Can Antioxidant-rich Berries Improve Risk Factors Associated with Cardiovascular Disease in Postmenopausal Smokers?

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CAN ANTIOXIDANT-RICH BERRIES IMPROVE RISK FACTORS ASSOCIATED WITH CARDIOVASCULAR DISEASE IN POSTMENOPAUSAL SMOKERS?
CAN ANTIOXIDANT-RICH BERRIES IMPROVE RISK FACTORS ASSOCIATED WITH CARDIOVASCULAR DISEASE IN POSTMENOPAUSAL SMOKERS?

A thesis submitted in partial fulfillment of the requirements for the degree of Master of Science in Food Science

By

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Bachelor of Science in Dietetics, 2009

December 2011
University of Arkansas
ABSTRACT

The purpose of this study was to examine the effects of smoking on risk factors associated with cardiovascular disease (CVD) and determine the efficacy of antioxidant-rich berries in ameliorating these risk factors in postmenopausal smokers. Menopause and cigarette smoking have been identified as major risk factors for CVD due to a decrease in antioxidant protection, increase in inflammation and oxidative stress, and adverse changes in serum lipids. Antioxidants, specifically flavonoid compounds found in blackberries and blueberries, have been shown to prevent low-density lipoprotein cholesterol oxidation and thus prevent downstream inflammation and oxidative stress. Healthy, postmenopausal nonsmokers (n=14) and smokers (n=31) were recruited to participate in the three-month study and smokers were randomly assigned to one of three groups: smoker control (n=12), smoker + 45 g/day freeze-dried blackberries (n=6), or smoker + 45 g/day freeze-dried blueberries (n=13). Body mass index, blood pressure, hemoglobin A1c, serum lipid profiles, glucose, and high-sensitivity C-reactive protein (hsCRP), and plasma thiobarbituric acid reactive substances (TBARS) were assessed at baseline and the end of the study. A two-sample t-test was performed on nonsmokers (n=14) and smokers (n=31) at baseline to assess the effects of smoking on CVD risk factors in postmenopausal women and are reported as means ± SD. Following treatment, data were analyzed as percent change by group using a randomized, one factor analysis of variance (ANOVA) followed up with a protected least significant differences (LSD) test. This study showed that smokers had significantly higher serum triglycerides and lower high-density lipoprotein cholesterol than nonsmokers; 130 ± 11 mg/dL vs. 87 ± 7 mg/dL (p=0.0010) and 58 ± 3 mg/dL vs. 66 ± 3 mg/dL (p=0.0384), respectively. There were no significant effects of blackberry or blueberry treatment
for three months in ameliorating risk factors associated with CVD in postmenopausal smokers.

The effects of berries on CVD risk factors needs to be elucidated in a larger sample size.
This thesis is approved for recommendation to the Graduate Council.

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Dr. Latha Devareddy

Thesis Committee:

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Dr. Sun-Ok Lee

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Dr. Edward Gbur, Jr.
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ACKNOWLEDGEMENTS

I would like to thank several people who have contributed to the successful completion of this thesis and my graduate degree. My major advisor, Dr. Latha Devareddy; my committee members, Dr. Lee and Dr. Gbur; fellow graduate students, Wil Gilbert and Sarah Graves; and a HUGE thank you to Lydia Kaume, whom helped me so much through this process. I am extremely grateful to my parents and sisters for their underlying support, encouragement, and understanding. My brother and sister-in-law, Jeremy and Jordan, and their wonderful two kids, Bram and Helen, gave me so much to look forward to on trips to Rogers and Dallas. To my sanity and happiness, I owe that to my patient, enduring other half, Tyler (whom many know as Joe). I am so grateful for the support and love I received from all who helped me through this process, for it will never be forgotten!
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CHAPTER 1. INTRODUCTION

Greater than 1 in 3 females in the United States live with cardiovascular disease (CVD) and it is the leading cause of mortality in women, with more than twice as many deaths than all forms of cancer combined [1]. Modifiable risk factors for CVD include diabetes mellitus, high serum lipids, high blood pressure, metabolic syndrome, overweight or obesity, physical inactivity, and tobacco use [2]. While rates of CVD mortalities have been declining over the years, the prevalence of risk factors remains high. Greater than 50% of people in the United States who have had a heart attack were not at high risk based on their cholesterol values below the ‘at risk’ threshold [3]. This has prompted many researchers and medical professionals to seek alternative CVD prediction risk models.

Menopause greatly increases a woman’s chance for developing CVD, which is attributed to lower levels of circulating estrogen and subsequent changes in lipid profile [4, 5]. Estrogen is thought to act as an antioxidant due to its phenolic ring structure similar to that of other antioxidant molecules [4]. Loss of estrogen leads to inflammation and reduced antioxidant protection in cells, especially of the vasculature, due to estrogen-estrogen receptor interactions. Following menopause, there is an unfavorable shift in lipid profile, such as an increase in low-density lipoprotein cholesterol (LDL-C) and triglycerides, a decrease in high-density lipoprotein cholesterol (HDL-C), and an overall increase in total cholesterol (TC) [5].

Furthermore, one in five women are cigarette smokers, which exacerbates the risk factors for CVD [1]. Cigarette smoking is the leading cause of preventable death in the US [6] and a direct cause of an increased risk for heart attack and coronary artery disease (CAD) [7]. This is associated with an increase in oxidative stress and inflammation, adverse changes in serum lipid levels, decreased nitric oxide availability, and activation of platelets [7, 8]. Smokers have been shown to have high inflammation and oxidative stress levels measured by C-reactive
protein (CRP) and thiobarbituric acid reactive substances (TBARS), respectively [8]. Epidemiological studies have shown that diets rich in fruits, vegetables, and whole grains significantly reduces the risk of developing cardiovascular diseases [9, 10]. This in part has been attributed to their antioxidant content, specifically phenolic compounds. Blackberries and blueberries contain significant amounts of anthocyanins and total phenolics [11, 12], which can serve to reduce inflammation and oxidative stress levels. Blueberries have shown to improve cardiovascular health in a rat model of hypercholesterolemia [13] and antioxidant levels in humans [14], while blackberries have shown to improve serum and liver lipids in an animal model of postmenopausal hypercholesterolemia [15]. Therefore, the goal of this study was to evaluate the efficacy of blackberries and blueberries in reducing CVD risk factors in postmenopausal smokers.

**Hypothesis**

The *hypothesis* of this study was that smoking significantly increases risk factors associated with CVD, such as serum cholesterol levels and markers of inflammation and oxidative stress; and that antioxidant-rich blackberries and blueberries would be effective in significantly reducing these risk factors.

**Objectives**

To test the hypothesis, we had two objectives:

1. To assess the effects of smoking on cardiovascular disease risk factors in postmenopausal women.
2. To determine the efficacy of antioxidant-rich berries in ameliorating risk factors associated with cardiovascular disease in postmenopausal smokers.
CHAPTER 2. REVIEW OF LITERATURE

Cardiovascular Disease

Cardiovascular disease (CVD) is the leading cause of mortality in the United States. It is prevalent in greater than 1 in 3 Americans and was responsible for 33.6% of deaths in 2007. CVD, or heart disease, is a broad term describing a class of diseases involving the heart or blood vessels, including hypertension, stroke, heart attack, and coronary heart disease (CHD). Although CVD deaths have been declining for years, the prevalence of CVD and its risk factors remains high [6]. It is a major financial burden, with estimated direct and indirect costs exceeding $503 billion dollars in 2010 [1]. The focus of preventing cardiovascular disease has been on alleviating factors that have been associated with an increased risk. One of the largest epidemiological studies to date, The Framingham Heart Study, began in 1948 with the objective of identifying risk factors that contribute to CVD. The ongoing study with more than three generations of participants has not only led to association of risk factors for CVD, but also how these factors increase the risk for CVD. The study identified major risk factors as high blood pressure, high blood cholesterol, smoking, obesity, diabetes, menopause, and physical activity, and the prevalence of these risk factors can be seen in all racial and ethnic groups [16, 17].

Recent data from 2007 showed that an estimated 34% of US adults had hypertension, 23% of men and 18% of women were cigarette smokers, 15% had total serum cholesterol levels $\geq 240$ mg/dL, 8% had diagnosed diabetes mellitus, and 37% had prediabetes [6]. These risk factors are of interest because they lead to an increase in inflammation and promote the formation of atherosclerosis, a condition in which fat, cholesterol, and other substances accumulate on the innermost layer of the artery [18]. This plaque formation can harden over time, impede blood flow, and cause coronary heart disease. Therefore, inflammation and
subsequent atherosclerosis formation play a pivotal role in the etiology of cardiovascular diseases through these various risk factors.

Rosamond and others [16] have identified optimal, elevated, or major risk factor levels associated with CVD. Optimal risk factors at age 50 for both men and women include a blood pressure at or below 120/80 mmHg, total cholesterol below 180 mg/dL, and no presence of diabetes or smoking, shown in Table 1. An elevated risk is defined as stage I hypertension – blood pressure of 140-159/90-99 mmHg, or cholesterol levels of 200 to 239 mg/dL. Major risk factors include stage II hypertension – blood pressure ≥160/≥100 mmHg, cholesterol above 240 mg/dL, current smoking, and diabetes. Men and women with optimal risk factors have greater than 10 years median life expectancy as compared with those with 2 or more major risk factors.

**Table 1. Optimal CVD Risk Factors at Age 50 for Men and Women**

<table>
<thead>
<tr>
<th>Risk Factor</th>
<th>Levels</th>
</tr>
</thead>
<tbody>
<tr>
<td>Blood Pressure</td>
<td>&lt;120/80</td>
</tr>
<tr>
<td>Total cholesterol</td>
<td>&lt;180 mg/dL</td>
</tr>
<tr>
<td>Fasting blood glucose</td>
<td>&lt;126 mg/dL</td>
</tr>
<tr>
<td>Nonsmoker</td>
<td></td>
</tr>
</tbody>
</table>

At age 40, the risk of developing coronary heart disease (CHD) is one in two for men and one in three for women. Comparable rates of first cardiovascular events occur 10 years later in life in women when associated with risk in men. This disparity diminishes with age and rates become higher in women after menopause [17, 19-21]. Menopause has been studied extensively in an attempt to elucidate the cause of increased risk for coronary heart disease compared to premenopausal age-matched women. Studies have shown an association between menopause and an unfavorable change in serum lipids, increase in inflammation and oxidative stress, and decreased antioxidant protection of cells [5, 22, 23].
Postmenopausal Cardiovascular Disease

The cardioprotective effects of estrogen can be seen by the lower rates of CVD in premenopausal women compared to age-matched male counterparts [1, 24, 25]. A large percentage of women entering menopause exert no signs of CVD, however, many develop the disease following the transition into postmenopausal status [26]. This increase is attributed to the loss of circulating estrogen, the predominant sex steroid hormone in women.

Endogenous circulating estrogen in the form of estradiol, or E2, plays crucial direct and indirect physiological effects within the vasculature shown in Table 2. Direct effects include an increase in nitric oxide (NO) synthesis, vasodilation, and endothelial cell growth, and a decrease in vascular injury and smooth muscle cell proliferation [5, 27, 28]. Indirect effects involve a decrease in atherosclerosis and vascular injury, an increase in endothelial cell growth, maintenance of healthy serum lipoprotein levels, and decreased lipid oxidation [5, 22, 23]. Therefore, E2 is involved in maintenance of the blood vessel lining, fluid and hormone balance, and proper lipoprotein levels.

The physiological roles of estrogen are possible through estrogen-receptor interactions. Estrogen receptor alpha (ERα) [27] and estrogen receptor beta (ERβ) [28] control the mechanisms of E2, however, ERα predominates in vascular cells of men and women, providing a good model for comparing the effects of hormonal differences linked to cardiovascular diseases [29]. Men lacking functional estrogen receptors have weakened vascular functioning and early calcification of the coronary artery [30]. Women with atherosclerotic coronary arteries have fewer estrogen receptors than those with normal coronary arteries [31]. The direct and indirect effects of estrogen are outlined below.
Table 2. Direct and Indirect Effects of Estrogen

<table>
<thead>
<tr>
<th>Direct</th>
<th>Indirect</th>
</tr>
</thead>
<tbody>
<tr>
<td>Increase nitric oxide synthesis</td>
<td>Decrease LDL-C</td>
</tr>
<tr>
<td>Increase in vasodilation</td>
<td>Increase triglycerides</td>
</tr>
<tr>
<td>Increase endothelial cell growth</td>
<td>Increase HDL-C</td>
</tr>
<tr>
<td>Decrease smooth muscle cell proliferation</td>
<td>Decrease lipid oxidation</td>
</tr>
</tbody>
</table>

Direct Effects of Estrogen

Estrogen is a critical cofactor in the nitric oxide synthase (NOS) pathway that directly induces vasodilation and initiates NOS genes [5, 32]. Fluctuations in blood pressure correlate with circulating estrogen levels in women during menstrual cycles and pregnancy [33], hence, estrogen is important for maintaining optimal blood pressure levels. Differences in blood pressure in age-matched men and women confirm that premenopausal women have lower blood pressure levels than age-matched counterparts. ERβ, although in proportionally smaller amounts in vascular tissue, plays a key role in blood vessels and functioning ERβ is also needed for normal dilation and blood pressure in men and women. Short-term estrogen administration in men has little effect on blood pressure, but long-term estrogen therapy improves vasodilation [34].

