDL-C (Jenkins et al. 1999). In a double-blind randomized study, 40 grams were given to postmenopausal women and compared with a wheat-base control for three months. The subjects on the flaxseed regimen exhibited a reduction in LDL-C by 4.7% and TG by 12.8%. In addition, apolipoprotein A-1 and apolipoprotein B concentrations were significantly reduced by 6% and 7.5%, respectively (Lucas et al. 2002). Lignans are being increasingly incorporated into the diet for their health properties, such as antitumorigenic

(Thompson et al. 1996), estrogenic or anti-estrogenic (Collins et al. 1997), and antioxidant properties (Prasad 1997; Prasad 2000; Kitts et al. 1999). Thus, uses of plant sources, such as nuts, legumes, soy, and vegetable oils, that provide lignans, may provide a variety of health benefits including prevention of CVD (Hu 2003). This may be partly due to incorporating high proportions of omega-3 fatty acids into serum lipids, which in turn is associated with a substantially reduced risk of death (Erkkila et al. 2003). In addition, diets low in saturated fat and cholesterol have been shown to promote weight loss and reduce cardiovascular markers (Yu-Poth et al. 1999). Lastly, various sources of protein have different effects on CVD; replacing refined carbohydrates with protein sources low in saturated fat has been shown to be beneficial in cardiovascular health (Hu 2005). Therefore, the use of plant sources low in saturated fat but rich in omega-3 fatty acids and protein may decrease CVD risk.

#### Soy Protein

A large body of evidence has established a role for soy in CVD risk reduction, particularly through lipid reduction (Clarkson 2002). Epidemiological studies have shown that the consumption of soy and/or isoflavones is inversely associated with circulating total (Nagata et al. 1998) or LDL-C (Ho et al. 2000) and TG (de Kleijn et al. 2001), and positively associated with HDL-C (Goodman-Gruen and Kritz-Silverstein 2001).

A meta-analysis reported that an average of 47 g soy protein per day resulted in reductions of 9.3% in circulating TC, 12.9% LDL-C, and 10.5% in TG (Anderson et al. 1995). This evidence contributed to the approval of the food label claim by the Food and Drug Administration for reduced risk of heart disease on foods that contain  $\geq 6.25$  g of soy protein per serving, assuming 4 servings or 25 g of soy protein intake daily (Food and Drug Administration 2002). The American Heart Association recommends individuals at high risk for CVD with elevated total and LDL-C to consume soy protein containing isoflavones along with other heart-healthy dietary modification and increased exercise (Krauss et al. 2001).

Epidemiological research has provided clear evidence that the consumption of soy foods may reduce the risk of CVD in Chinese women (Zhang et al. 2003). The Shanghai Women's Health Study reported that the usual intake of soy foods was inversely associated with both systolic and diastolic blood pressure, especially in elderly Chinese women (Yang et al. 2005). A population-based cross-sectional study of men and women in the Hong Kong

Chinese population also found higher soy intake was related to a less atherogenic plasma lipid profile (Ho et al. 2000). Interestingly, soy and isoflavone intake has been shown to be associated with a reduced risk of cancer; ovarian cancer in southeastern Chinese women (Zhang et al. 2004). In addition, it is speculated that breast cancer protection in Asian women consuming a traditional soy-containing diet is derived from early exposure to soybean products containing genistein (Lamartiniere 2000).

Flavonoids and isoflavones, components of soy, may be responsible for protecting individuals from CVD either by favorably affecting blood lipids or by reducing oxidative stress, acting as antioxidants. Among the Japanese population, flavonoid and isoflavone intake is the main component among phytochemicals with antioxidant potential in the diet. Evidence suggests a high consumption of both flavonoids and isoflavones by Japanese women may contribute to a lower incidence of CVD compared with women in western countries (Arai et al. 2000). The Oxford arm of the European Prospective Investigation into Cancer and Nutrition showed associations of moderate intakes of soyfoods, as part of a regular diet, with favorable blood cholesterol concentrations. Mean plasma LDL-C concentration in women with a soy protein intake  $\geq 6$  g/d was 12.4% lower than in women who consumed  $< 0.5$  g/d (Rosell et al. 2004).

Hypercholesterolemic subjects given 30-50 g of soy protein/day along with a cholesterol-lowering diet significantly reduced LDL-C and total homocysteine concentrations without increasing lipoprotein (a) concentrations, suggesting an antiatherosclerotic effect (Tonstad et al. 2002). However, Engleman et al. (2005) reported soy protein isolate with isoflavones had no significant effect in reducing oxidative damage or favorably altering blood lipids in postmenopausal women. Likewise, Vega-Lopez et al. (2005) concluded that diets high in soy protein or isoflavones derived from soy had little effect on plasma antioxidant capacity or biomarkers of oxidative stress.

Soy protein was shown to affect serum lipid profiles in a beneficial direction for CVD risk in healthy, young men (McVeigh et al. 2006). The consumption of 20 grams of soy protein per day for six weeks, instead of animal protein, reduced non-HDL-C by 2.6% (Teixeira et al. 2000). In addition, male type 2 diabetics benefited from isolated soy protein compared with casein by reductions in the TC-to-HDL-C and LDL-C-to-HDL-C ratios and

increased HDL-C concentration (Teixeira et al. 2004). These findings suggest that isolated soy protein may help patients at risk for CVD.

There are several mechanisms by which soy protein may affect lipid metabolism. Soy protein peptide chains may up-regulate LDL receptors, thereby stimulating gene expression of key enzymes and proteins in lipid metabolism (Tovar et al. 2002; Iqbal et al. 2002; Iritani et al. 1997). It is also speculated that soy protein may improve blood lipid profiles by altering LDL receptor quantity or activity (Baum et al. 1998). Soy protein peptides have been shown to regulate cholesterol homeostasis in vitro (Lovati et al. 2000). Lastly, the amino acid profile of soy differs from that of animal protein and thereby may have an effect on lipid metabolism. The amino acids lysine and methionine have been shown to have hypercholesterolemic effects in rabbits, while arginine showed the opposite effect (Kurowska and Carroll 1994, Huff et al. 1977). This evidence led to the hypothesis that because soy has a higher ratio of arginine-to-lysine and methionine, it may explain the hypocholesterolemic effect of soy (Erdman 2000).

It has been shown that soy protein compared to whey protein supported lean mass gain and prevented a training-induced drop in antioxidant capacity, suggesting that soy foods prevent the risk of exercise-induced increase in oxidative stress (Brown et al. 2004). In addition, soy beverage consumption for three weeks (two servings/day, 40 grams protein, 44 mg of isoflavones/day) was shown to exert antioxidant effects and reduced exercise-induced oxidant stress in young men (Brown et al. 2004). Moderate intensity, weight resistance exercise, combined with 4 weeks of soy consumption, lowered serum peroxide values in men (Hill et al. 2004). Similarly, moderate intensity exercise, along with four weeks of soy intake, exerted a lowering effect on serum lipid peroxide production in women (Box et al. 2005).

The non-protein components of soy, such as saponins, isoflavones, and phytic acid, may also have health benefits by affecting serum TC (Greaves et al. 2000). However, isoflavones are the most studied component of soy and will be discussed in more detail below.

### Soy Isoflavones

*Structure and function:* Isoflavones are phytoestrogens, a group of non-steroidal plant chemicals (genistein, daidzein, and glycitein) that possess estrogen-like activity. The chemical and structural similarity of  $17\beta$ -estradiol to equol, a metabolite of daidzein, has led to the hypothesis that isoflavones may be responsible for the hypocholesterolemic effect of soy (Setchell 1998). Specifically, it is the presence of the phenolic ring and the distance between the hydroxyl groups that makes the isoflavones similar to estrogen (shown in Figure 6).

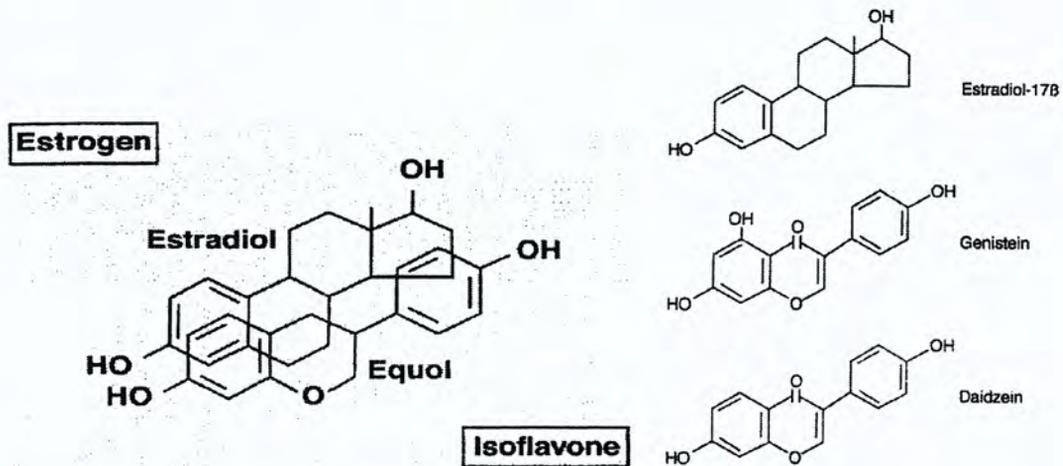


Figure 6. Chemical and structural similarity of estrogen and isoflavones.

Genistein and daidzein, the two predominant isoflavones in soy, have been estimated to exert  $10^{-2}$  to  $10^{-3}$  of the activity of  $17\beta$ -estradiol (Miksiceck 1994). However, the isoflavone concentration in circulation among those consuming the amount of soy present in traditional Japanese diets (50-80mg/d) may be 100-fold higher than the concentration of endogenous estrogens (Setchell and Cassidy 1999). Thus, the plasma concentration of these isoflavones would place them in a biologically effective range, comparable with that of endogenous estrogen. However, dietary isoflavones have less biologically relevant estrogenic activity in vivo compared to estradiol in postmenopausal women (Teede et al. 2004).

*Isoflavones and CVD:* A cross-sectional study examining the association between usual dietary isoflavone intake and CVD risk factors (including lipids and lipoproteins, BMI

and fat distribution, blood pressure, glucose, and insulin) in 208 postmenopausal women reported that subjects with the highest genistein daily intake had significantly lower BMI, waist circumference, and fasting insulin than those with no genistein consumption (Goodman-Gruen and Kritz-Silverstein 2001). In addition, genistein, daidzein, and total isoflavone intake were each positively associated with higher HDL-C and negatively associated with post challenge insulin, suggesting a protective role of dietary isoflavones from soy intake against CVD risk factors in postmenopausal women. Intake of soy protein with low and high isoflavone (1.39 mg and 2.25 mg isoflavones/g soy protein, respectively) content for six months decreased risk factors (6% reduction in TC and 7% reduction in non-HDL-C) associated with CVD in postmenopausal women (Potter et al. 1998). Similarly, the Framingham Offspring Study (de Kleijn et al. 2001) provided evidence that high intakes of phytoestrogens in postmenopausal women were associated with reduced risk of CVD. However, estimated dietary intake of phytoestrogens in healthy postmenopausal American Caucasian women is <1 mg/day. In contrast, a 24-week study indicated that isoflavone-rich and isoflavone-poor soy protein had no effect on lipid profiles in perimenopausal women (Dent et al. 2001), suggesting that postmenopausal women may be more responsive to soy isoflavones than perimenopausal women. Soy protein with intact isoflavones, compared to soy protein devoid of isoflavones, increased HDL-C and apolipoprotein A-I concentrations, but did not influence LDL-C or fibrinogen in normolipidemic, healthy subjects (Sanders et al. 2002). Wangen et al. (2001) concluded that although the consumption of isoflavones as a constituent of isolated soy protein resulted in insignificant improvements in the lipid profile of normocholesterolemic and mildly hypercholesterolemic postmenopausal women, the consumption of soy foods over many years in conjunction with other lipid-lowering strategies may lower CVD risk.

Soy protein, independent of its isoflavone component, shifted LDL particle distribution to a less atherogenic pattern in hypercholesterolemic men and women (Desroches et al. 2004). A meta-analysis by Zhuo et al. (2004) showed that with identical soy protein intake, a high isoflavone intake led to a significantly greater decrease in LDL-C than a low isoflavone intake, demonstrating that isoflavones have LDL-C-lowering effect independent of soy protein. Lastly, in a more recent meta-analysis of twenty-three

randomized controlled trials from 1995 to 2002, soy protein containing isoflavones were shown to significantly reduce serum TC, LDL-C, and TG, and significantly increase HDL cholesterol. Importantly, the changes were related to the level and duration of soy isoflavones intake, the sex and initial serum lipid concentration of the subjects (Zhan and Ho 2005). Conversely, another meta-analysis of ten studies concluded that the consumption of soy-associated isoflavones is not related to change in LDL-C or HDL-C (Weggemans and Trautwein 2003). Likewise, another meta-analysis of seventeen clinical trials confirmed that isoflavones in the form of tablets, isolated soy protein, or soy diets with up to 150 mg/day had no statistical or clinical benefit on serum lipids (Yeung and Yu, 2003). Therefore, the effect of isoflavones on serum lipids warrants further investigation.

Discrepancies among the findings of these clinical studies may be attributed to individual variability, baseline cholesterol concentrations, dietary intake of other foods/nutrients, use of medications, isoflavone metabolizing capacity, and genetics. Equol (shown in Figure 6) a gut bacteria metabolite of daidzein, has a greater binding affinity to estrogen receptors and a greater antioxidant capacity compared to daidzein (Bingham et al. 2003). Interestingly, individuals vary in their ability to synthesize equol (Rowland et al. 2000), suggesting that responsiveness to isoflavones may vary depending on an individual's equol-synthesizing capacity (Setchell et al. 2002). A large (n=117) randomized, double-blind, placebo-controlled, crossover dietary intervention trial demonstrated that isoflavone supplementation had no effect on lipid or other biomarkers of CVD risk in postmenopausal women, but increased HDL-C in an estrogen receptor  $\beta$  gene-polymorphic subgroup (Hall et al. 2006). This study suggests that polymorphisms in genes may determine an individual's response to estrogen or isoflavone supplementation.

Mechanisms responsible for the effects of soy isoflavones on the lipid profile are currently being explored. Isoflavones from intact soy foods may act as a natural selective estrogen receptor modulator, and exert an effect on lipid metabolism similar to endogenous estrogen. Results from a six-month trial with postmenopausal women indicated that soy protein, with different amounts of isoflavones (56 vs. 90 mg of isoflavones), decreased the risk of CVD by improving blood lipid profiles (Baum et al. 1998). Researchers reported that

the mechanism by which apolipoprotein B-containing lipoproteins were depressed may be due to alterations in LDL receptor quantity or activity.

Isoflavones may have an effect on lipogenesis and lipolysis. Incubation of isolated rat adipocytes with genistein restricted glucose conversion to total lipids, either in the absence or presence of insulin (Szkudelska et al. 2000). Thus, the anti-lipogenetic action of genistein may be related to an alteration in glucose transport and metabolism, in addition to its effect on restricting fatty acid synthesis.