Nitric oxide synthase can be induced (iNOS) or is constitutively expressed in the endothelial (eNOS) cells of blood vessels [35]. The eNOS isoform functions to dilate blood vessels, prevent leukocyte adhesion, and inhibit proliferation of smooth muscle cells [32]. The expression of eNOS can be increased or decreased by stress and other environmental agents. A complex of an estrogen receptor and eNOS is needed for a functional signal and diminished availability of estrogen causes a decrease in eNOS and an increase in blood pressure and leukocyte adhesion. Factors such as smoking, diabetes, and hypertension lead to excess production of a superoxide (O₂⁻) reactive oxygen species (ROS) that interacts with NO to form
peroxynitrite, a highly reactive molecule. This biochemical cycle is continuous because peroxynitrite causes eNOS to become reactive and begins generating superoxide ions itself [36]. The production of superoxide ROS can increase levels of oxidative stress if antioxidant protective mechanisms are not sufficient enough to scavenge the highly reactive molecules. Estrogen exhibits antioxidant effects that may indirectly resist oxidative stress. In addition, estrogen indirectly effects the expression of apolipoprotein gene expression and levels of serum lipoproteins.

Indirect Effects of Estrogen

The abnormal changes in serum lipoprotein and triglyceride levels in postmenopausal women are due to apolipoprotein gene expression in the liver and altered lipoprotein metabolism. Apolipoproteins are proteins that bind to cholesterol to form lipoproteins. Apolipoprotein A (Apo A) is the major protein in HDL-C and apolipoprotein B (Apo B) is the predominant protein in LDL-C. ApoA and ApoB play a role in the development of atherosclerosis. Lipoprotein a (Lp(a)), discovered in the 1960’s, is linked to transport of LDL-C and initiation of the blood clotting process. Lp(a) is comprised of LDL-C and the Apo A protein and selectively found in certain individual’s serum, but not all. There is a positive correlation between serum Lp(a) concentration and risk for heart attack, leading to the idea that Lp(a) may be an independent risk factor for atherosclerosis [37].

Studies have shown that postmenopausal women experience undesirable changes in serum lipids, increasing the risk of developing CVD [22, 23]. This includes a rise in low-density lipoprotein cholesterol and triglyceride levels, and a decrease in high-density lipoprotein cholesterol levels [5, 38]. Significant changes occur during the menopausal period and time since menopause correlates with increased atherogenesis and other vasculature changes [39].
Atherogenesis promotes inflammation because of the cascade of factors released by plaque and foam cell accumulation on the arterial wall. Within three years following natural menopause, there is a significant increase in total cholesterol and LDL-C [40], compared with an increase after six weeks following ovariectomy [41]. Approximately five to eight years following menopause, there is a significant increase in intima-media thickening and formation of plaque deposits [42], contributing to atherogenesis.

A prospective study by Matthews et al. [43] examined changes in coronary heart disease risk factors within a year of the final menstrual period. Participants were 1,054 women from the Study of Women’s Health Across the Nation. Women were examined annually during the early follicular phase of the menstrual cycle to normalize for fluctuating hormones. They saw substantial increases in total cholesterol and LDL-C within the one-year interval before and after the final menstrual period. Therefore, researchers have suggested that ApoB, the major protein that comprises LDL-C, and Apo A, the major protein that comprises HDL-C, also be part of the cardiovascular screening process [20] because of the role they play in lipoprotein levels.

Preventing changes in cholesterol levels and formation of atherosclerotic plaque should be of importance before total cessation of menses as physiological changes may have taken place during the period of diminishing estrogen levels and before the final menstrual period. The menopausal transition can take up to 3 years before menses ceases and during this time, estrogen levels are slowly diminishing. The diminished circulating estrogen affects the entire vascular system and decreases the antioxidant protection in cells.

Estrogen as an Antioxidant
It is proposed that estrogen acts as an antioxidant because of its phenolic hydroxyl group [4]. Short- and long-term administration of 17β-estradiol in postmenopausal women has shown to decrease oxidation of LDL-C [5, 44]. In vivo mice studies demonstrate that ovariectomy causes an increase in serum and liver lipid peroxide levels within one month and a significant difference is seen between ovariectomized and sham control mice within three months [45]. Serum or liver lipid peroxide levels are sensitive markers of LDL-C oxidation that induces oxidative stress. One year later, a follow-up clinical trial [46] compared 18 premenopausal women with 10 naturally postmenopausal women and saw a significant difference (p<0.05) in serum lipid peroxide levels at the beginning of the study. After 7 of the premenopausal women underwent ovariectomy, serum lipid peroxide levels were examined 15, 30, and 60 days after the operation to assess changes. Sixty days after surgery, serum from five subjects confirmed an increase in levels (p<0.05). The increased serum lipid peroxide levels observed in postmenopausal and ovariectomized women compared to premenopausal women demonstrates estrogen’s role as an antioxidant and its preventative effect against oxidative damage. Further complicating the consequences of menopause, smoking can greatly influence the timing and onset of menopause, severity of symptoms, and contributes to the development of CVD by many of the same mechanisms.

**Smoking and Cardiovascular Disease**

Roughly 21.1 million, or 1 in 5, American women are cigarette smokers and the risk for ischemic stroke in smokers is double that for nonsmokers [47]. Smoking is the leading cause of preventable disease and deaths in the United States and a major cause of cardiovascular disease among women [6]. It is the single most important factor in the development and progression of
atherosclerosis [7, 48]. Smoking leads to an increase in inflammation and oxidative stress, a reduction in nitric oxide (NO) availability, and activation of blood clotting platelets [8]. There is an unfavorable shift in blood lipids of long-term smokers and a continuous activated immune response that leads to an increase in chronic inflammatory markers, such as C-reactive protein. Postmenopausal smokers are at serious risk for developing heart disease and its related comorbidities.

Vasomotor Dysfunction

Smoking is associated with a decrease in vasomotor dysfunction through damaged nitric oxide molecules. Cigarette smoke causes a 20 to 25% increase in blood leukocyte count [49] as well as elevated L-selectin expression, an adhesion molecule that brings white blood cells (WBC) to the endothelium of inflamed tissue [50]. Slight systemic changes are seen in levels of inflammatory intermediates in the lungs and circulation of healthy smokers. NO controls inflammation through leukocytes, platelets, and thrombocytes [51]. There is a strong correlation between cigarette smoking and acute phase proteins, such as fibrinogen and C-reactive protein [52-54], a marker of chronic inflammation. Inflammation has been shown to produce reactive oxygen species, which promotes oxidative stress [55].

The tar and gas phases of cigarette smoke contain more than $10^{17}$ and $10^{15}$ free radicals, respectively. The free radicals contained in the tar phase are long lasting compared to the radicals contained in the gas phase. Cigarette smokers are exposed to 8% tar and 92% gas components [56] while actively engaging in smoking. Second-hand smoke contains 85% of the side stream smoke and 15% of the mainstream smoke from smokers [57], however, the second-hand smoke contains higher concentrations of toxic gas components than mainstream smoke because of the lack of filter [58]. To further damage healthy cells, smoking decreases the
bioavailability of NO through generation of ROS and reduction of the antioxidant capacity of the cell [48]. The free radicals from cigarette smoke and activated endogenous free radicals, such as uncoupled nitric oxide synthase (NOS), xanthine oxidase, and nicotinamide adenine dinucleotide phosphate (NADPH) oxidase, trigger an increase in lipid peroxidation [7]. The imbalance between oxidative stress and the internal antioxidant protection increases oxidation of lipids, proteins, and DNA and creates an impaired protection mechanism in cells [59]. This has negative implications on serum lipoproteins as seen in the differences in lipid profiles in smokers when compared to age-matched nonsmokers.

Changes in Lipid Profile

Alterations in serum cholesterol levels may be the primary reason for the development of atherosclerosis in smokers. Smokers have increased levels of total serum cholesterol, triglycerides, and LDL-C, and decreased high-density lipoprotein cholesterol (HDL-C) [60]. This creates an unfavorable lipid profile in which the total cholesterol:HDL-C ratio drops, a strong risk factor for the development of atherosclerosis. Abnormalities of serum lipids could be caused from a number of notable differences among smokers compared to sex- and age-matched non-smokers. For instance, smokers have significantly lower serum antioxidant vitamin C, beta-carotene, and beta-cryptoxanthin levels, even after accounting for dietary intake [61]. These are antioxidant vitamins that could, at effective levels, protect against the damage of lipid oxidation. Cigarette smoke also oxidizes glutathione (GSH), one of the predominant endogenous antioxidants that functions to rid the body of harmful peroxides and maintains vitamins C and E in their reduced forms so they are accessible to cells [62]. Prevention and treatment of CVD should focus on maintaining normal levels of biomarkers of inflammation and oxidative stress or alleviating the cause of high levels of such biomarkers.
The Importance of Measuring Inflammation and Oxidative Stress

Greater than 60% of people who develop coronary events have one or less traditional risk factors present and more than 50% have normal or slightly increased lipid values [18]. This has prompted many researchers to ask that high-sensitivity C-reactive protein (hsCRP) be used in predicting risk of cardiovascular disease events. Inflammation is an important element in the development and progression of atherosclerosis. The Physicians’ Health Study [63] and the Framingham Heart Offspring Study [64] showed clinical significance in using hsCRP as independent predictors for CVD risk. The American Heart Association and Centers for Disease Control published guidelines on assessing risk according to levels of hsCRP; below 1 mg/L correlates with low risk, medium risk is levels between 1 and 3 mg/L, and high risk is levels over 3 mg/L (Clinical Chemistry 55:2 2009). Individuals may be more accurately classified as ‘at risk’ if hsCRP was added to clinical risk prediction criteria for CVD. Inflammation and oxidative stress are closely linked and one can further increase the other.

Very simply, oxidative stress is the imbalance between free radical production and the body’s antioxidant defenses and has been implicated in a number of conditions, such as cardiovascular disease, cancer, diabetes, rheumatoid arthritis, and aging [65, 66]. Animal models support the hypothesis that oxidative stress plays a causal role in vascular diseases such as atherosclerosis. Large human investigations support this hypothesis because oxidative stress is implicated in many of the risk factors for CVD [67]. Oxidative stress can be quantified by measuring levels of lipid peroxidation. There are several methods for measuring lipid peroxidation, however, thiobarbituric acid reactive substances (TBARS) assay remains the most widely used assay [68]. TBARS is specific for malondialdehyde (MDA), which is a naturally occurring produce of lipid peroxidation in cells and tissues [68, 69]. Lipid peroxides, derived from polyunsaturated fatty acids, can easily degrade to smaller compounds, i.e. MDA. The assay
procedure consists of forming an adduct of MDA, in this case contained in plasma, and thiobarbituric acid (TBA) in conditions of high temperature and acid, i.e. 100°C and acetic acid.

Normal TBARS concentration in human plasma is 1.86 – 3.94 µM MDA [68]. Orhan and others [70] found that TBARS were significantly elevated in smokers when compared to nonsmoker controls (p<0.01). Ochs-Balcom [71] and colleagues measured TBARS levels in 1,197 men and 1,149 women ages 35-79 with no significant differences in body mass index or pack-years smoking and found that men had a higher TBARS value than women (1.44 vs. 1.33 nmol/mL, p<0.01). They concluded that the lower TBARS values in women could be due to the antioxidant activity of estrogen, however, Ide and others [72] concluded that plasma estrogen did not explain differences in TBARS levels. The debate remains about estrogen’s ability as an antioxidant, but women have shown to have higher levels of antioxidant vitamins such as vitamin E and β-cryptoxanthin [71]. The higher levels of antioxidant vitamins in healthy premenopausal women compared to age-matched men could be due to the fact estrogen quenches free radicals and other antioxidant vitamins remain in circulation until otherwise needed.

Another method of measuring lipid peroxidation is isoprostane levels by gas chromatography-mass spectrometry, a highly reliable and specific method [73]. Specifically, F₂-isoprostanes, measurable in plasma, serum, and urine, are prostaglandin-like compounds that are the products of lipid peroxidation of arachidonic acid [74]. Isoprostanes are greatly influenced by the amount of cigarettes smoked, so they are a reliable marker for an accurate quantification of inflammation. The drawbacks to measuring isoprostanes for quantifying oxidative stress levels are cost of buying the isoprostane standard and prolonged time validating a method for GC-MS specifically for F₂-isoprostanes. Pilz and others [75] observed the
isoprostane levels in 47 adult smokers. Baseline levels of plasma, serum, and urinary 8-epi-PGF$_{2\alpha}$ were significantly increased in smokers compared to nonsmokers. A reduction in levels was seen only a few days following cessation and reached normal levels after 4 weeks [76]. These results indicate that individuals may benefit from smoking cessation and levels of oxidative stress may return to normal [77].

Smoking and menopause are risk factors for CVD because they increase levels of inflammation and oxidative stress and over time this can cause vasomotor dysfunction, changes in lipid profile, an exhausted immune system, and decreased protection in cells throughout the body.

It is of concern to health professionals to prevent the onset of cardiovascular disease by maintaining optimal levels of CVD risk factors. Because it is the number one cause of mortality in the United States, it remains a burden to a large proportion of US adults and is a focus for many scientific professionals as well.

**Current Prevention and Treatment of CVD**

Menopause is an unavoidable process for women. Those who have experienced menopause report the occurrence of hot flashes, sleep disturbance, emotional and sexual imbalance, nervousness, and vasomotor symptoms [78]. Smoking has been reported to negatively affect the timing of menopause and its related symptoms [79]. The Women's Health Initiative showed that hormone replacement therapy (HRT) increases the risk for heart disease, stroke, and cancer of the breast and endometrium [80], and is no longer recommended. Current recommendations for preventing heart disease in women include smoking cessation, maintaining a healthy body weight, exercise, controlling medical conditions, and eating well.
However, treatment is generally not recommended until CVD risk factor levels have already transitioned into the ‘at risk’ or ‘high risk’ categories, or following a cardiovascular event. The traditional method of treating CVD includes maintaining a healthy diet, exercising regularly, and pharmaceutical applications.

Pharmaceutical Therapy for CVD

There are several pharmaceuticals that have been developed to target risk factors of CVD. Statins, the most commonly prescribed class of drugs, target high cholesterol levels through an enzyme that inhibits cholesterol synthesis in the liver. Although rare, cases have been reported of liver failure and other common side effects include nausea, vomiting, diarrhea, and constipation [81]. Nitrates are potent vasodilators that target high blood pressure and use of nitrates has been linked to sudden drops in blood pressure, fainting, dizziness, and heart palpitations. Other medications – ACE inhibitors, calcium channel blockers, diuretics, and blood thinners – have their own lists of side effects [82]. Therefore, compliance is low and patients do not want to cope with the side effects of drug treatment or the financial burden associated with the cost of prescription. Consequently, many women are looking for a natural, safe alternative to drug therapies. Risk factors for CVD, such as high cholesterol, obesity, hypertension, and diabetes, have shown to be significantly influenced by diet and lifestyle and these factors have been increasingly popular targets of CVD treatment.

Dietary Therapy for CVD

The American Heart Association has outlined diet recommendations for reducing the risk of cardiovascular disease [83]. These include consuming a diet rich in fruits and vegetables, choosing whole grains over refined grains, eating high-fiber foods, consuming oily fish at least
twice a week, limiting saturated and trans fat intake, choosing lean meats, and limiting intake of beverages and foods with added sugars. These recommendations evolved from studies that examined the effects of dietary patterns on CVD risk and risk factors [84, 85]. Studies have shown that dietary consumption of fruits, vegetables, and grains significantly reduces the risk of developing CVD, type 2 diabetes, aging-related diseases, and some cancers [10, 86, 87].

Specifically, fruits and vegetables have been implicated in a number of population-based studies as having beneficial effects on biomarkers associated with risks of cardiovascular disease, primarily LDL-C. These positive results have been attributed, in part, to the non-macronutrient composition, such as vitamins, minerals, fiber, and phytochemicals, such as phenolic compounds. Blackberries and blueberries are predominantly carbohydrates, a good source of fiber, extremely low in sodium, contain very little fat in the form of healthy unsaturated fatty acids, and a large number of phenolic compounds [88], which are known to act as potent free-radical scavenging antioxidants.

Antioxidants

Antioxidant-rich foods have become popular in recent years due to the growing concern over the negative consequences of oxidative stress and dietary antioxidants counteract the formation of free radicals [89]. Free radicals are generated by factors such as the body's metabolism, UV rays from sunlight, air pollution, cigarette smoke, pesticides, and drugs. Free radicals are molecules that contain an unpaired electron in their outer orbital shell [65]. They are unstable, extremely reactive, and have a strong tendency to stabilize themselves by “stealing” electrons from other molecules, such as proteins, lipids, and nucleic acids. Thus, free radicals cause cellular injury or death to otherwise healthy cells in the body. Antioxidant
availability in vivo would prevent free radicals from starting the cascade of oxidative stress formation.

The most common free radicals are oxygen-derived, including superoxide, hydroxyl, peroxyl, alkoxyl, and hydroperoxyl, belonging to a group classified as reactive oxygen species (ROS) [66]. These ROS produced by endothelial cells, smooth muscle cells, and macrophages oxidize LDL-C [67]. Oxidized LDL-C molecules form foam cells, a major component of atherosclerotic plaque that accumulates on the vascular wall. In addition, oxidized LDL-C stimulates expression of adhesion molecules and chemotactic factors that help monocytes and other leukocytes attach to the arterial wall [90], further promoting ROS production. Foam cells and leukocytes cause fatty streaks, smooth muscle proliferation, and lead to an unstable fibrous plaque susceptible to rupture [67]. Without sufficient levels of antioxidants, the generation of ROS and increase in oxidative stress levels could hinder cardiovascular health.

Current antioxidants used in the prevention of cardiovascular disease include vitamins E, C, and β-carotene [91]. Multivitamins are commonly recommended by physicians; however, those containing α-tocopherol and β-carotene and taken on a regular basis may cause adverse effects because these vitamins are fat-soluble and stored readily by the body. Furthermore, they show no effect in decreasing the risk for cardiovascular mortality and may cause negative consequences on lipid concentrations [91, 92].

The Heart Protection Study was a randomized, placebo-controlled study [91] in 20,536 high-risk individuals investigating statin medication and vitamin use. They found no significant differences in cardiovascular mortality with vitamin E, vitamin C, and β-carotene supplementation for five years. There was increased plasma concentrations of these nutrients and dosage level appeared to have no adverse effects at the end of the study period. The lack of
significant positive findings as a consensus for antioxidant supplementation in CVD suggests that antioxidants may be more beneficial when consumed from whole foods and that separate classes and/or subclasses of antioxidants, such as phenolic compounds, may exhibit more potent \textit{in vivo} effects than antioxidant vitamins C, E, and \(\beta\)-carotene.

**Phenolic Compounds**

Phenolic compounds have been shown to resist platelet aggregation, reduce proinflammatory mediators, and decrease expression of adhesion molecules, making them antithrombotic [93]. Phenolic compounds are secondary metabolites present in all plants [94], however, flavonoids are the most abundant phenolic compounds in plant foods. Table 3 shows flavonoid subclasses, compounds, and typical foods with high content taken from the United States Department of Agriculture’s databases of selected foods [95-97].
### Table 3. Flavonoid Subclasses, Compounds, and Good Food Sources

<table>
<thead>
<tr>
<th>Subclass</th>
<th>Compounds</th>
<th>Good food sources</th>
</tr>
</thead>
<tbody>
<tr>
<td>Flavonols</td>
<td>Quercetin, Kaempferol, Myricetin, Isorhamnetin</td>
<td>Onions, broccoli, apples, berries, tomato, grapes, tea, red wine</td>
</tr>
<tr>
<td>Flavones</td>
<td>Luteolin, Apigenin, Tangeritin</td>
<td>Celery, parsley</td>
</tr>
<tr>
<td>Flavanones</td>
<td>Hesperetin, Naringenin, Erdidictyol, Homoeriodictyol</td>
<td>Oranges, grapefruit, lemons</td>
</tr>
<tr>
<td>Flavan-3-ols</td>
<td>Catechin, Gallocatechin, Epicatechin, Epigallocatechin, Epicatechin 3-gallate, \ Theaflavin, Theaflavin 3-gallate, Theaflavin 3'-gallate, \ Theaflavin 3,3’ digallate, Thearubigins</td>
<td>Apples, blackberries, red wine, green tea, black tea</td>
</tr>
<tr>
<td>Anthocyanidins</td>
<td>Cyanidin, Delphinidin, Malvidin, Pelargonidin, Peonidin, Petunidin</td>
<td>Blackberries, blueberries, raspberries, red wine</td>
</tr>
<tr>
<td>Proanthocyanidin</td>
<td>Monomers and polymers of flavonoids</td>
<td>Apples, chocolate, red seeded grapes</td>
</tr>
<tr>
<td>Isoflavones</td>
<td>Daidzein, Genistein, Glycitein</td>
<td>Tofu, soymilk</td>
</tr>
</tbody>
</table>

Adapted from the US Department of Agriculture databases for isoflavone, flavonoid, proanthocyanidin content of selected foods [95-97]

Flavonoids improve endothelial function and inhibit platelet adherence [98-100], and therefore, are thought to reduce the oxidation of LDL-C [101, 102]. Flavonoids have been shown to inhibit lipid peroxidation via the phospholipid bilayer by trapping radicals at the interface of the membrane and preventing a chain reaction of generating free radicals and damaging other lipids [103]. Furthermore, they can chelate metal ions that could generate ROS and inhibit lipoxygenase reactions [104]. These effects are thought to contribute to flavonoids’ role in reducing oxidative stress and inflammation, and reducing the risk of CVD.

Epidemiological studies show conflicting results between flavonoid intake and stroke or CVD mortality. Several epidemiological studies have found a significant inverse relationship between flavonoid intake and cardiovascular disease in women [105-107]. The Zutphen Elderly Study [105] showed that a flavonoid intake of 30 mg/day was associated with a 50% reduction in CHD mortality when compared to a diet with less than 19 mg/day. Knekt and colleagues [108]
in a cohort study of 10,054 Finnish men and women concluded that higher intake of quercetin, a specific flavonoid, was associated with a lower mortality from ischemic heart disease ($p=0.02$). Some studies have disputed this relationship [109, 110]. Yochum and others [110] found no association between flavonoid intake and stroke mortality in 34,492 postmenopausal women.

The discrepancies associating flavonoid intake with CVD rates and mortality may be due to the varying amount of flavonoid-containing foods in certain populations, the variability of amount of flavonoids within a food [94], and the statistical approach when analyzed. For example, Hertog et al. [111] combined total flavonoid intake, saturated fat, and smoking in a multivariant analysis of coronary heart disease. Knekt and others [108] analyzed only one flavonoid compound and concluded that higher intakes of quercetin was associated with decreased mortality from ischemic heart disease.

Anthocyanins are a class of flavonoids which have been studied exclusively and shown to be potent antioxidants with anti-inflammatory and cell regulatory properties [112]. Blackberries and blueberries are rich in anthocyanins and have been shown to improve the cardiovascular system in an animal model [13, 15].

Anthocyanins

The in vivo effects of anthocyanins are thought to be largely dependent upon stability and bioavailability. Anthocyanins are comprised of phenolic ring structures known as anthocyanidins and their sugar moieties. In vitro studies [113] show that anthocyanins in their aglycone form are extremely unstable at neutral conditions and relatively stable in acidic environments. The stability is also dependent upon the attachments at $R_1$ and $R_2$ on the B-ring of the anthocyanidin structure. Hydroxy and methoxy groups attached on the aglycone structure decrease the stability, therefore, pelargonidin remains the most stable anthocyanidin. Cyanidin and peonidin
contain hydrogen molecules at $R_2$ and hydroxy and methoxy groups at $R_1$, respectively and are slightly stable. The basic structure of anthocyanidins is shown in Figure 1.

![Figure 1. Structure of Anthocyanidins](image)

<table>
<thead>
<tr>
<th>Anthocyanidin</th>
<th>$R_1$</th>
<th>$R_2$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pelargonidin</td>
<td>H</td>
<td>H</td>
</tr>
<tr>
<td>Cyanidin</td>
<td>OH</td>
<td>H</td>
</tr>
<tr>
<td>Delphinidin</td>
<td>OH</td>
<td>OH</td>
</tr>
<tr>
<td>Peonidin</td>
<td>OCH$_3$</td>
<td>H</td>
</tr>
<tr>
<td>Petunidin</td>
<td>OCH$_3$</td>
<td>OH</td>
</tr>
<tr>
<td>Malvidin</td>
<td>OCH$_3$</td>
<td>OCH$_3$</td>
</tr>
</tbody>
</table>

Anthocyanin-monoglucosides are stable at neutral pH because the sugars prevent degradation. However, incubating anthocyanin-3-glucosides such as cyanidin, malvidin, and peonidin 3-glucosides with human fecal microflora resulted in more than 90% degradation after 2 hours indicating that gut microflora are largely responsible for the degradation of anthocyanins into phenolic acids. Bacterial biotransformation of the microflora is responsible for cleaving the sugar to leave the aglycone anthocyanin and because the pH of the colon can reach 8.0, this may be an unstable environment for anthocyanidins [114]. It was previously
believed that dietary flavonoids were minimally absorbed, however, studies have been shown that some are absorbed better than others.

The sugar moiety of anthocyanins and other flavonoids is also an important component affecting the absorption and bioavailability in vivo. Hollman and others [114] observed absorption of quercetin, which is a flavonol in both blackberries and blueberries, in nine patients who had undergone ileostomy. After following a 12 day quercetin-free diet, subjects consumed one of three supplemented diets (100 mg) over a 12 day period: 1) fried onions with quercetin glucosides; 2) pure quercetin rutinoside; or 3) pure quercetin aglycone. They found that absorption of quercetin aglycone was lower than quercetin glycosides (24% vs. 52%), indicating the sugars increased absorption of quercetin when compared to the aglycone form. In a single-blind study conducted by Kay and Holub [14], consumption of 100 g of freeze-dried blueberry powder with a high-fat diet led to an increase in serum antioxidant levels shown by the oxygen radical absorbance capacity (ORAC) assay. Compared to a control high-fat diet fed one-week prior, after consuming the 100 g blueberry powder, there was an 8.5% increase in serum ORAC after 1 hour ($p=0.02$) and a 15% increase after 4 hours ($p=0.009$). Conversely, when six healthy women consumed 189 g of frozen blueberries containing 690 mg anthocyanins in 315 mL water, anthocyanins were difficult to detect in urine. Approximately 0.004% of the 690 mg anthocyanin consumed was detected in urine. There were no anthocyanins detected in any of the plasma samples at any given time point (10 minutes-24 hours). This may be because there are over 25 different anthocyanins in blueberries [115] and such small amounts of each that they are all degraded rather quickly in circulation or because of their poor lipophilic properties.