*Isoflavones and inflammation:* Chronic inflammation is a major contributor to atherosclerosis and CVD (Libby et al. 2002). An important marker of inflammation is elevation in serum CRP (Bassuk et al. 2004), an acute-phase reactant secreted by hepatocytes in response to proinflammatory cytokines such as IL-6. In several epidemiologic studies, CRP was shown to be a strong, independent predictor of CVD risk in both men and women (Ridker et al. 2001; Rifai et al. 2002; Ridker et al. 2002, Ridker et al. 1997). The mechanism by which inflammation increases CVD risk is not known. However, it is possible that inflammation may adversely affect lipid metabolism. During periods of acute inflammation, lipid metabolism is altered to reflect a proatherogenic profile including: increased TG, decreased HDL-C, and the appearance of small, dense LDL particles (Khovidhunkit et al. 2004; Khovidhunkit et al. 2000; Cabana et al. 1989).

Recently, isoflavones have been shown to have a beneficial effect on CRP concentration, a classic downstream marker of inflammation which mediates the initiation and propagation of atherosclerotic plaques (Hall et al. 2005). However, isoflavones have not been shown to have similar effects on other inflammatory markers in postmenopausal women. For example, in a different study with hypercholesterolemic adults on a cholesterol-lowering diet, the treatment of added soy with and without isoflavones, or milk protein, did not affect lipids or inflammatory markers (Hilpert et al. 2005). Regardless of the protein source, individuals with low CRP responded with a significant decrease in LDL-C and in the LDL:HDL ratio, whereas those with high CRP had a significant increase in LDL-C, the LDL:HDL ratio, apolipoprotein B, and Lp(a) compared with the initial run-in diet. Therefore, inflammation may not only attenuate lipid responses, but also aggravate dyslipidemia in hypercholesterolemic individuals.

Based on the relationship between inflammation and vascular function, some studies evaluating the effect of soy isoflavones on vascular function have shown protective effects (Squadrito et al. 2002; Squadrito et al. 2003; Cuevas et al. 2003), while others have not (Hale et al. 2002; Simons et al. 2000; Teede et al. 2001). However, the majority of studies were short-term and enrolled a small number of subjects. Evidence from a long-term study with monkeys showed that soy protein consumption with modest isoflavone content (0.94-1.88 mg of isoflavones/g protein) inhibited the early progression of coronary artery atherosclerosis (Adams et al. 2005). In one study of postmenopausal women, the daily consumption of soy protein with isoflavones resulted in positive vascular effects that were independent of lipid and antioxidant effects (Steinberg et al. 2003). Likewise, the beneficial effects of isoflavones in postmenopausal women could be related to platelet function, as evidenced by a negative relation between isoflavone treatment and platelet thromboxane A<sub>2</sub> receptor density (Garrido et al. 2006). These novel effects may be above and beyond those of improving the more common CVD risk factors, such as blood pressure and TC. However, in a large, one-year, double-blind clinical trial, Kreijkamp-Kaspers et al. (2005) reported that soy protein with isoflavones provided no beneficial effects on vascular function in older postmenopausal women. Likewise, a recent eight-week study in 89 healthy postmenopausal women given fruit cereal bars with and without isoflavones (50 mg/d) reported no effect on plasma Hcy concentration, an independent risk factor for CVD, which also may be related to vascular function (Reimann et al. 2006).

*Isoflavones and oxidative stress:* Although many in vitro studies have shown that flavonoids in legumes and soybean products inhibit the oxidation of lipoproteins [via inhibiting the oxidative modification of LDL by macrophages (Kapiotis et al. 1997) and enhancing resistance of LDL to oxidation (Kanazawa et al. 1995)], there is no clear evidence of free radical-scavenging antioxidant effects of flavonoids or their metabolites in vivo. Two rat studies by Khan and Sultana (2004) suggest that isoflavones act as potent chemoprotective agents against potassium bromate- and ferric nitriloacetate-mediated renal oxidative stress, toxicity, and subsequent cell proliferation. However, isoflavone supplementation for three weeks in diabetic rats had no effect on reducing oxidative stress (Hsu et al. 2003). Similarly, no effect on erythrocyte antioxidant enzyme activities was

found in a six-month study of isoflavone supplementation in postmenopausal women (Hsu et al. 2001). Isoflavones in soy protein isolate consumed for six weeks were not shown to exert a significant effect in reducing oxidative damage in postmenopausal women (Engleman et al. 2005).

However, some studies support the antioxidant effects of isoflavones. Genistein was shown to be beneficial in preventing oxidative stress in cortical neuronal cells in vitro (Sonee et al. 2004), providing evidence that isoflavones may play a protective role in the central nervous system. In a three-week pilot study, isoflavones were shown to decrease oxidative DNA damage in men and women, but had no effect on 8-isoprostane levels (Djuric et al. 2001). Thus, isoflavones appear to have free radical quenching ability (Mitchell et al. 1998; Arora et al. 1998); genistein and daidzein have been shown to prevent 8-OHdG formation in cells and DNA exposed to oxidants (Giles and Wei 1997). Moreover, the protective effect of both genistein and daidzein against DNA oxidative damage are exhibited at concentrations reachable in plasma after soy consumption (Foti et al. 2005). This demonstrates that the antioxidant activity of isoflavones could contribute to the health benefits of soy.

The antioxidant action of isoflavones and their metabolites inhibited the oxidation of LDL, exerting an antiatherosclerotic effect, in a rabbit model (Yamakoshi et al 2000). The long-term supplementation of soy isoflavones to rats resulted in decreased oxidative stress, improved endothelial function, and reduced blood pressure (Mahn et al. 2005). Oxidative damage was decreased in men who consumed one liter of soy milk daily for four weeks (Mitchell and Collins 1999). Isoflavones may also decrease oxidative stress indirectly by improving antioxidant status (Cai and Wei 1996). Likewise, soy product supplementation in humans has been shown to decrease the rate at which lipids are oxidized (Tikkanen et al. 1998 and Wiseman et al. 2000). Collectively, these results indicate that a long-term soy isoflavone supplement trial in humans is warranted.

Mechanisms responsible for the effects of soy isoflavones on oxidative stress are still unclear. Wiseman et al. (2000) demonstrated that the consumption of naturally occurring isoflavones from soy foods reduced lipid peroxidation in vivo and increased the resistance of LDL to oxidation, measured by plasma concentration of F<sub>2</sub>-isoprostanes (8-epi-prostaglandin F<sub>2</sub>α) and resistance of LDL to copper-ion-induced oxidation. These results suggest that

isoflavones may function as an antioxidant to reduce formation of the preformed lipid hydroperoxides. Similarly, Nhan et al. (2005) reported in a small study of premenopausal women that isoflavones from soymilk supplementation reduced lipid peroxidation, measured by urinary F<sub>2</sub>-isoprostanes, in an age-dependent manner, with greater effects in older women and with lower doses of isoflavones. In vitro studies suggest that genistein and daidzein inhibit LDL oxidation in the subendothelium of vessels in a similar manner to that of vitamin E (Hodgson et al. 1996). Studies in rats indicated that feeding an isoflavone-rich soy protein isolate significantly inhibited LDL oxidation after LDL particles are isolated from serum (Anderson et al. 1999). Thus, isoflavones may be transported in LDLs and may act like antioxidant vitamins to inhibit oxidative damage. The protective effect from soy isoflavones may be due to altering LDL particle size, which may influence resistance to oxidation (Tikkanen et al. 1998). Interestingly, esterified isoflavones have been shown to be incorporated into LDL ex vivo, resulting in greater oxidative resistance. However, this effect has not been validated in vivo (Meng et al. 1999). Therefore, the hypocholesterolemic effect of soy foods is likely dependent on the presence of soy protein with isoflavones intact (Wagner et al. 2003). However, it is important to note that there is evidence that the substitution of soyfoods for animal products, regardless of isoflavone concentration, reduces CVD risk because of reductions in blood lipids, oxidized LDL, Hcy, and blood pressure (Jenkins et al. 2002).

*Adverse effects of isoflavones:* Although there are many benefits of soy consumption, there is still concern regarding potential adverse effects of over consumption. It is important to note that some researchers have demonstrated that genistein and daidzein may be carcinogenic in estrogen-sensitive organs, although isoflavones are generally chemoprotective compounds. In vitro evidence indicates that oxidative DNA damage due to isoflavone supplements plays a role in tumor initiation and cell proliferation via estrogen receptor-estrogen response element binding, thereby inducing tumor promotion and/or progression, resulting in cancer of estrogen-sensitive organs (Murata et al. 2004). Likewise, studies have shown that genistein and/or daidzein induce cancers of reproductive organs in rodents, such as the uterus (Newbold et al. 2001) and vulva (Thigpen et al. 2001). In addition, genistein was shown to have tumor-enhancing effects on breast tissue (Allred et al.

2001) and the colon (Rao et al. 1997). Thus, estrogen-like substances, such as isoflavones and their metabolites, may participate in tumor initiation and promotion by causing DNA damage and cell proliferation, potentially leading to carcinogenesis. Epidemiological and clinical studies suggest that estrogens may exert carcinogenic actions in humans (Bernstein and Ross 1993). A meta-analysis revealed that postmenopausal users of estrogen have an increased risk of breast and endometrial cancer (Nelson et al. 2002). Hence, like endogenous estrogens, genistein and daidzein may have the capacity to produce not only beneficial actions, but also adverse carcinogenic effects. However, the amount of isoflavones given in animal studies is important to consider. Although these adverse effects have been reported from animal studies, there is no evidence of comparable intakes in human studies.

### **Summary**

Understanding the effect of isoflavones on menopause-associated risk factors for CVD will provide clarity on alternative therapies to hormone treatment. Given that hormone therapies increase the risk for breast cancer and other cardiovascular complications, alternative therapies hold promise. Long-term consumption of isoflavones is important to study to determine their effect on CVD risk factors, such as: oxidative stress, body iron stores, and total Hcy concentration, in postmenopausal women. In addition, it is important to study the inter-relationship among these factors.

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## EXCESS IRON IS RELATED TO CENTRAL ADIPOSITY BUT NOT TO OXIDATIVE STRESS IN POSTMENOPAUSAL WOMEN

A paper to be submitted to the Journal of Nutrition

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### ABSTRACT

**Background:** Postmenopausal women are at risk of atherosclerotic cardiovascular disease (CVD) due to a decrease in circulating estrogen levels, compromised antioxidant status, and an increase in iron stores, oxidative damage, and abdominal fat accumulation.

**Objective:** To determine the relationship among excess iron, oxidative stress, and adiposity in postmenopausal women.

**Design:** Healthy postmenopausal women were recruited for the Soy Isoflavones for Reducing Bone Loss study, a randomized, double-blind, clinical trial designed to examine the effect of soy isoflavones on bone. Antioxidant enzymes (superoxide dismutase, catalase, glutathione peroxidase), iron status (serum ferritin [SF] and other indices), oxidative stress (oxidized LDL [oxLDL], urinary isoprostanes [PGF<sub>2α</sub>], protein carbonyls, DNA damage), and body size and composition (body mass index [BMI], waist circumference [WC], waist-to-hip ratio [WHR], sagittal diameter, androidal-to-gynoidal fat mass ratio [AGF ratio]) were measured at baseline (n=122). Insulin resistance was calculated using the homeostasis model assessment (HOMA). Pearson correlation and multiple regression analyses were used to determine the contributing CVD risk factors to oxidative stress and central adiposity.

**Results:** Iron status was normal based on median SF (53.1 ng/mL), hemoglobin (13.7 g/dL), and transferrin saturation (24%) in these women. The median BMI=24.9 kg/m<sup>2</sup> and WHR=0.75; fasting insulin (12.8 mU/mL) and glucose (87.0 mg/dL) were within normal limits. Excess iron was not related to oxidative stress but was related positively to central adiposity indices (AGF ratio [r=0.28, P<0.01], waist fat mass [r=0.24, P<0.01], sagittal diameter [r=0.18, P<0.05], WHR [r=0.33, P<0.001]), age (r=0.23, P<0.05), and HOMA

( $r=0.16$ ,  $P<0.09$ ). Lipid oxidation measures were also associated positively with central adiposity. Multiple regression analysis revealed age, LDL cholesterol, and AGF ratio were contributors to the overall variance ( $R^2=34\%$ ;  $P\leq 0.0001$ ) in oxLDL, whereas oxLDL, time since menopause, HOMA, HDL cholesterol,  $PGF_{2\alpha}$ , and SF contributed to the overall variance ( $R^2=33\%$ ;  $P\leq 0.0001$ ) in AGF ratio.

**Conclusion:** Iron excess was positively associated with central adiposity, possibly mediating insulin resistance in these postmenopausal women, but not related to increased oxidative stress. Maintaining a favorable lipid profile, antioxidant and iron status, as well as minimal central adiposity, may protect postmenopausal women from chronic disease.

**KEY WORDS** Cardiovascular disease, oxidative stress, iron, adiposity, insulin resistance

## INTRODUCTION

Body iron stores, oxidative stress, blood lipids, and body fat typically increase with age, especially after menopause due to the loss of endogenous estrogen production. The cessation of menses in postmenopausal women contributes to elevated serum ferritin (SF) (Nordbo et al. 1994), as evidenced by postmenopausal women having three-fold greater SF compared to premenopausal women (Masse et al. 2004). Although controversial, some epidemiological studies show a positive correlation between body iron stores and risk for atherosclerotic cardiovascular disease (CVD) (Salonen et al. 1992; Danesh et al. 1999; Klipstein-Grobusch et al. 1999), while others state that there is insufficient evidence for this relationship (Corti et al. 1997; Eichner et al. 1998). Aspirin intake, shown to reduce the incidence of myocardial infarction, was associated with low SF in the Framingham Heart Study, indicating a potential role of iron in CVD (Fleming et al. 2001). Finally, elevated SF concentration in postmenopausal women was associated with an increased risk of ischemic stroke, possibly due to the interaction of other CVD risk factors that accelerate atherogenesis by stimulating oxidative stress (Van der et al. 2005; Klipstein-Grobusch et al. 1999).

Oxidative stress or compromised antioxidant status has been suggested to be involved in the etiology of diseases including cancer, CVD, cataracts, and the aging process (Institute of Medicine, 2000). Favorable oxidative status is defined as a balance between reactive oxygen species and the antioxidant defense system. Transition metals, such as iron and copper, catalyze free radical oxidation of lipids and proteins. Iron, as a prooxidant, may contribute to atherosclerosis by promoting oxidative modification of LDL, a key step that leads to arterial scarring and inflammation. In the Fenton reaction, free iron catalyzes hydrogen peroxide to the harmful hydroxyl radical, resulting in lipid peroxidation. Once lipid hydroperoxides accumulate, free iron may directly initiate additional lipid peroxidation (Steinberg et al. 1989).

Some studies disagree regarding the role of iron in oxidative stress. Iron supplementation did not increase oxidized LDL (oxLDL) in women with low iron status (Derstine et al. 2003; Binkoski et al. 2004). Similarly, Sempos et al. (2001) found no evidence that body iron stores were directly related to CHD or indirectly related to oxLDL. However, women with high iron stores and elevated total cholesterol (TC) were shown to be at greater risk for atherosclerosis compared to those with only elevated iron stores (Kiechel et al. 1994). Thus, in a healthy population, iron excess may not be a major concern; however, in persons with high oxidative stress and hyperlipidemia, iron excess may place them at greater risk for CVD (Reddy and Clark, 2004). Hence, the iron hypothesis first proposed by Sullivan (1981), although controversial, suggests that elevated iron stores are causally linked to CVD. This hypothesis suggests that oxidative imbalance is the central biologic mechanism for the greater incidence of heart disease in men and postmenopausal women compared with the lower incidence in premenopausal women.