Anthocyanins from blackberries have been shown to be slightly absorbed in humans. Four healthy women consumed 720 mg anthocyanins derived from elderberries in 500 mL
water. Cyanidin 3-glucoside, the predominant anthocyanin in blackberries, was found in urine and plasma samples from all subjects within 4 hours following consumption [116]. Consumption of 200 g blackberries (89.2% cyanidin 3-glucoside) concurrently with 15 g sugar, 60 g of bread, and 10 g of butter resulted in the highest identifiable cyanidin 3-glucoside peak and its related metabolites in urine between 2 and 4 hours following consumption. A 24-hour urine collection revealed that excretion of the native cyanidin 3-glucoside accounted for just 0.023% of total ingested amount and metabolites accounted for 0.16% of ingested amount [117]. Cyanidin 3-glucoside and other anthocyanins may be metabolized into methylated and glucuronidated conjugates and these need to be analyzed as metabolites in plasma and urine as well as the native anthocyanins [116], however, many of these metabolites have not yet been identified.

This chapter has focused on the risk factors for cardiovascular disease, specifically menopause and smoking, and how they contribute to the etiology of CVD. Additionally, how inflammation and oxidative stress play a pivotal role in the development of cardiovascular disease in postmenopausal smokers and how antioxidant-rich berries may alleviate these risk factors. This was a pilot clinical trial and to our knowledge, this is the first study to examine the effects of blackberry and blueberry treatment on CVD risk factors in postmenopausal smokers.
CHAPTER 3. METHODS

Study Design and Criteria for Participation

This study was approved by the University of Arkansas Institutional Review Board (IRB) (Appendix 1) and Washington Regional Medical Center (Fayetteville, AR). Guidelines for a clinical trial were followed for the entire duration of the study. The study design is shown in Figure 2. Healthy, postmenopausal women were recruited from the Northwest Arkansas Region by mail, radio, flyer, brochures, and email to participate in the three-month study. They were required to meet the following criteria: have no personal history of heart disease, refrained from using hormone replacement therapy (HRT) within three months prior to beginning the study, and not be on any medication that would affect the outcome of the study. Smokers were moderate smokers defined by smoking 1-20 cigarettes/day. Nonsmokers were recruited for a control group and smokers were recruited and randomly assigned to one of three groups: smoker control, smoker + blackberries, and smoker + blueberries. Participants signed an informed consent (Appendix 2) and completed a full medical history questionnaire (Appendix 3) including personal history of skeletal health, cardiovascular function, hypertension, diabetes, gastrointestinal/digestive problems, liver disease, respiratory problems, thyroid disorder, medication or drug use, and physical activity.

Blackberry and Blueberry Treatment

Women assigned to the blackberry and blueberry treatment groups were required to consume 45 grams of freeze-dried berries per day and it was suggested replacing a snack with the berries so that normal daily caloric intake was not altered. Freeze-dried blackberries and blueberries were purchased from WaterShed Foods (Gridley, IL). Forty-five grams of berries
were weighed and individually vacuum-packaged to retain nutritive value and for ease of storage and transportation. Participants were given the sufficient amount of vacuum-packaged berries needed until the final visit. Calendars were provided to participants in the treatment groups to record compliance.

**Analysis of Macronutrient Composition and Flavonoid Content of Freeze-Dried Berries**

Upon arrival, freeze-dried blackberries and blueberries were sent to the Central Analytical Laboratory (University of Arkansas Poultry Science Center; Fayetteville, AR) for proximate analysis of fat, protein, dry matter, ash, and crude fiber. Percent carbohydrates were calculated by subtracting percent fat and protein from 100. Flavonoid content was analyzed in triplicates by high performance liquid chromatography (HPLC) using a previously outlined method [118]. Blackberry flavonoid profiles were followed up and confirmed by mass spectroscopy, as it was difficult to detect specific peaks when compared to the blackberry standard.

**Clinical Assessment**

Participants reported to the study center at baseline and following the three-month treatment period. They were required to fast 8-12 hours overnight and came in for collection of laboratory values. Height, weight, body mass index (BMI), and blood pressure were recorded. BMI was calculated as \[\text{[weight (kg)}]/[\text{height (m)}]^2\]. Venous blood was collected by a skilled phlebotomist in separate BD Vacutainer® (BD Diagnostics; New Franklin, NJ) collection tubes designated for plasma, serum, and hemoglobin A1c (HbA1c) collection. Whole blood was
centrifuged in a Beckman Coulter (Brea, CA) centrifuge at 1200 x g at 4°C for 20 minutes. Plasma and serum supernates were aliquoted into separate tubes and stored at -80°C until analysis. HbA1c was analyzed by LabCorp, Inc. (Fayetteville, AR).

**Dietary Assessment**

A 114-item food frequency questionnaire (FFQ) (Appendix 4) was administered at each visit with measuring cups and spoons as references. Participants were read a list of items and asked to report portion size and frequency of consumption over the previous 7 days. The FFQ was analyzed by Nutrition Pro (Axxya Systems; Stafford, TX) software based on USDA References. The following nutrients were of interest: kilocalories, total fat, saturated fat, polyunsaturated fat, monounsaturated fat, dietary cholesterol, total carbohydrates, dietary fiber, and protein.

**Serum Lipid Profiles**

Serum total cholesterol, HDL-cholesterol, and triglycerides were analyzed on an ACE Alera clinical chemistry system (Alfa Wasserman, Inc.; West Caldwell, NJ) in duplicates using commercially available kits, quality controls, and calibrators (Alfa Wasserman, Inc.; West Caldwell, NJ). LDL-cholesterol was then calculated using the Friedewald formula [119] as follows: 

\[(LDL \text{ cholesterol}) = (total \text{ cholesterol}) - (HDL \text{ cholesterol}) - \left[\frac{\text{triglycerides}}{5}\right],\]

whereas all values are given in mg/dL.

**Inflammation and Oxidative Stress Markers**
Inflammation was quantified by high-sensitivity C-reactive protein (hsCRP) using a latex particle enhanced immunoturbidimetric assay measured on an ACE Alera clinical chemistry system (Alfa Wasserman, Inc.; West Caldwell, NJ) using commercially available reagents (Alfa Wasserman, Inc.; West Caldwell, NJ). A four-point calibration curve was developed using known concentrations of human C-reactive protein and used to determine sample concentration in serum based on absorbance at 570 nm. The kit was sensitive for human hsCRP between 0.1 – 160 mg/L.

Oxidative stress levels were determined by thiobarbituric acid reactive substances (TBARS) assay kit (Cayman Chemical; Ann Arbor, Michigan). A volume of 100 µL of plasma was used for the assay. Sodium dodecyl sulfate was added to lyse cellular membranes. Thiobarbituric acid dissolved in a mixture of full-strength glacial acetic acid and sodium hydroxide was added and samples were boiled at 100°C for one hour, cooled on ice, and centrifuged 1600 x g at 4°C for 10 minutes. The supernates were taken for analysis of concentration of plasma LDL-C peroxide products, predominantly malondialdehyde (MDA), and read at an absorbance of 540 nm on a Synergy HT (BioTek; Winooski, VT) microplate reader. Sample concentrations of lipid peroxidation were determined as MDA equivalents by interpolation from the standard curve.

**Statistical Methods**

Baseline analysis was performed in JMP (Version 9, SAS® Institute Inc.; Cary, NC) between controls and treatment groups to determine if groups were significantly different in regard to age, years postmenopausal, and BMI using one-way analysis of variance (ANOVA). Additionally, one-way ANOVA was performed on smoker control and treatment groups to ensure smoker groups were not significantly different from one another. If ANOVA indicated
significance ($\alpha=0.05$), differences in means were determined using a protected least significant difference procedure (LSD). A baseline two-sample t-test of nonsmokers and smokers was analyzed to assess the effects of smoking on risk factors for cardiovascular disease. Risk factors of interest were BMI, blood pressure, HbA1c, glucose, serum total cholesterol, LDL-C, triglycerides, and HDL-C, hsCRP, and TBARS. Baseline results are reported as means ± standard deviation.

Percent change of all variables after the treatment period was performed using SAS® (Version 9.2, SAS Institute Inc.; Cary, NC). Randomized, one factor analysis of variance (ANOVA) was performed and if indicated significance ($\alpha=0.05$), was followed up by a protected LSD procedure. Percent change results are reported as means ± standard error of the mean.
CHAPTER 4. RESULTS

Macronutrient Composition of Freeze-Dried Berries

The proximate analysis showed that blackberries contained 6.3% fat, 87.3% carbohydrates of which 27.7% was crude fiber, and 6.4% protein. Blueberries contained 2.3% fat, 94.2% carbohydrates, 12.5% crude fiber, and 3.6% protein (Table 4). This provided a calculated 195 kcal, 3 g fat, 39 g carbohydrates, 12 g fiber, and 3 g protein per day for participants consuming blackberries and 185 kcal, 1 g fat, 42 g carbohydrates, 6 g fiber, and 2 g protein per day for participants consuming blueberries.

Flavonoid Content of Freeze-Dried Berries

The HPLC analysis of the freeze-dried blackberries and blueberries is shown in Tables 5 and 6, respectively. Blackberries contained 631.3 ± 8.7 mg anthocyanins, 74.4 ± 0.5 mg flavonols, and 248.8 ± 1.6 ellagitannins per 100 g blackberries, yielding a total flavonoid content of approximately 955 mg/100 g. Cyanidin 3-glucoside comprised more than 50% of the flavonoid composition in the freeze-dried blackberries. Blueberries contained 1449.2 ± 119.7 mg anthocyanins, 79.8 ± 6.8 mg flavonols, and 162.1 ± 4.2 mg chlorogenic acid per 100 g with an approximate 1691 mg total flavonoids/100 g. Freeze-dried blueberries contained 29 flavonoid compounds, approximately 85% as anthocyanins. The approximate intake of anthocyanins per day attributed to the freeze-dried blackberries and blueberries was 276 mg and 652 mg, respectively, and the total flavonoid intake was 430 mg and 761 mg.

Participant Characteristics
Forty-five participants completed the study: 14 nonsmoker controls, 12 smoker controls, 6 smoker + blackberry treatment participants, and 13 smoker + blueberry treatment participants. A total of 65 participants were recruited for the baseline visit. Twelve did not return for the final visit, one was excluded for being 14 years postmenopausal, one due to complications with the baseline blood draw, one after admitted being on hormone replacement therapy, and five based on high glucose and HbA1c values.

The average age of study participants was 55.8 ± 3.9 years old and they were 6.9 ± 3.0 years postmenopausal. Baseline values for smokers were analyzed to ensure that CVD risk factors were not significantly different among smoker control and smoker treatment groups (data not shown). No significant differences were observed between smoker control and smoker treatment groups at baseline.

**Effects of Smoking on CVD Risk Factors in Postmenopausal Women**

Non-smokers and smokers did not differ in regard to BMI, blood pressure, total cholesterol, LDL-C, glucose, hsCRP, or TBARS at the beginning of the study. Non-smokers had significantly higher HbA1c values than smokers (5.8% ± 0.1 vs. 5.6% ± 0.1; \( p=0.0468 \)) (Table 7). Figure 3 shows the effect of smoking on serum lipid profiles. Smokers had significantly higher triglycerides (130 ± 11 mg/dL vs. 87 ± 7 mg/dL; \( p=0.0010 \)) and lower HDL-C (58 ± 3 mg/dL vs. 66 ± 3 mg/dL; \( p=0.0384 \)) values than non-smokers. Smokers had higher levels of inflammation and oxidative stress shown by hsCRP and TBARS than non-smokers, albeit, not significant (Figure 4). The daily dietary intake of non-smokers and smokers is shown in Table 8. Smokers had significantly lower dietary fiber intake than non-smokers (21 ± 2 g/day vs. 28 ± 2 g/day; \( p=0.0166 \)).
Effects of Antioxidant-Rich Berries on CVD Risk Factors in Postmenopausal Smokers

Following three months of daily treatment with 45 grams of freeze-dried blackberries or blueberries, there were no significant changes in CVD risk factors, excluding diastolic blood pressure (Table 9). There was a $16.08 \pm 5.18\%$ ($p=0.0035$) increase in diastolic blood pressure observed following blackberry treatment.

Figures 5 illustrates the effects of berries on total cholesterol, LDL-C, triglycerides, and HDL-C. There were no significant changes in serum lipids in either blackberry or blueberry treatment groups. There was an increase in total cholesterol, LDL-C, and triglycerides in both treatment groups, although insignificant. There was a slight insignificant increase in HDL-C in the blackberry treatment group and a small decrease in HDL-C in the blueberry treatment group. Figure 6 shows changes in inflammation and oxidative stress shown by hsCRP and TBARS following blackberry or blueberry treatment. There were no changes in the inflammation or oxidative stress markers, however, it is notable that the smoker control group increased inflammation and oxidative stress by $31.12 \pm 20.33\%$ and $20.17 \pm 10.82\%$, respectively. These increases are not significant when compared to treatment or nonsmoker control groups.

Table 10 shows the dietary intake of control and treatment groups at the end of the study. Following three-months consumption of blackberries or blueberries, there were no significant changes in dietary intake. It is notable, however, that blackberries and blueberries caused an increase in fiber, $49.30 \pm 37.79\%$ and $80.96 \pm 25.67\%$, respectively, compared with an $18.87 \pm 26.72\%$ increase in fiber consumption in smoker controls, however, these results are not significant.
CHAPTER 5. DISCUSSION AND CONCLUSIONS

The aim of this study was to investigate the effects of smoking on CVD risk factors in postmenopausal women and evaluate the effect of antioxidant-rich berries in ameliorating those risk factors.