Less research has been conducted on the relationship between excess iron and body fat distribution, despite the independent association of each factor with CVD risk. A positive association between SF and indices of central adiposity (Urakawa et al. 2003; Gillum 2001), insulin resistance (Sheu et al. 2003; Iwasaki et al. 2005), and metabolic syndrome (Jehn et al. 2004) suggests a role of excess iron in obesity and CVD. The objective of our study was to determine the relationship among atherosclerotic CVD risk factors, particularly oxidative stress indices, central adiposity, and body iron stores in postmenopausal women.

## SUBJECTS AND METHODS

### *Study Design*

Postmenopausal women were recruited for the **Soy Isoflavones for Reducing Bone Loss (SIRBL)** study, a randomized, double-blind, three-year clinical trial that was designed to examine the effects of two doses of soy isoflavones on bone loss in postmenopausal women. The aim of this ancillary study was to determine the relationship among specific CVD risk factors at baseline including body iron stores, oxidative stress, and central adiposity.

### *Subject Selection*

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 (see Figure 1 for subject enrollment flow chart). Trained graduate assistants conducted telephone interviews (n=2,411) to screen potential postmenopausal women to ensure that they met inclusion/exclusion criteria: postmenopausal women,  $\leq 65$  years of age, had their last menstrual cycle at least 12 months ago (cessation of menses from one to eight years), had a body mass index (BMI) ( $\text{kg}/\text{m}^2$ ) ranging from 18.5 through 29.9, free of chronic diseases, non-smoking (not smoked in the past 6 months), willing to consume one of three treatments daily for three years, willing to discontinue all supplements and take only study supplements provided for three years, willing to avoid isoflavone-rich foods, and able to visit our testing facility at designated intervals (pre-baseline, baseline, 6 mo, 1 yr, 2 yrs, 3 yrs). We excluded women from the study if they had a history of or currently had any of the following conditions: removal of the ovaries, hysterectomy, urolithiasis, bone disease, renal disease, cancer, CVD, diabetes mellitus, respiratory disease, parathyroid disease, thyroid disease, or liver disease. In addition, women were excluded if they used any of the following (currently or within last 12 months): hormone or estrogen therapy (by mouth or skin patch), selective estrogen receptor modulators (SERMs), or other hormones. Exclusion criteria also included the use of

estrogen or progestogen creams and/or calcitonin currently or within the last six months. We also excluded subjects from the study who had any use of bisphosphonates (currently or in the past) or current (or within the last three months) use of antibiotics. Likewise, the current use of the following was grounds for exclusion: cholesterol-lowering or anti-hypertensive medications, herbal therapies, nutritional/dietary supplements, or excessive alcohol intake (limit to  $\leq 7$  drinks/wk with no alcohol intake four days prior to blood draws). We also excluded subjects with excessive vasomotor symptoms from the study. Lastly, we excluded vegans and subjects who had a first-degree relative with breast cancer from the study.

### *Data Collection*

At baseline, we obtained from each subject a health and medical history and a nutrition history using interviewer-administered questionnaires. Using a food frequency questionnaire from Block Dietary Data Systems (Berkeley, CA), we assessed usual dietary intake. We evaluated antioxidant intake both as dietary ingestion only and dietary plus supplemental use. Fasted (at least nine hours) blood samples were drawn between 7 and 8 a.m. and processed as serum, plasma, and erythrocyte samples; we separated serum and plasma from whole blood by centrifuging for 15 minutes at (4°C) at 1000 x g and stored aliquots at -80°C until analyses. Each subject collected a 24-hour urine sample; we measured the total volume and froze aliquots at -20°C until analysis. A certified clinical reference laboratory (Laboratory Corporation of America, Kansas City, MO) analyzed each subject's serum and plasma for the blood lipid profile (total cholesterol [TC], low-density lipoprotein cholesterol [LDL-C], high-density lipoprotein cholesterol [HDL-C], triacylglycerol [TG]) and other blood chemistry markers. We measured oxidative stress indices (protein carbonyls, oxidized low-density lipoproteins (oxLDL), urinary isoprostanes, specifically 15-isoprostane  $F_{2\alpha}$  (PGF $_{2\alpha}$ ), 8-hydroxy-2'-deoxyguanosine (8-OHdG), antioxidant enzymes (catalase [CAT], glutathione peroxidase [GPX], superoxide dismutase [SOD]), insulin, and SF in the laboratory at Iowa State University. We calculated the intra-assay and inter-assay variability for each method using a pooled sample as a quality control; these values are listed in Table 1.

We used the following procedures to analyze oxidative stress indices and antioxidant enzymes. To measure antioxidant enzyme (CAT, GPX, and SOD) activity, we used erythrocyte lysate and to determine protein carbonyls (expressed as per mg creatinine), we

used heparinized plasma (Cayman Chemical Company; Ann Arbor, MI). We measured plasma oxLDL (ALPCO Diagnostics; Windham, NH) and urinary isoprostanes, measured as 15-isoprostanes  $F_{2\alpha}$  (PGF $_{2\alpha}$ ) (Oxford Biomedical Research; Oxford, MI) using enzyme-linked immunoassay (ELISA) kits. Urine is better than blood to quantify PGF $_{2\alpha}$  because some factors, such as aspirin, may suppress the circulating concentration (Reilly et al. 1996) and urine may be stored for a longer period than serum/plasma without degrading. We measured urinary 8-OHdG to assess DNA damage using an ELISA kit (Stressgen Bioreagents; Victoria, BC, Canada). We measured SF to determine iron stores using a radioimmunoassay (RIA) kit (Ramco Diagnostics; Stafford, TX). We measured fasting insulin using an RIA kit (LINCO Research; St. Charles, MO) and calculated the homeostatic model assessment (HOMA) of insulin resistance for each subject using the following formula: fasting serum insulin ( $\mu\text{U/mL}$ ) x fasting plasma glucose (mg/dL)/405 (Matthews et al. 1985).

Both dual-energy x-ray absorptiometry (DXA; Delpi W Hologic Inc; Waltham, Massachusetts) and anthropometric measurements were used as indices of central adiposity. A certified DXA operator conducted a whole body scan on each subject. We used this technology to assess central (waist plus hip fat mass=androidal fat) and gynoidal (thigh fat mass=gynoidal fat) adiposity using subregion analysis (Discovery Version 12.3:7). We calculated the androidal-to-gynoidal fat mass (AGF ratio) ratio for each subject. A trained anthropometrist 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.); we calculated waist-to-hip ratio (WHR) for each subject.

### *Statistical Analysis*

Statistical analyses were performed with SAS (Version 9.0). For all results, we considered  $P < 0.05$  as the level of statistical significance. Pearson correlation analysis was used initially to examine the correlations among the CVD risk factors for entry into the multiple regression models. Multiple regression analysis with protein carbonyls selection was used to determine the factors related to oxLDL and AGF ratio. The initial variables in the regression model for oxLDL included: age, AGF ratio, SF, LDL-C, and dietary intake of

selenium, vitamin E, vitamin C, and beta-carotene. The initial variables in the regression model for AGF ratio included: age, oxLDL, time since menopause, HDL-C, LDL-C, SF, HOMA, and PGF<sub>2α</sub>.