Menopause has been shown to significantly increase the risk of cardiovascular disease [120]. There is a twofold increase in CVD rates in postmenopausal women compared to age matched premenopausal women [121]. The current study population had high levels of total cholesterol, LDL-C, and hsCRP (Figures 3 and 4) when compared to optimal values recommended for women [122]. Higher levels of serum total cholesterol, triglycerides, LDL-C [123, 124] and CRP [21, 125] have been observed in postmenopausal women. This creates an unfavorable atherogenic lipid profile putting persons at risk for heart attack and stroke, thus could be the cause of increased rates of coronary heart disease in postmenopausal women. Derby and colleagues [126] showed that menopausal women in the highest estradiol quartile had the lowest levels of total cholesterol, LDL-C, and triglycerides and the highest level of HDL-C. Studies on the effects of estrogen hormone-receptor interactions, the continuous decrease in circulating estradiol during the shift to postmenopausal status affects lipoprotein levels and these changes have been associated with lipoprotein metabolism [41, 127].

Additionally, we observed that smoking negatively influenced CVD risk factors in postmenopausal women. Smokers had significantly higher triglycerides and lower HDL-C than nonsmokers (Figure 3). These findings agree with previous studies [7, 121]. In a study of 25,689 healthy Japanese men 40-59 years old, heavy smokers had significantly lower total cholesterol, HDL-C, and LDL-C, and higher triglycerides and atherogenic index than nonsmokers and light smokers [128]. The increase in total cholesterol, LDL-C, and triglycerides, and decrease in
HDL-C in smokers could be due to lower serum antioxidant levels observed in smokers [61], although the study did not examine diet or physical activity and this may have shown significant differences in dietary intake of fiber, vitamin A, vitamin C, or other nutrients in smokers that may affect blood lipid levels.

The smokers in this study had higher levels of inflammation and oxidative stress than nonsmokers (Figure 4), although insignificant. Several studies have shown a positive association between cigarette smoking and CRP, a marker of chronic inflammation [129, 130]. Tracy and colleagues [131] found a strong association between CRP levels and pack-years among smokers among 400 men and women free of clinical CVD. These findings are not in agreement with the current study findings and this could be due to the differences in sample sizes or participant characteristics. Their population had an average age of 77 and included both men and women.

Inflammation is a cascade of events that promotes oxidative stress and reactive oxygen species in turn will influence inflammation. It has been proposed that smoking increases oxidative stress levels, i.e. thiobarbituric acid reactive substances (TBARS), through the deposit of heavy metals from cigarette smoking, such as cadmium, on the arterial wall [132, 133]. The level of cadmium in the aorta of smokers increases proportionally to the pack-years of cigarettes smoked [133]. Other studies involving larger populations have observed significantly higher TBARS and other lipid peroxidation values in smokers compared to nonsmokers [7, 134].

Prevention and treatment options for CVD are widely available, however, most are not preferred or recommended due to side effects, safety, and cost [122]. Risk factors for CVD have shown to be significantly altered by dietary habits and therefore could be a natural alternative to drug therapy. A 6-month intervention trial [135] involving 12 healthy,
hypercholesterolemic postmenopausal women consuming high-fat diets and 13 postmenopausal women controls consuming low-fat diets was conducted to examine the effects of a low-fat monounsaturated-rich diet via consumption of oleic acid-rich peanuts. Women consuming the peanuts for 6 months experienced a significant decrease in serum cholesterol and LDL-C. This could be attributed to the decreased intake of total fat, increase in healthy monounsaturated fat, or increase in dietary fiber. The study population differed from ours in that the postmenopausal women were already at elevated risk based on total fat intake and serum cholesterol levels prior to intervention and the study period lasted 6 months, while ours was a 3-month duration.

Fiber is undoubtedly an important component of a healthy diet, however, in the following 3-month study did not show any effects on CRP levels, an independent risk factor for CVD. King and others [136] supplemented 7 or 14 g psyllium fiber for 3 months in 158 overweight volunteers 40-65 years old with no personal history of heart disease. At the end of the study, there was no change in CRP, fibrinogen, or white blood cell counts in either dosage group. This may show that fiber itself is not effective in reducing inflammation and CVD risk, but that diets naturally high in fiber would contain additional bioactive compounds that would alleviate risk factors. Our study supplemented 12.5 and 6.0 grams of fiber for women consuming blackberries or blueberries, respectively, which is very similar to the fiber supplemented in this study. Furthermore, the study population recruited was overweight classified by BMI >25 kg/m², which may have been an additional burden to cardiovascular health. Our study population had an average BMI of 26.1 kg/m², classified as overweight, and could explain increased CVD risk factors at the beginning of the study.

The growing popularity of antioxidants in recent years is due to their health promoting properties as a result of the ability to quench free radicals and lower oxidative stress levels.
Berries have been shown to be anti-inflammatory [112] and inflammation is a known component in the etiology of CVD [18, 137]. Blueberries and blackberries have been shown to improve cardiovascular health in animal models [13, 15], however, the effects in humans are inconsistent [138-140]. Blackberries and blueberries used in this study are rich sources of anthocyanins and total flavonoids. A comparable study [141] involving healthy men and women, 45-61 years old, assessed CRP and blood lipids after 14 and 28 days consumption of 280 g fresh bing sweet cherry consumption. There was no changes in CRP seen after 14 days, however, there was a 25% reduction in CRP levels after 28 days. No changes were seen in plasma total cholesterol, HDL-C, LDL-C, triglycerides, or particle subfractions of HDL-C or LDL-C after 14 or 28 days treatment. The amount of total phenolics in the sweet bing cherries consumed daily was comparable to the daily phenolic intake of the blackberry treatment group in our study, however, cherries are predominantly hydroxycinnamate and therefore, the anthocyanin consumption is not comparable between this study and our current study. It is notable that major anthocyanins in cherries are cyanidin 3-rutinoside and cyanidin 3-glucoside, and the major anthocyanin in blackberries is cyanidin 3-glucoside. This may explain why we saw slightly better improvements in hsCRP following blackberry treatment compared to blueberry treatment, albeit not significant.

Karlsen and others showed that 300 mg/day isolated anthocyanins from bilberries equivalent to 100 g fresh bilberries per day showed no effect on CRP, total cholesterol, HDL-C, LDL-C, or plasma antioxidant levels after 3 week supplementation in 118 men and women ages 40-74 [142]. In a follow-up study [143] with 330 mL bilberry juice derived from steam processing whole bilberries, they did observe a significant decrease in CRP levels after 4 weeks of treatment. An additional study involving 255 mg/day flavonoids, predominantly cyanidin anthocyanins, extracted from chokeberries [144] showed a significant reduction CRP levels
after 6 weeks of treatment. Blackberries in this study are also predominantly cyanidin anthocyanins and may modulate CRP levels similar to that of chokeberries.

Studies examining the absorption of anthocyanins in blackberries [117] and blueberries [140, 145] have shown that anthocyanins from these two berries greatly differ in recovery in serum and urine after ingestion, and that blackberry anthocyanins are better absorbed (0.16%) [117] compared to blueberry anthocyanins (0.002-0.003%) [140]. While we did not examine the recovery of anthocyanins in biological fluids or serum antioxidant status following consumption of blackberries or blueberries, there was an amount of flavonoids that have shown to improve CVD risk factors in previous studies. Naruszewicz [144] and colleagues observed a significant reduction in CRP levels in postmenopausal women following 6 weeks of daily supplementation of 255 mg/day flavonoid extract from chokeberries. Blackberries and blueberries provided daily flavonoid amounts of 430 and 760 mg for 3 months.

Lack of positive findings from this study compared to previous literature on flavonoid intake could be due to the specific flavonoids. Furthermore, studies have shown that the most commonly consumed foods with highest flavonoid content are teas, apples, onions, and broccoli [19, 105]. We did not observe any effects after three months treatment of blackberries or blueberries on CVD risk factors in postmenopausal smokers. This may be attributed to the small sample size of treatment groups, dose of treatment, or particular population.

**Limitations and Future Research**

The main limitations of this study include not having premenopausal control or a nonsmoker treatment group. This made it difficult to compare the effects of antioxidant-rich berries in a population that may be at lower risk for CVD. Furthermore, the potency of
antioxidant-rich foods is dependent upon absorption and bioavailability in vivo, which were not measured in this current study. Type of anthocyanin [113], composition of gut microflora [114], and ingestion with other foods, such as those containing fat [14] contributes substantially to availability of anthocyanins. Future studies examining the effects of antioxidant-rich foods in reducing CVD risk factors in a population should investigate bioavailability, as this can vary person-to-person and may present as confounding in human studies. Furthermore, we recruited a total of sixty-five participants for this study and concluded the three-month study with forty-five participants; of which we lost seven nonsmoker controls, ten smoker controls, two smokers + blackberries, and one smoker + blueberries participants. Therefore, our group sizes were relatively small and did not allow us to have strong statistical power.
REFERENCES


23. Miller VT: Effects of Estrogen or Estrogen/Progestin Regimens on Heart-Disease Risk-Factors in Postmenopausal Women - the Postmenopausal Estrogen/Progestin


# TABLES

**Table 4. Proximate Analysis of Freeze-Dried Blackberries & Blueberries**

<table>
<thead>
<tr>
<th></th>
<th>Blackberries (%)</th>
<th>Blueberries (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Carbohydrates</td>
<td>87.3</td>
<td>94.1</td>
</tr>
<tr>
<td>Crude Fiber</td>
<td>27.7</td>
<td>12.5</td>
</tr>
<tr>
<td>Protein</td>
<td>6.4</td>
<td>3.6</td>
</tr>
<tr>
<td>Fat</td>
<td>6.3</td>
<td>2.3</td>
</tr>
</tbody>
</table>

Data represents the mean percentage. Upon arrival, blackberries and blueberries were analyzed in duplicates by the Central Analytical Laboratory (University of Arkansas Poultry Science Center; Fayetteville, AR). Percent carbohydrates was calculated using the following: 100 – percent protein – percent fat.
Table 5. Flavonoid Content of Freeze-Dried Blackberries

<table>
<thead>
<tr>
<th>Flavonoid Compound</th>
<th>Identification</th>
<th>Mean mg/100 g</th>
<th>SEM mg/100 g</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anthocyanins</td>
<td>Cyanidin 3-glucoside</td>
<td>549.1</td>
<td>9.7</td>
</tr>
<tr>
<td></td>
<td>Cyanidin 3-rutinoside</td>
<td>11.7</td>
<td>2.0</td>
</tr>
<tr>
<td></td>
<td>Cyanidin 3-xyloside</td>
<td>22.1</td>
<td>1.2</td>
</tr>
<tr>
<td></td>
<td>Cyanidin 3-malonylglucoside</td>
<td>17.0</td>
<td>0.9</td>
</tr>
<tr>
<td></td>
<td>Cyanidin 3-dioxalylglucoside</td>
<td>31.4</td>
<td>0.9</td>
</tr>
<tr>
<td></td>
<td><strong>Total anthocyanins</strong></td>
<td><strong>631.3</strong></td>
<td><strong>8.7</strong></td>
</tr>
<tr>
<td>Flavonols</td>
<td>Quercetin 3-rutinoside</td>
<td>29.3</td>
<td>0.1</td>
</tr>
<tr>
<td></td>
<td>Quercetin 3-galactoside</td>
<td>11.6</td>
<td>0.1</td>
</tr>
<tr>
<td></td>
<td>Quercetin 3-glucoside</td>
<td>4.3</td>
<td>0.1</td>
</tr>
<tr>
<td></td>
<td>Quercetin 3-glucuronide</td>
<td>4.5</td>
<td>0.3</td>
</tr>
<tr>
<td></td>
<td>Quercetin-3-O-[6&quot;-(3-hydroxy-3methylglutaroyl)]-b-galactoside</td>
<td>12.0</td>
<td>0.2</td>
</tr>
<tr>
<td></td>
<td>Quercetin 3-glucosylpentoside</td>
<td>2.0</td>
<td>0.1</td>
</tr>
<tr>
<td></td>
<td>Quercetin 3-oxalylpentoside</td>
<td>2.5</td>
<td>0.1</td>
</tr>
<tr>
<td></td>
<td>Quercetin 3-rhamnoside</td>
<td>3.2</td>
<td>0.1</td>
</tr>
<tr>
<td></td>
<td>Unidentified</td>
<td>3.1</td>
<td>0.1</td>
</tr>
<tr>
<td></td>
<td>Quercetin</td>
<td>1.9</td>
<td>0.1</td>
</tr>
<tr>
<td></td>
<td><strong>Total flavonols</strong></td>
<td><strong>74.4</strong></td>
<td><strong>0.5</strong></td>
</tr>
<tr>
<td>Ellagitannins</td>
<td>Lambertianin C isomer</td>
<td>99.3</td>
<td>2.6</td>
</tr>
<tr>
<td></td>
<td>Sanguin H-6/ lambertianin A</td>
<td>111.8</td>
<td>0.3</td>
</tr>
<tr>
<td></td>
<td>Galloyl-bis-HHDP glucose isomer</td>
<td>13.0</td>
<td>0.1</td>
</tr>
<tr>
<td></td>
<td>Ellagic acid</td>
<td>24.4</td>
<td>1.6</td>
</tr>
<tr>
<td></td>
<td><strong>Total ellagitannins</strong></td>
<td><strong>248.8</strong></td>
<td><strong>1.6</strong></td>
</tr>
<tr>
<td><strong>Total flavonoids</strong></td>
<td></td>
<td><strong>=954.5 mg/100 g</strong></td>
<td></td>
</tr>
</tbody>
</table>

Data represents mean ± SEM. Analysis was performed in triplicates by high-performance liquid chromatography by a previously outlined method [118] and confirmed by mass spectroscopy.
Table 6. Flavonoid Content of Freeze-Dried Blueberries

<table>
<thead>
<tr>
<th>Flavonoid Compound</th>
<th>Identification</th>
<th>Mean mg/100 g</th>
<th>SEM mg/100 g</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Anthocyanins</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cyanidin 3-arabinoside</td>
<td></td>
<td>13.9</td>
<td>1.6</td>
</tr>
<tr>
<td>Cyanidin 3-galactoside</td>
<td></td>
<td>21.0</td>
<td>1.9</td>
</tr>
<tr>
<td>Cyanidin 3-glucoside</td>
<td></td>
<td>14.7</td>
<td>1.0</td>
</tr>
<tr>
<td>Delphinidin 3-acetylglucoside</td>
<td></td>
<td>36.4</td>
<td>1.8</td>
</tr>
<tr>
<td>Delphinidin 3-arabinoside</td>
<td></td>
<td>153.6</td>
<td>13.1</td>
</tr>
<tr>
<td>Delphinidin 3-galactoside</td>
<td></td>
<td>185.4</td>
<td>15.8</td>
</tr>
<tr>
<td>Delphinidin 3-glucoside</td>
<td></td>
<td>119.9</td>
<td>8.9</td>
</tr>
<tr>
<td>Malvidin 3-acetylglucoside</td>
<td></td>
<td>51.4</td>
<td>2.0</td>
</tr>
<tr>
<td>Malvidin 3-arabinoside</td>
<td></td>
<td>154.5</td>
<td>13.2</td>
</tr>
<tr>
<td>Malvidin 3-galactoside</td>
<td></td>
<td>194.8</td>
<td>18.7</td>
</tr>
<tr>
<td>Malvidin 3-glucoside</td>
<td></td>
<td>151.8</td>
<td>11.2</td>
</tr>
<tr>
<td>Peonidin 3-arabinoside</td>
<td></td>
<td>17.6</td>
<td>1.9</td>
</tr>
<tr>
<td>Peonidin 3-galactoside</td>
<td></td>
<td>20.6</td>
<td>1.5</td>
</tr>
<tr>
<td>Petunidin 3-acetylglucoside</td>
<td></td>
<td>24.6</td>
<td>1.0</td>
</tr>
<tr>
<td>Petunidin 3-arabinoside</td>
<td></td>
<td>85.9</td>
<td>8.4</td>
</tr>
<tr>
<td>Petunidin 3-galactoside</td>
<td></td>
<td>106.2</td>
<td>9.6</td>
</tr>
<tr>
<td>Petunidin 3-glucoside</td>
<td></td>
<td>96.8</td>
<td>8.4</td>
</tr>
<tr>
<td><strong>Total anthocyanins</strong></td>
<td></td>
<td>1449.2</td>
<td>119.7</td>
</tr>
<tr>
<td><strong>Flavonols</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Myricetin 3-galactoside/glucoside</td>
<td></td>
<td>13.0</td>
<td>0.6</td>
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<tr>
<td>Quercetin 3-acetylRhamnoside</td>
<td></td>
<td>5.5</td>
<td>0.5</td>
</tr>
<tr>
<td>Quercetin 3-galactoside</td>
<td></td>
<td>18.9</td>
<td>2.3</td>
</tr>
<tr>
<td>Quercetin 3-glucoside</td>
<td></td>
<td>5.4</td>
<td>0.7</td>
</tr>
<tr>
<td>Quercetin 3-rutinoside</td>
<td></td>
<td>3.6</td>
<td>0.3</td>
</tr>
<tr>
<td>Unidentified 1</td>
<td></td>
<td>6.0</td>
<td>0.3</td>
</tr>
<tr>
<td>Unidentified 2</td>
<td></td>
<td>6.0</td>
<td>0.5</td>
</tr>
<tr>
<td>Unidentified 3</td>
<td></td>
<td>0.5</td>
<td>0.0</td>
</tr>
<tr>
<td>Unidentified 4</td>
<td></td>
<td>12.3</td>
<td>1.5</td>
</tr>
<tr>
<td>Unidentified 5</td>
<td></td>
<td>4.0</td>
<td>0.3</td>
</tr>
<tr>
<td>Unidentified 6</td>
<td></td>
<td>4.4</td>
<td>0.1</td>
</tr>
<tr>
<td><strong>Total flavonols</strong></td>
<td></td>
<td>79.8</td>
<td>6.8</td>
</tr>
<tr>
<td><strong>Other polyphenols</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Chlorogenic acid</td>
<td></td>
<td>162.1</td>
<td>4.2</td>
</tr>
<tr>
<td>Total other polyphenols</td>
<td></td>
<td>162.1</td>
<td>4.2</td>
</tr>
<tr>
<td><strong>Total flavonoids</strong></td>
<td></td>
<td>≈1691.1 mg/100 g</td>
<td></td>
</tr>
</tbody>
</table>

Data represents mean mg/100g ± SEM. Analysis was performed in triplicates by high-performance liquid chromatography by a previously outlined method [118].
Table 7. Effects of Smoking on CVD Risk Factors

<table>
<thead>
<tr>
<th></th>
<th>Nonsmoker (n=14)</th>
<th>Smoker (n=31)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean</td>
<td>Mean</td>
<td></td>
</tr>
<tr>
<td>Age (years)</td>
<td>57.2 1.0</td>
<td>55.2 0.7</td>
</tr>
<tr>
<td>Years postmenopausal</td>
<td>8.0 0.8</td>
<td>7.0 0.5</td>
</tr>
<tr>
<td>Cigarettes/day (#)</td>
<td>-- --</td>
<td>17 2</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>25.3 1.2</td>
<td>26.5 0.9</td>
</tr>
<tr>
<td>Systolic BP (mmHg)</td>
<td>110 3</td>
<td>115 4</td>
</tr>
<tr>
<td>Diastolic BP (mmHg)</td>
<td>71 3</td>
<td>67 3</td>
</tr>
<tr>
<td>Glucose (mg/dL)</td>
<td>96 3</td>
<td>97 1</td>
</tr>
<tr>
<td>Hemoglobin A1c (%)*</td>
<td>5.8 0.1</td>
<td>5.6 0.1</td>
</tr>
</tbody>
</table>

*Data analysis was run on baseline values. *p=0.0468. BMI = body mass index; BP = blood pressure.

Table 8. Effect of Smoking on Dietary Intake

<table>
<thead>
<tr>
<th></th>
<th>Nonsmoker (n=14)</th>
<th>Smoker (n=30)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean</td>
<td>Mean</td>
<td></td>
</tr>
<tr>
<td>Calories (kcal)</td>
<td>2042 179</td>
<td>1950 113</td>
</tr>
<tr>
<td>Total Fat (g)</td>
<td>81 9</td>
<td>82 5</td>
</tr>
<tr>
<td>Saturated Fat (g)</td>
<td>27 3</td>
<td>27 2</td>
</tr>
<tr>
<td>PUFA (g)</td>
<td>17 3</td>
<td>16 1</td>
</tr>
<tr>
<td>MUFA (g)</td>
<td>32 4</td>
<td>32 2</td>
</tr>
<tr>
<td>Cholesterol (mg)</td>
<td>303 38</td>
<td>323 66</td>
</tr>
<tr>
<td>Sodium (mg)</td>
<td>2155 170</td>
<td>2040 155</td>
</tr>
<tr>
<td>Potassium (mg)</td>
<td>3971 306</td>
<td>3556 184</td>
</tr>
<tr>
<td>Carbohydrates (g)</td>
<td>239 24</td>
<td>201 14</td>
</tr>
<tr>
<td>Total Fiber (g)*</td>
<td>28 2</td>
<td>21 2</td>
</tr>
<tr>
<td>Protein (g)</td>
<td>91 7</td>
<td>88 7</td>
</tr>
</tbody>
</table>

*Data analysis was run on baseline values. *p=0.0166. PUFA = polyunsaturated fatty acids; MUFA = monounsaturated fatty acids. A smoker participant was removed from analysis due to outlier values for calories, carbohydrates, and total fiber.
Table 9. Effects of Berries on BMI, Blood Pressure, Blood Glucose, and HbA1c

<table>
<thead>
<tr>
<th></th>
<th>Nonsmoker Control (n=14)</th>
<th>Smoker Control (n=12)</th>
<th>Smoker + Blackberries (n=6)</th>
<th>Smoker + Blueberries (n=13)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean % SEM %</td>
<td>Mean % SEM %</td>
<td>Mean % SEM %</td>
<td>Mean % SEM %</td>
</tr>
<tr>
<td>BMI</td>
<td>0.60 1.85</td>
<td>0.16 1.07</td>
<td>2.89 1.44</td>
<td>-0.31 0.97</td>
</tr>
<tr>
<td>Systolic BP</td>
<td>-4.51 3.34</td>
<td>-4.86 3.61</td>
<td>6.93 5.11</td>
<td>-2.89 3.47</td>
</tr>
<tr>
<td>Diastolic BP</td>
<td>-3.69a 3.39</td>
<td>-2.21a 3.66</td>
<td>16.08b 5.18</td>
<td>-3.97a 3.52</td>
</tr>
<tr>
<td>Glucose</td>
<td>-0.02 3.15</td>
<td>3.85 3.40</td>
<td>0.12 4.80</td>
<td>1.45 3.40</td>
</tr>
<tr>
<td>HbA1c</td>
<td>-0.27 5.8</td>
<td>-0.02 5.18</td>
<td>-2.3 2.70</td>
<td>0.40 4.98</td>
</tr>
</tbody>
</table>

Data represents percent change from baseline. Values in a row not connected by a common letter are significantly different (p<0.05). A positive value indicates an increase and a negative value indicates a decrease. BMI = body mass index; BP = blood pressure; HbA1c = hemoglobin A1c.

Table 10. Effect of Berries on Dietary Intake

<table>
<thead>
<tr>
<th></th>
<th>Nonsmoker Control (n=14)</th>
<th>Smoker Control (n=11)</th>
<th>Smoker + Blackberries (n=6)</th>
<th>Smoker + Blueberries (n=13)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean % SEM %</td>
<td>Mean % SEM %</td>
<td>Mean % SEM %</td>
<td>Mean % SEM %</td>
</tr>
<tr>
<td>Energy</td>
<td>3.91 8.45</td>
<td>7.50 9.13</td>
<td>19.20 12.91</td>
<td>1.57 8.77</td>
</tr>
<tr>
<td>Total Fat</td>
<td>8.00 10.41</td>
<td>7.38 11.25</td>
<td>17.37 15.91</td>
<td>-3.23 10.81</td>
</tr>
<tr>
<td>Saturated Fat</td>
<td>2.42 11.59</td>
<td>8.35 12.51</td>
<td>14.45 17.70</td>
<td>-6.01 12.02</td>
</tr>
<tr>
<td>PUFA</td>
<td>17.45 14.38</td>
<td>1.93 15.53</td>
<td>28.00 21.96</td>
<td>10.74 14.92</td>
</tr>
<tr>
<td>MUFA</td>
<td>12.06 11.39</td>
<td>13.11 12.30</td>
<td>16.53 17.40</td>
<td>-1.72 11.82</td>
</tr>
<tr>
<td>Cholesterol</td>
<td>-9.50 10.56</td>
<td>3.71 11.41</td>
<td>12.18 16.14</td>
<td>-5.67 10.96</td>
</tr>
<tr>
<td>Potassium</td>
<td>-2.93 14.67</td>
<td>-38.31 16.60</td>
<td>23.66 22.40</td>
<td>-18.89 15.22</td>
</tr>
<tr>
<td>Carbohydrates</td>
<td>8.85 10.37</td>
<td>17.81 11.20</td>
<td>35.88 15.84</td>
<td>18.14 10.76</td>
</tr>
<tr>
<td>Total Fiber</td>
<td>8.42 24.74</td>
<td>18.87 26.72</td>
<td>49.30 37.79</td>
<td>80.96 25.67</td>
</tr>
<tr>
<td>Protein</td>
<td>-2.92 8.73</td>
<td>0.94 9.43</td>
<td>4.44 13.34</td>
<td>-2.11 9.06</td>
</tr>
</tbody>
</table>

Data represents percent change from baseline. A positive value indicates an increase and a negative value indicates a decrease. Kcal = kilocalories; MUFA = monounsaturated fatty acids; PUFA = polyunsaturated fatty acids. One smoker control was eliminated due to extreme outliers for energy, total fat, carbohydrates, and total fiber.
Figure 2. Study Design

Recruiting participants

| Non-smoker Control | Smoker Control | Smoker + Blackberries | Smoker + Blueberries |

Baseline and final visits; 8-12 hour overnight fast

Height, weight, blood pressure, venous blood draw, food frequency questionnaire

Blood analyses and data organization

Statistical analyses of baseline data and percent change following treatment
Bars represent mean ± SEM from baseline data. Groups of bars that do not share a common letter are significantly different ($p<0.05$). TC = total cholesterol; LDL-C = low-density lipoprotein cholesterol; HDL-C = high-density lipoprotein cholesterol.
Figure 4. Effects of Smoking on Inflammation & Oxidative Stress

Bars represent mean ± SEM from baseline data. Non-smoker: n=14; Smoker: n=31. hsCRP = high-sensitivity C-reactive protein; TBARS = thiobarbituric acid reactive substances; MDA = malondialdehyde.
Data represents mean percent change ± SEM. TC = total cholesterol; LDL-C = low-density lipoprotein cholesterol; HDL-C = high-density lipoprotein cholesterol.
Figure 6. Effects of Berries on Inflammation & Oxidative Stress

Data represent mean percent change ± SEM. Nonsmoker control: n=14; Smoker control: n=12; Smoker + Blackberries: n=6; Smoker + Blueberries: n=13. hsCRP = high-sensitivity C-reactive protein; TBARS = thiobarbituric acid reactive substances.
MEMORANDUM

TO: Latha Devareddy  
    Ro DiBrezzo

FROM: Ro Windwalker  
      IRB Coordinator

RE: New Protocol Approval

IRB Protocol #: 08-09-067

Protocol Title: The Role of Anti-Oxidant Rich Berries in Prevention of Smoking-Induced Bone Loss in Postmenopausal Women

Review Type: ☑ EXPEDITED ☐ EXEMPT ☐ FULL IRB

Approved Project Period: Start Date: 09/11/2008  Expiration Date: 09/10/2009

Your protocol has been approved by the IRB. Protocols are approved for a maximum period of one year. If you wish to continue the project past the approved project period (see above), you must submit a request, using the form Continuing Review for IRB Approved Projects, prior to the expiration date. This form is available from the IRB Coordinator or on the Compliance website (http://www.uark.edu/admin/rssinfo/compliance/human-subjects/index.html). As a courtesy, you will be sent a reminder two months in advance of that date. However, failure to receive a reminder does not negate your obligation to make the request in sufficient time for review and approval. Federal regulations prohibit retroactive approval of continuation. Failure to receive approval to continue the project prior to the expiration date will result in Termination of the protocol approval. The IRB Coordinator can give you guidance on submission times.