## RESULTS

### *Subject Characteristics*

The subjects' descriptive characteristics and daily nutrient intakes at baseline are listed in Table 2. The median age of subjects in this study was 55 y (range 47-62 y). The range of time since menopause was 1 through 8 y with a median of 3.3 y. The women's BMI ranged from normal to overweight (18.9-29.8 kg/m<sup>2</sup>), with a median of 24.9 kg/m<sup>2</sup>. A BMI greater than 25.0 kg/m<sup>2</sup>, considered as overweight, was found in 47% of women in this study. Dietary intake of macronutrients and selected antioxidant nutrients (vitamins A, C, E, and selenium) was within the recommended dietary allowances for these women. Descriptive data for iron, adiposity, and oxidative stress indices, and antioxidant enzyme, blood lipid, glucose and insulin concentrations, as well as insulin resistance (HOMA) are presented in Table 3.

### *Iron Indices*

Median values for SF (53.1 ng/mL), hemoglobin (Hb) (13.7 g/dL), and transferrin saturation (24%) were within the normal range, suggesting that these women did not have excess iron conditions. However, a small percentage (3) of women had SF above 140 ng/mL and 9% had transferrin saturation under 15%. With the exception of one woman with a Hb of 11.4 g/dL, none of them had iron deficiency anemia.

### *Oxidative Status*

It is difficult to state whether oxidative status was normal in these women; however, values were in the normal range based upon the manufacturers' guidelines. There was a wide range of antioxidant enzyme activity among the subjects, but the median values were the within normal range for each enzyme: CAT (41.3; 5.9-112.5 nmol/min/mL), SOD (755.6; 27.3-1874.2 U/mL), and GPX (188.5; 91.7-430.4 nmol/min/mL). Similarly, the oxidative stress indices varied among subjects, but the median values were normal: oxLDL (73.7;

31.8–130.6 U/L),  $\text{PGF}_{2\alpha}$  (2.44; 1.26–5.82 ng/mL), protein carbonyls (6.0; 0.06–67.7 nmol/mg protein), and 8OHdG (43.7; 14.5–194.8 ng/mL)].

#### *Adiposity and Insulin Resistance*

More than a 10-fold variation was found in androidal fat mass (1.09–10.12) compared to a 4-fold variation in gynoid fat mass (2.44–8.97). The median AGF ratio was 1.0 kg (range 0.45–2.20 kg). The median WHR was 0.75, a level which is not associated with a high risk of chronic diseases (Yusuf et al. 2005). Fasting insulin (median 12.8 mU/mL, range=4.5–47.2) and glucose (median=87.0 mg/dL, range=66.0–108.0) concentrations were also within normal limits. Four of the 122 women had an insulin concentration above 20 mU/mL and 8 had a glucose concentration above 100 mg/dL, but these women did not have values in the diabetic range. The calculated median HOMA was 2.7 (range 0.83–10.8).

#### *Correlation among CVD Risk Factors*

Pearson correlation analysis among SF, oxLDL,  $\text{PGF}_{2\alpha}$ , central adiposity, age, and HOMA is shown in Table 4. Serum ferritin was not significantly associated either with oxidative stress or antioxidant indices. However, it was related to various indices of central adiposity; the highest positive correlation ( $r=0.33$ ;  $P<0.001$ ) was observed between SF and WHR. In addition, SF was correlated with AGF ratio ( $r=0.23$ ;  $P<0.01$ ), WaistFM ( $r=0.24$ ;  $P<0.01$ ), SagDiam ( $r=0.18$ ;  $P<0.01$ ), age ( $r=0.23$ ;  $P<0.05$ ), and HOMA ( $r=0.16$ ;  $P<0.09$ ). The lipid peroxidation indicators, oxLDL and  $\text{PGF}_{2\alpha}$ , were also positively associated with central adiposity indices. Specifically, oxLDL was positively associated with AGF ratio ( $r=0.23$ ,  $P<0.05$ ), WaistFM ( $r=0.24$ ,  $P<0.05$ ), and age ( $r=0.23$ ,  $P<0.05$ ). Similarly,  $\text{PGF}_{2\alpha}$  was correlated with AGF ratio ( $r=0.32$ ,  $P<0.01$ ), WaistFM ( $r=0.27$ ,  $P<0.01$ ), WHR ( $r=0.33$ ,  $P<0.01$ ), and HOMA ( $r=0.21$ ,  $P<0.05$ ).

#### *Contributors to Oxidative Stress and Central Adiposity*

Multiple regression analysis with backward selection was performed to examine the potential contributors to factors to lipid peroxidation and central adiposity. The results are shown in Table 5. To determine the contributing factors to oxidative stress, we chose oxLDL to represent oxidative damage. The variables included in this model are: age, AGF ratio, SF, dietary antioxidants (vitamin C, beta-carotene, vitamin E, and selenium). These variables contributed 39.3% of the variability to oxLDL. However, only age (1.6%), LDL-C

(24.8%), and AGF ratio (2.2%) were the only significant contributors to the overall variance ( $R^2=34.4\%$ ;  $P\leq 0.0001$ ) for oxLDL. In this model, SF was not a significant contributor to oxLDL. To determine the contributing factors of central adiposity, we choose AGF ratio to represent abdominal adiposity because it is assessed via DXA, a gold-standard method for body composition assessment. Variables included in the AGF ratio model are: age, oxLDL, time since menopause, HOMA, HDL-C, LDL-C,  $\text{PGF}_{2\alpha}$ , and SF. These variables accounted for 33% in the variability but only oxLDL (3.0%), time since menopause (1.8%), HDL-C (5.7%), SF (4.4%), HOMA (2.6%), and  $\text{PGF}_{2\alpha}$  (3.5%) showed a significant contribution ( $R^2=37.9\%$ ;  $P\leq 0.0001$ ).

## DISCUSSION

Iron exerts catalytic activity to produce free radicals causing oxidative damage, increasing CVD risk. Men and postmenopausal women are at increased CVD risk, which may be attributed to elevated iron stores (Nordbo et al. 1994). Serum ferritin is a commonly used marker of iron status that accurately reflects iron stores, which may differ by age and sex (Zacharski et al. 2000). Postmenopausal women have higher SF levels compared to premenopausal women, both in blood donors (43.4 vs. 23.1  $\mu\text{g/L}$ ) and in nondonors (71.7 vs. 32.8  $\mu\text{g/L}$ ) (Berge et al. 1994). In our previous study with perimenopausal women, the median SF value was lower (27.5  $\mu\text{g/L}$ ) (Swain et al. 2002) compared to postmenopausal women (45.6  $\mu\text{g/L}$ ) (Hanson et al. 2006, In press).

Epidemiological data, although controversial, suggests a positive correlation between excess iron stores and risk for CVD (Salonen et al. 1992; Danesh et al. 1999). Based on the association of aspirin with reduced risk of myocardial infarction and reduced iron stores, suggests the role of iron in the etiology of CVD. The Framingham Heart Study reported that aspirin supplementation lowered SF, supporting epidemiologic studies regarding associations between elevated SF and CVD (Fleming et al. 2001; Salonen et al. 1992; Danesh et al. 1999). In the presence of classic CVD risk factors, SF may adversely affect ischemic heart disease (Klipstein-Grobusch et al. 1999). The Bruneck Study also showed that SF was one of the strongest indicators of carotid artery disease in both men and women. In addition the predictive significance of SF was found to be synergistic with hypercholesterolemia (Kiechl

et al. 1994). Since iron stores, TC, and LDL-C increase with age (Berge et al. 1994), this synergistic effect is of concern in postmenopausal women.

However, in normal, healthy individuals, free iron is not readily available because body iron is either bound to transferrin or stored as ferritin (Meyers 2000). We found no significant relationship between SF and oxidative stress or antioxidant enzymes, suggesting iron was not involved in free radical generation in these women. The women in our study were early postmenopausal, with a median SF concentration of 53 ng/mL, which is not considered an iron excess state. Thus, iron excess may not be of major concern in a healthy population. However, individuals with high oxidative stress combined with hyperlipidemia may be at high risk for CVD (Reddy and Clark, 2004). In women with low iron status, iron supplementation did not increase the susceptibility of LDL to oxidative modification (Binkoski et al. 2004), suggesting that subjects who need iron supplementation should not be concerned about oxidization of LDL. In addition, iron status was not associated with LDL oxidative susceptibility in healthy men and women (Derstine et al. 2003). Sempos et al. (2001), in an extension review, concluded that excess iron stores were not related to oxLDL or coronary heart disease. It should be noted that the women in our study were not severely hypercholesterolemic (LDL cholesterol  $\leq 160$  mg/dL, TG  $\leq 200$  mg/dL), nor had elevated iron stores, and thus were not likely to be very susceptible to oxidative damage.

Although iron was not related to oxLDL, we found excess iron was positively associated with the AGF ratio (Table 4 and 5b). Since central adiposity is a well-known risk factor for atherosclerotic CVD, the association of SF to the AGF ratio suggests a role for iron in androidal fat accumulation. Our results are supported by data indicating a direct correlation of elevated SF concentration with visceral fat and subcutaneous fat area using computed tomography (Iwasaki et al. 2005). Further, elevated SF is associated with decreased insulin sensitivity and increased fasting plasma insulin and glucose, abnormalities which may lead to increased adiposity (Gillum 2001). Similarly, our results showing the relationship (trend,  $p=0.09$ ) between SF and HOMA also suggest that iron status may play a role in insulin resistance. Likewise, data from the Third National Health and Nutrition Examination Survey shows that elevated iron stores, reflected by SF, was positively associated with the prevalence of the metabolic syndrome and insulin resistance (Jehn et al.

2004). In addition, SF concentration was also related to the degree of insulin resistance in Chinese women, after adjusting for age, BMI, and menopausal status (Sheu et al. 2003). In contrast, Chambers et al. (2006) found that serum iron was inversely correlated with BMI, waist circumference, and fat mass among Hispanic women. However, SF was not measured in this study and hence the authors caution against over interpretation of their results. In addition, ethnic or cultural differences may influence the association between iron stores and indices of adiposity.

One possible mechanism might be that excess liver iron stores may reduce the ability of insulin to suppress hepatic glucose production and interfere with hepatic insulin clearance, thereby leading to peripheral hyperinsulinemia (Fernandez-Real et al. 1998; Niederau et al. 1984). In addition, excess iron may reduce pancreatic insulin secretion (Wilson et al. 2003), further contributing to insulin resistance. Finally, elevated free fatty acids may damage pancreatic beta cells, interfere with muscle glucose uptake, and contribute to insulin resistance (Tuomainen et al. 1997).

There is a clear association between iron overload conditions and insulin resistance, which may result in diabetes. For example, type 2 diabetes is a common problem in hemochromatosis (Witte et al. 1996) and thalassemia (Cario et al. 2003) patients, conditions of iron overload. However, elevated SF also has been observed in individuals without diabetes, but with decreased insulin sensitivity (Tuomainen et al. 1997; Haap et al. 2003) and in women with gestational diabetes (Lao et al. 2001), as well as individuals with type 2 diabetes (Fernandez-Real JM et al. 2002). Finally, elevated SF concentration has been shown to precede the development of diabetes in two prospective studies of Finnish men (Salonen et al. 1998) and U.S. nurses (Jiang et al. 2004). Although the subjects in our study were healthy, we nonetheless found that both HOMA and SF contributed to central adiposity, confirming the interrelationship among iron, adiposity, and insulin resistance. The HOMA model has been used in cohort and epidemiological studies as a useful tool to assess insulin resistance. HOMA has an advantage over other models because it requires only a single plasma sample for insulin and glucose determination (Wallace et al. 2004).

Adipose tissue is highly metabolic and abdominal fat induces oxidative stress, which in turn has been shown to cause insulin resistance in men (Urakawa et al. 2003). In both men

and women, a high waist circumference was associated with elevated oxLDL, independent of BMI (Weinbrenner et al. 2006), illustrating that the abdominal fat mass may induce a greater degree of oxidative stress. Our results support the idea that, in addition to high LDL-C, central adiposity (AGF ratio) is one of the key factors related to elevated oxLDL.

As expected, our study showed that oxLDL, time since menopause, HOMA, HDL-C, and  $\text{PGF}_{2\alpha}$  contributed to central adiposity (Table 4B), but interestingly SF was one of the significant contributing factors to the AGF ratio and also was positively correlated with other central adiposity indices. Perhaps excess iron exacerbates central adiposity by aggravating insulin resistance, but it is also possible that central adiposity may contribute to excess iron stores. On the other hand, SF is an acute-phase reactant or inflammatory marker and may be elevated in conditions not associated with excess body iron stores. Overweight and obese individuals are characterized as having a low-grade inflammatory response (Vega 2004). Since obesity is an inflammatory state, it may increase holo ferritin rather than iron-bound ferritin. Yet, we do not have a method to differentiate holo and iron-bound ferritin. Thus, increased SF may reflect obesity-induced inflammation, a relatively new factor in the etiology of CVD (van der et al. 2005). Since iron is involved in the immune process, an increased demand for iron may result in low serum iron (Ganz 2003). An inverse relationship of serum iron with BMI, waist circumference, and fat mass supports this concept (Chambers et al. 2006).

We demonstrated an association among CVD risk factors, but since this was an observational study, we cannot establish cause and effect. However, future research is needed to understand the mechanism(s) involving iron, insulin resistance, and obesity. Individuals with hypercholesterolemia, type 2 diabetes, excess iron stores, and/or smokers (at increased risk of oxidative stress) may be good candidates for future research in this area.

In conclusion, iron excess was positively associated with central adiposity, possibly mediating insulin resistance in these postmenopausal women. Nonetheless, excess iron stores were not related to increased oxidative stress (oxidative damage indices or antioxidant enzymes). Our study suggests that maintaining a favorable lipid profile, antioxidant and iron status, as well as minimal central adiposity, may protect postmenopausal women from chronic disease.

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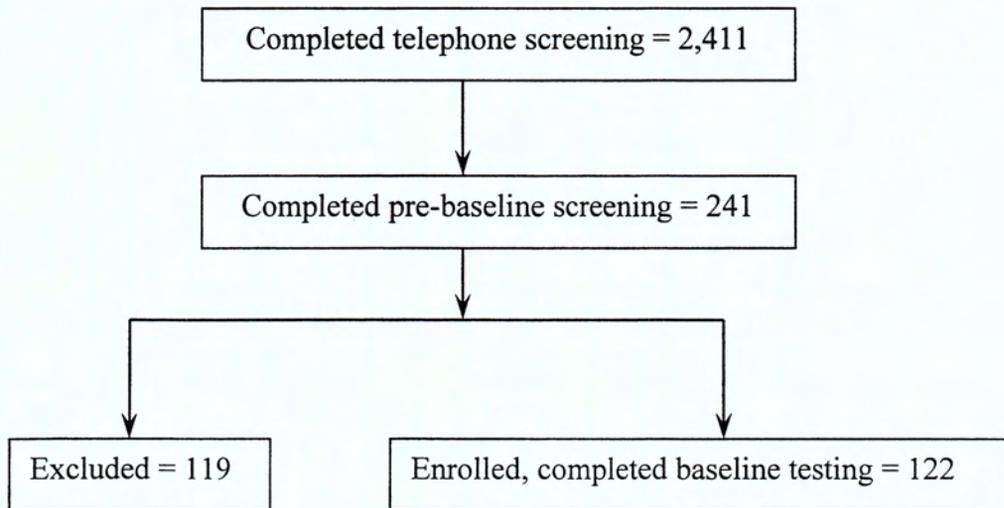
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**FIGURE 1****Subject enrollment flow chart**