If you wish to make any modifications in the approved protocol, you must seek approval prior to implementing those changes. All modifications should be requested in writing (email is acceptable) and must provide sufficient detail to assess the impact of the change.

If you have questions or need any assistance from the IRB, please contact me at 120 Ozark Hall, 5-2208, or irb@uark.edu.

The University of Arkansas is an equal opportunity/affirmative action institution.
INFORMED CONSENT

Title: The Role of Antioxidant Rich Berries in Prevention of Smoking-Induced Bone Loss in Postmenopausal Women

Description: This is a research study with two primary objectives:

1) To evaluate the extent to which smoking aggravates bone loss in postmenopausal women, and

2) Determine the role of antioxidant-rich berries such as blueberries and blackberries in prevention of this smoking-induced bone loss. The specific aims of the study are as follows:

Aim 1: To determine the extent to which smoking aggravates postmenopausal bone loss and to assess the bone protective effects of blueberries and blackberries by measuring:
   (a) Bone mineral density and bone mineral content of lumbar spine (L1-L4), whole body, hip, and forearm
   (b) Two biomarkers of bone formation (serum bone-specific alkaline phosphatase, ALP, BSAP and osteocalcin, OC) and bone resorption (urinary deoxypyridinoline, Dpd and helical peptide, HP).

Aim 2: To identify potential modes by which these dried fruits modulate bone metabolism by assessing:
   (a) urinary calcium and phosphorus excretion
   (b) serum and urinary F₂-isoprostanes as well as other serum assays reflective of antioxidant properties including superoxide dismutase, glutathione peroxidase, and glutathione reductase.

As a participant in this study, you will fill out a quick Food Frequency Questionnaire (FFQ) to help assess nutrition indices known to influence skeletal mass. These include dietary intake of minerals such as calcium, magnesium, and boron, vitamins D and K, protein, and fiber as well as relevant medical history and lifestyle variables. You may be recruited for the study if you are postmenopausal woman with mild to moderate bone loss as will be determined by your bone scan using the dual energy x-ray absorptiometry (DEXA). For the scan, you will be asked to lie still on the DEXA table while the DEXA machine moves over you. This painless procedure is simple, non-invasive and takes about 15 minutes to complete. The amount of radiation used by the DEXA machine is about one eighth what you would receive from a chest x-ray. You will then be assigned to one of the four treatment groups.
These groups will be

I. Non-smoking controls,
II. Postmenopausal women who smoke daily,
III. Postmenopausal women who smoke daily - assigned to eat 45 grams of dried blueberries and
IV. Postmenopausal women who daily - assigned to eat 45 grams of dried blackberries.

The duration of treatment will be for a period of nine months, a period sufficient to see changes in bone mineral density (BMD) and bone mineral content (BMC). After this period, your BMD and BMC will be assessed again using the DEXA machine. Fasting venous blood (20 ml, which is approximately 4 teaspoons) will be drawn for serum analyses will be collected between 8:00-10:00 AM by a phlebotomist on designated dates at baseline, 3, 6, and 9, months. You will also be instructed on how to collect second void urine samples on the same dates before your visit to the department at baseline, 3, 6, and 9.

**Duration of Involvement:** Approximately 9 months

**Number of participants:** Approximately 60 postmenopausal women will participate in this study.

**Risks and Benefits:** A small risk associated with the use of the DEXA machine is the exposure to a small amount of ionizing radiation which is not known to have side effects and no radiation remains in a patient's body after examination. The amount received during a DEXA test is about the same as six (6) days of normal background radiation in Northwest Arkansas. The great benefits include: free accurate and vital health information from the DEXA bone density testing. This is the most accurate method available for the diagnosis of osteoporosis and is also considered an accurate estimator of fracture risk. The risk associate with blood is bruising. Blood will be drawn by a certified phlebotomist who will strictly follow the protocol. Bruising can be prevented by keeping pressure on the site for several minutes after the needle is withdrawn without bending their elbow.

**Voluntary Participation:** Participation in the research project is voluntary. You are free to deny or withdraw from the study at any time if you so desire. Withdrawing from the study or not participating will have no repercussions.

**Removal from the Research Project:** The principal investigator (P.I) or the sponsor may also remove you from the project at any time and for any reason. Based on the assessment of the P.I., some of the reasons that you might be removed from the project are, but are not limited to the following:

- If you are not following instructions of your P.I. or her assistants
- If the study is terminated or
- For any reason at the discretion of the investigator

If you are removed from the project for any reason, your P.I. will ask you to have a final evaluation. This evaluation could include any of the assessments/tests previously mentioned in this document and any other procedures that the project P.I. feels are medically necessary. You may also be asked questions about your experience with the project.
Confidentiality: You will be assigned a code number, and this will be the only information used for tracking purposes. Your testing information will be accessed only by persons directly involved in the testing and will be stored in a locking filing cabinet. If your bone density score is greater than 1.5 standard deviations below the norm for your age, gender, and race, you will be alerted to address the risk for osteoporosis. Any other information used in this study will be reported as group data without identifying information.

Compensation: US $ 25.00 per visit. If you develop health problems during the course of the study, University of Arkansas will not provide compensation and will not provide medical treatment without charge for any medical charges as a result of this research investigation.

Inclusion Criteria: Healthy women 1 to 5 years postmenopausal, whose lumbar spine BMD t-score is between 0.5 and 2 SD below the mean, and not on HRT and/or other pharmacological agents known to affect bone, will be enrolled in this proposed study. The subjects will be postmenopausal women (i.e. follicle stimulating hormone; FSH >40 IU/mL) who have not been on HRT or other osteoporosis related medications for at least six months prior to the initiation of the study. Subjects who meet the inclusion criteria will be considered for the study regardless of ethnicity and race.

Exclusion Criteria: The subjects who will be recruited for the study should not be receiving endocrine (e.g., prednisone, other glucocorticoids) or neuroactive (e.g. dilantin, phenobarbital) drugs or any drugs known to influence bone and calcium metabolism. Women whose BMD t-score at any site falls below 2.5 SD of the mean will be excluded from the study and referred to their primary care physician. Furthermore, subjects treated with calcitonin, bisphosphonates, raloxifene, sodium fluoride, anabolic agents, e.g. PTH and growth hormone, or steroids for less than 6 months prior to the start of the study will be excluded. In addition, subjects with metabolic bone disease, renal disease, a history of urolithiasis, cancer, cardiovascular disease, diabetes mellitus, respiratory disease, gastrointestinal disease, liver disease, or other chronic diseases will be excluded. Women with endometriosis, pelvic inflammatory disease, endometrial polyps, and significant leiomyomata uteri will also be excluded from the study. Also women who regularly consume blueberries or blackberries will not be accepted into the study.

Right to Withdraw: You are free to refuse to participate in the study and to withdraw at any time. Your decision to withdraw will bring no penalty to you.

Who do I call if I have questions?

The study: Dr. Latha Devareddy at 479-575-4474

A study-related injury: Dr. Latha Devareddy at 479-575-4474

My rights as a person in the study: Washington Regional Medical Center Institutional Review Board at 1125 N. College Ave, Fayetteville, AR 72703 (479) 713-7667 or (479) 463-1000.
Informed Consent: I, ____________________________________________, have read the description of this program, including the purpose of the program, the procedures to be used, the potential risks and side effects, the confidentiality, as well as the option to withdraw from the program at any time. The investigator has explained each of these items to me. The investigator has answered all of my questions regarding the program, and I understand what is involved. My signature below indicates that I freely agree to participate in this program and that I have received a copy of this agreement if desired from the investigator.

I will receive a copy of this signed consent if desired.

Signature:  
By signing this consent form, I have not waived any of the legal rights, which I otherwise would have as a participant in a research study

_________________________________________  ___________________________________________  ______
Participant's Signature                  Participant's Printed Name                  Date

_________________________________________  ___________________________________________  ______
Legal Representative Signature                Legal Representative Printed Name            Date
(If necessary)

IF SIGNED BY A PERSONAL REPRESENTATIVE OF THE INDIVIDUAL, DESCRIBE THE REPRESENTATIVE’S LEGAL AUTHORITY TO ACT ON BEHALF OF THE INDIVIDUAL (e.g. father):
_________________________________________
_________________________________________
_________________________________________

_________________________________________  ___________________________________________  ______
Person Obtaining Consent Signature          Obtaining Consent Printed Name              Date
Subject ID: ____________________  Interviewer: ____________________  Date______________

HEALTH AND MEDICAL HISTORY QUESTIONNAIRE

Age________ Height_______ Weight_______ BMI__________

I.  Medical History

A.  Skeletal Health

Personal history of skeletal disorders:

1. Not known ____________________
2. Yes: uncontrolled ________________
3. Yes: Medications ________________
4. Yes: Exercise program ________________
5. Yes: Modified diet ________________
6. Yes: Surgery ________________
7. Yes: Combined program ________________
Give details ______________________________________________________
Type of Medication(s)_____________________________________________
Current dosage ________________ Years taken _________________________
How does this condition affect your activity?

Family history of skeletal disorders:

1. None _____
2. One parent _____
3. Both parents _____
4. One close relative _____
5. More than one close relative _____

Relative(s) ______________________________________________________
Comments: _______________________________________________________

B. Cardiovascular Function

Personal history of cardiovascular disease:

1. Not known ________________
2. Yes: uncontrolled ________________
3. Yes: Medications ________________
4. Yes: Exercise program ________________
5. Yes: Modified diet ________________
6. Yes: Surgery ________________
7. Yes: Combined program __________
   Give details ______________________________________________________
   Type of Medication(s)______________________________________________
   Current dosage ___________ Years taken ______________________________
   How does this condition affect your activity?

Family history of cardiovascular disease:
   1. None ______
   2. One parent ______
   3. Both parents ______
   4. One close relative ______
   5. More than one close relative ______

   Relative(s) ______________________________________________________
   Comments: ________________________________________________________

C. Hypertension
   1. None known __________________
   2. Yes: uncontrolled __________
   3. Yes: Medications ______________
   4. Yes: Exercise program ______________
   5. Yes: Modified diet __________________
   6. Yes: Yes: combined program __________
   7. Most recent blood pressure __________

   Explain ____________________________________________________________________
   Type of Medication(s)________________________________________
   Current dosage ________________ Years taken _________________________
   Ever taken thiazide diuretics? __________

D. Diabetes
   1. No record or indication ______
   2. In past, but not now ______
   3. Yes, well controlled ______
   4. Yes, not controlled ______

   Explain __________________________________________________________________
   Type of Medication(s)____________________________________
   Current dosage ________________ Years taken _________________________
   How does this condition affect your activity? _________________________

E. Gastrointestinal/Digestive Problems
   1. No record or indication ______
   2. In past, but not now ______
3. Yes, well controlled ____
4. Yes, not controlled ____
   Explain ____________________________________________________
Type of Medication(s)__________________________________________
Current dosage _____________________Years taken________________

Ever taken steroids (i.e., prednisone)? __________________________
Currently taking antacids? ________________________________
How does this condition affect your activity? ____________________

F. Liver Disease/ Problems
   1. No record or indication ____
   2. In past, but not now ____
   3. Yes, well controlled ____
   4. Yes, not controlled ____
   Explain ____________________________________________________
Type of Medication(s)__________________________________________
Current dosage _____________________Years taken________________
How does this condition affect your activity? ____________________

G. Respiratory Problems
   1. No record or indication ____
   2. In past, but not now ____
   3. Yes, well controlled ____
   4. Yes, not controlled ____
   Explain ____________________________________________________
Type of Medication(s)__________________________________________
Current dosage _____________________Years taken________________
How does this condition affect your activity? ____________________

H. Thyroid Disorder
   1. No record or indication ____
   2. In past, but not now ____
   Hyper? ________ Hypo? ___
   Explain ____________________________________________________
Type of Medication(s)__________________________________________
Ever taken thyroid hormones (i.e., Synthroid)? ______

I. Reproductive history
   LMP_____________; No. of children__________________________
II. Medication or Drug Use

A. Previous or Present Use of Any of the Following (Specify):

1. Anabolic steroids _____________
2. Corticosteroids or glucocorticoids _____ prednisone _____
3. Thiazide diuretics _____ Other diuretics _____
4. Vitamin D
5. Medications for bone:
   Fosamax: ___________________________
   Evista (Roloxaphin): __________________
   Miacalcin (Calcitonin): _________________
   Teriparatide (Forteo):___________________
6. Others:
   _______________________________________
   _______________________________________
   _______________________________________

B. Previous or Present Use of Alcoholic Beverages (Beer, wine, hard liquor)
   Please indicate:
   Frequency of intake (Times/week or times/month): _____
   Number of servings at a sitting: _____
   Number of years of use: _____

C. Estrogen or Hormone Replacement (ERT or HRT)
   1. Never ___ move along to section D.
   2. Yes, in past ___ at what age? ___ & How long _____

D. Currently /previously a smoker?
   _____If yes, number of cigarettes per day___________

III. Physical Activity

A. Occupational Intensity (respond to 1, 2, 3, or 4):
   1. Majority of time: Sitting ____ Standing ____ Walking ____
   2. Equal amount of time:
      Sitting and Standing ____
      Walking and Sitting ____
      Standing and Walking ____
   3. Combination: Sitting, Standing, and Walking ____
   4. Much of time: Lifting & Carrying
Vitamin and Mineral Supplement(s)

1. Do you take any vitamin or mineral supplement(s)?  Yes _____  No _____
2. If yes, please list all names of vitamins or mineral supplements and how often you take the supplement(s)?