**TABLE 1****Intra-assay and inter-assay variability<sup>1</sup>**

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<b>Assay</b>	<b>Intra-assay (%)</b>	<b>Inter-assay (%)</b>
Protein Carbonyls	2.5	3.4
Oxidized LDL	4.8	5.2
Urinary Isoprostanes	3.2	17.0
8-hydroxy-2'-deoxyguanosine	2.5	3.2
Catalase	5.3	8.4
Glutathione Peroxidase	4.0	7.5
Superoxide Dismutase	8.7	10.9
Serum Ferritin	4.9	5.6

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<sup>1</sup> Calculated from a pooled sample as a quality control in each assay

**TABLE 2**  
**Subject characteristics**

<b>Measurement (n = 122)</b>	<b>Median<sup>1</sup></b>	<b>Range (min-max)</b>
<b>Age (y)</b>	55.2	(47.3-61.8)
<b>Time Since Menopause (y)</b>	2.7	(1.0-7.9)
<b>Weight (kg)</b>	68.1	(47.8-89.2)
<b>Height (cm)</b>	165.6	(150.6-178.4)
<b>BMI (kg/m<sup>2</sup>)</b>	24.9	(18.9-29.8)
<b>Dietary Intake/d<sup>2</sup></b>		
Energy (KJ)	6649	(1171-19109)
Protein (g)	64	(20-168)
Carbohydrate (g)	195	(33-476)
Fat (g)	67	(17-247)
Saturated Fat (g)	20	(5-65)
Iron (mg)	11.4	(3.1-45.6)
Polyunsaturated Fatty Acids (g)	16.1	(2.5-70.4)
Vitamin A (RE)	1286	(360-5163)
Vitamin C (mg)	100	(19-325)
Vitamin E (mg)	9	(2-38)
Selenium (µg)	75	(23-227)

<sup>1</sup> Median values are reported for subject characteristics and dietary intake, since most were not normally distributed

<sup>2</sup> Dietary intake assessed using Food Frequency Questionnaire (Block Dietary Data Systems)

**TABLE 3****Baseline descriptive data (n=122)**

<b>Measurement</b>	<b>Median<sup>1</sup></b>	<b>Range (min-max)</b>
<b>Iron indices</b>		
Ferritin (ng/mL)	53.1	(4.8-175.1)
Hemoglobin (g/dL)	13.7	(11.4-16.0)
Hematocrit (%)	40.8	(33.5-47.6)
Serum Iron (ug/dL)	80.0	(31.0-177.0)
Transferrin Saturation (%)	24.0	(7.0-56.0)
<b>Oxidative stress indices</b>		
8-hydroxy-2'-deoxyguanosine (ng/mL)	43.7	(14.5-194.8)
Urinary Isoprostanes (ng/mL)	2.44	(1.26-5.82)
Oxidized LDL (U/L)	73.7	(31.8-130.6)
Protein Carbonyls (nmol/mg protein)	6.0	(0.06-67.7)
<b>Antioxidant enzymes<sup>2</sup></b>		
Catalase (nmol/min/mL)	41.3	(5.9-112.5)
Superoxide Dismutase (U/mL)	755.6	(27.3-1874.2)
Glutathione Peroxidase (nmol/min/mL)	188.5	(91.7-430.4)
<b>Central adiposity indices</b>		
Waist Circumference (cm) <sup>3</sup>	76.7	(62.8-98.6)
Sagittal Diameter (cm) <sup>3</sup>	18.6	(14.4-24.8)
Waist-to-hip Ratio	0.75	(0.64-0.91)
Waist Fat Mass (kg) <sup>4</sup>	2.24	(0.39-5.34)
Androidal fat mass (kg)	5.37	(1.09-10.12)
Gyndoidal fat mass (kg)	5.26	(2.44-8.97)
AGF ratio	1.00	(0.45-2.20)
<b>Blood lipids</b>		
Total cholesterol (mg/dL)	205	(142-285)
Total triacylglycerides (mg/dL)	81	(31-290)

LDL cholesterol (mg/dL)	122	(62-173)
HDL cholesterol (mg/dL)	69	(42-103)
TC/HDL cholesterol ratio	3.0	(1.9-4.9)
<b>Other</b>		
Fasting Insulin (mU/mL)	12.8	(4.5-47.2)
Fasting Glucose (mg/dL)	87.0	(66.0-108.0)
HOMA <sup>5</sup>	2.72	(0.83-10.8)

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<sup>1</sup> Median values are reported for baseline descriptive data, since most were not normally distributed

<sup>2</sup>n=117 because data for 5 subjects are missing due to processing errors of erythrocytes at time of collection

<sup>3</sup>Assessed by anthropometric measurements

<sup>4</sup>Assessed by dual energy x-ray absorptiometry

<sup>5</sup>Homeostasis Model Assessment of Insulin Resistance (HOMA) calculated as follows:

Fasting insulin (mU/mL) x Fasting glucose (mg/dL) / 405 (Matthews et al. 1985)

**TABLE 4****Correlations<sup>1</sup> between body fat measurements, age, and HOMA and SF, oxLDL, PGF<sub>2α</sub>**

	<b>SF</b>	<b>oxLDL</b>	<b>PGF<sub>2α</sub></b>
<b>AGF ratio</b>	0.28**	0.27**	0.32**
<b>WaistFM</b>	0.24**	0.26**	0.27**
<b>SagDiam</b>	0.18*	0.08	0.17
<b>WHR</b>	0.33***	0.23*	0.12
<b>Age</b>	0.23*	0.23**	0.09
<b>HOMA</b>	0.21	0.02	0.21*
<b>SF</b>	---	0.06	0.09

<sup>1</sup>Pearson correlation coefficients (\* p<0.05, \*\* p<0.01, \*\*\* p<0.001)

SF = serum ferritin; oxLDL= oxidized low-density lipoproteins; PGF<sub>2α</sub> = urinary isoprostanes

AGF ratio = androidal to gynoidal fat mass ratio; WaistFM = waist fat mass;

SagDiam = sagittal diameter; WHR= waist-to-hip ratio; WaistCirc = waist circumference

HOMA = Homeostasis Model Assessment of Insulin Resistance

There were no significant associations between ferritin and oxidative stress indices

TABLE 5

Regression analyses<sup>1</sup>: Cardiovascular disease risk factors**A. Factors related to oxidative stress, specifically oxLDL**Overall Model R<sup>2</sup> = 36.0% (Adj R<sup>2</sup> = 34.4%); F (3,118) = 22.15; (*p* ≤ 0.0001)

Independent Variable	Parameter	Percentage	<i>P</i> value <sup>1</sup>	Variance
	Estimate	Variance		Inflation
<i>Intercept</i>	-23.12			
Age	0.7326	1.6	0.086	1.04
LDL-C	0.3987	24.8	<0.0001	1.06
AGF ratio	0.8262	2.20	0.046	1.06

Variables eliminated from the model were: serum ferritin, dietary antioxidants (selenium, beta-carotene, vitamin E, vitamin C)

**B. Factors related to central adiposity, specifically AGF ratio**Overall Model R<sup>2</sup> = 29.49% (Adj R<sup>2</sup> = 32.98%); F (5,116) = 9.43; (*p* ≤ 0.0001)

Independent Variable	Parameter	Percentage	<i>P</i> value <sup>1</sup>	Variance
	Estimate	Variance		Inflation
<i>Intercept</i>	0.7138			
OxLDL	0.0033	2.97	0.026	1.07
Time since Menopause	0.0231	1.80	0.081	1.05
HOMA	0.0398	2.61	0.037	1.11
HDL-C	-0.0055	5.74	0.002	1.04
Urinary Isoprostanes	0.0759	3.46	0.016	1.11
Serum ferritin	0.0017	4.37	0.007	1.04

Variables eliminated from the model were: age and LDL-C

<sup>1</sup> Multiple regression analysis with stepwise selection. Variables left in model are significant at *P* ≤ 0.05 level