Name __________________________  How often?  _____ per day  _____ per week
Name __________________________  How often?  _____ per day  _____ per week
Name __________________________  How often?  _____ per day  _____ per week
Name __________________________  How often?  _____ per day  _____ per week
Name __________________________  How often?  _____ per day  _____ per week
Name __________________________  How often?  _____ per day  _____ per week
Name __________________________  How often?  _____ per day  _____ per week
Name __________________________  How often?  _____ per day  _____ per week

Food Frequency Questionnaire

This questionnaire asks you about your consumption of foods and beverages over the past week, which includes the time from exactly one week ago until the last meal you had before you fill out this questionnaire. The quantity column is the amount of food/beverage and the interval is how often (day/week).

<table>
<thead>
<tr>
<th>Description</th>
<th>Amnt</th>
<th>Unit</th>
<th>Quantity</th>
<th>Day/Week</th>
</tr>
</thead>
<tbody>
<tr>
<td>Breads Cereals and Grain Products</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Whole grain breads (whole wheat, rye, pumpernickel)</td>
<td>1.00</td>
<td>sl.</td>
<td></td>
<td></td>
</tr>
<tr>
<td>White breads (french - 1 slice, burger/hot dog bun-1/2 item)</td>
<td>1.00</td>
<td>svg.</td>
<td></td>
<td></td>
</tr>
<tr>
<td>English muffin, bagel, pita bread</td>
<td>0.50</td>
<td>item</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Whole grain crackers: Triscuits, Wheat Thins, etc. (4-6 each)</td>
<td>5.00</td>
<td>item</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Other crackers: Saltines, Ritz, etc. (4-6 each)</td>
<td>5.00</td>
<td>item</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tortilla, corn, 6 inch diameter (medium)</td>
<td>1.00</td>
<td>item</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Muffins</td>
<td>1.00</td>
<td>item</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pancakes (2), waffles (1-7 inch diameter)</td>
<td>1.00</td>
<td>svg.</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Whole grain hot cereal: rolled oats, rolled wheat</td>
<td>0.50</td>
<td>c.</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Instant or quick hot cereal: cream of wheat, cream of rice</td>
<td>0.50</td>
<td>c.</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cold cereals: shredded wheat, raisin bran, or bran flakes</td>
<td>0.75</td>
<td>c.</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cold cereals: Frosted Flakes, Sugar Smacks, etc.</td>
<td>0.75</td>
<td>c.</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Rice, cooked</td>
<td>0.50</td>
<td>c.</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pasta, cooked</td>
<td>0.50</td>
<td>c.</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Fruits and Juices
<table>
<thead>
<tr>
<th>Item</th>
<th>Price</th>
<th>Unit</th>
</tr>
</thead>
<tbody>
<tr>
<td>Apple or pear, fresh, medium</td>
<td>1.00</td>
<td>item</td>
</tr>
<tr>
<td>Banana, medium</td>
<td>1.00</td>
<td>item</td>
</tr>
<tr>
<td>Orange (1 item) or grapefruit (1/2 item)</td>
<td>1.00</td>
<td>svg.</td>
</tr>
<tr>
<td>Peach (1), nectarine (1/2) or apricots (2)</td>
<td>1.00</td>
<td>svg.</td>
</tr>
<tr>
<td>Berries (in season)</td>
<td>0.75</td>
<td>c.</td>
</tr>
<tr>
<td>Cantaloupe, medium (in season)</td>
<td>0.25</td>
<td>c.</td>
</tr>
<tr>
<td>Other melon (watermelon, honeydew, casaba)</td>
<td>1.00</td>
<td>c.</td>
</tr>
<tr>
<td>Pineapple, fresh</td>
<td>0.50</td>
<td>c.</td>
</tr>
<tr>
<td>Dried fruits: raisins (2 tbsp), dates (2), prunes (2), dried apricots (4)</td>
<td>0.25</td>
<td>c.</td>
</tr>
<tr>
<td>Canned fruit or frozen fruit</td>
<td>0.50</td>
<td>c.</td>
</tr>
<tr>
<td>Orange or grapefruit juice</td>
<td>0.50</td>
<td>c.</td>
</tr>
<tr>
<td>Tomato juice or vegetable juice</td>
<td>0.50</td>
<td>c.</td>
</tr>
<tr>
<td>Other juices: apple, grape, pineapple, or cranberry</td>
<td>0.50</td>
<td>c.</td>
</tr>
<tr>
<td>Fruit drinks: lemonade, punch, Koolaid</td>
<td>0.50</td>
<td>c.</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Fats and Oils</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Vegetable oils: corn, safflower, soy, etc</td>
<td>1.00</td>
</tr>
<tr>
<td>Olive oil</td>
<td>1.00</td>
</tr>
<tr>
<td>Shortening</td>
<td>1.00</td>
</tr>
<tr>
<td>Lard</td>
<td>1.00</td>
</tr>
<tr>
<td>Margarine</td>
<td>1.00</td>
</tr>
<tr>
<td>Butter</td>
<td>1.00</td>
</tr>
<tr>
<td>Mayonnaise</td>
<td>1.00</td>
</tr>
<tr>
<td>Regular salad dressings</td>
<td>1.00</td>
</tr>
<tr>
<td>Low-calorie dressings</td>
<td>1.00</td>
</tr>
<tr>
<td>Sour cream</td>
<td>1.00</td>
</tr>
<tr>
<td>Cream cheese</td>
<td>1.00</td>
</tr>
<tr>
<td>Half &amp; Half, table cream</td>
<td>1.00</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Milk, Yogurt and Cheeses</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Skim milk or low fat milk</td>
<td>1.00</td>
</tr>
<tr>
<td>Whole milk</td>
<td>1.00</td>
</tr>
<tr>
<td>Chocolate milk</td>
<td>1.00</td>
</tr>
<tr>
<td>Yogurt</td>
<td>1.00</td>
</tr>
<tr>
<td>Cheese: cheddar, Colby, American, Monterey Jack, etc.</td>
<td>1.00</td>
</tr>
<tr>
<td>Other cheeses: Swiss, mozzarella, ricotta, string, etc.</td>
<td>1.00</td>
</tr>
<tr>
<td>Cottage cheese</td>
<td>0.50</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Vegetables</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Salads: lettuce, celery, green peppers, onions</td>
<td>1.00</td>
</tr>
<tr>
<td>Dark green leafy vegetables, raw or cooked</td>
<td>0.50</td>
</tr>
<tr>
<td>Carrots, raw or cooked</td>
<td>0.50</td>
</tr>
<tr>
<td>Tomatoes, fresh, medium</td>
<td>1.00</td>
</tr>
<tr>
<td>Food Description</td>
<td>Amount</td>
</tr>
<tr>
<td>--------------------------------------------------------------------------------</td>
<td>--------</td>
</tr>
<tr>
<td>Starchy vegetables, cooked: corn, peas, mixed vegetables</td>
<td>0.50</td>
</tr>
<tr>
<td>Other vegetables, cooked: green beans, beets, zucchini</td>
<td>0.50</td>
</tr>
<tr>
<td>Cauliflower, broccoli, brussel sprouts, cabbage</td>
<td>0.50</td>
</tr>
<tr>
<td>Winter squash, cooked: acorn, butternut, hubbard</td>
<td>0.50</td>
</tr>
<tr>
<td>White potato, baked, broiled, or mashed</td>
<td>1.00</td>
</tr>
<tr>
<td>Sweet potatoes or yams, cooked</td>
<td>0.50</td>
</tr>
</tbody>
</table>

**Beverages**

<table>
<thead>
<tr>
<th>Beverage Description</th>
<th>Amount</th>
<th>Unit</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cola drinks (1 can = 12 fl. oz)</td>
<td>12.00</td>
<td>fl.oz.</td>
</tr>
<tr>
<td>Diet cola drinks (1 can = 12 fl. oz)</td>
<td>12.00</td>
<td>fl.oz.</td>
</tr>
<tr>
<td>Non-cola drinks: 7-Up, Sprite, Slice, etc. (1 can/12 fl. oz)</td>
<td>12.00</td>
<td>fl.oz.</td>
</tr>
<tr>
<td>Diet non-cola drinks (1 can = 12 fl. oz)</td>
<td>12.00</td>
<td>fl.oz.</td>
</tr>
<tr>
<td>Coffee or tea (1 cup = 8 fl. oz)</td>
<td>8.00</td>
<td>fl.oz.</td>
</tr>
<tr>
<td>Decaffeinated coffee or teas: Sanka, herbal tea, etc.</td>
<td>8.00</td>
<td>fl.oz.</td>
</tr>
<tr>
<td>Hot chocolate or cocoa</td>
<td>1.00</td>
<td>c.</td>
</tr>
<tr>
<td>Beer (1 can = 12 fl. oz)</td>
<td>12.00</td>
<td>fl.oz.</td>
</tr>
<tr>
<td>Wine, dry or table (red, white, or blush)</td>
<td>4.00</td>
<td>fl.oz.</td>
</tr>
<tr>
<td>Liquor: vokda, whiskey, gin, rum, etc.</td>
<td>1.50</td>
<td>fl.oz.</td>
</tr>
</tbody>
</table>

**Protein Foods**

<table>
<thead>
<tr>
<th>Protein Description</th>
<th>Amount</th>
<th>Unit</th>
</tr>
</thead>
<tbody>
<tr>
<td>Legumes: lentils, pinto beans, navy beans, cooked</td>
<td>1.00</td>
<td>c.</td>
</tr>
<tr>
<td>Nuts and seeds: peanuts, almonds, sunflower seeds, etc.</td>
<td>0.25</td>
<td>c.</td>
</tr>
<tr>
<td>Peanut butter, nut butters</td>
<td>1.00</td>
<td>T.</td>
</tr>
<tr>
<td>Tofu or other meat substitutes</td>
<td>3.00</td>
<td>oz.</td>
</tr>
<tr>
<td>Beef: rib roast, steak, pot roast, veal, etc.</td>
<td>3.00</td>
<td>oz.</td>
</tr>
<tr>
<td>Beef, ground, cooked</td>
<td>3.00</td>
<td>oz.</td>
</tr>
<tr>
<td>Pork: chops, roast, ham</td>
<td>3.00</td>
<td>oz.</td>
</tr>
<tr>
<td>Lamb: chops, roast</td>
<td>3.00</td>
<td>oz.</td>
</tr>
<tr>
<td>Poultry: chicken, turkey, duck</td>
<td>3.00</td>
<td>oz.</td>
</tr>
<tr>
<td>Fish, canned with oil: tuna, sardines</td>
<td>3.00</td>
<td>oz.</td>
</tr>
<tr>
<td>Tuna, water packed</td>
<td>3.00</td>
<td>oz.</td>
</tr>
<tr>
<td>Fish, fresh or frozen, no breading: trout, halibut, sole, etc.</td>
<td>3.00</td>
<td>oz.</td>
</tr>
<tr>
<td>Shellfish: shrimp, scallops, lobster, clams</td>
<td>3.00</td>
<td>oz.</td>
</tr>
<tr>
<td>Eggs, whole, large</td>
<td>1.00</td>
<td>item</td>
</tr>
<tr>
<td>Egg substitutes or egg whites</td>
<td>0.25</td>
<td>c.</td>
</tr>
<tr>
<td>Lunch meats: bologna, salami, etc.</td>
<td>1.00</td>
<td>item</td>
</tr>
<tr>
<td>Frankfurters or sausage link (4 in x 1 1/8 in)</td>
<td>1.00</td>
<td>item</td>
</tr>
</tbody>
</table>

**Desserts and Sweets**

<table>
<thead>
<tr>
<th>Dessert Description</th>
<th>Amount</th>
<th>Unit</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cookies: chocolate chip, oatmeal, peanut butter, etc.</td>
<td>2.00</td>
<td>item</td>
</tr>
<tr>
<td>Brownies, 2 in.</td>
<td>1.00</td>
<td>item</td>
</tr>
<tr>
<td>Doughnut or sweet roll</td>
<td>1.00</td>
<td>item</td>
</tr>
<tr>
<td>Cake, 1/12 of 9 in.</td>
<td>1.00</td>
<td>sl.</td>
</tr>
</tbody>
</table>
Granola bars (1 item) or granola (1/2 cup) | 1.00 | item
---|---|---
Pie, 1/8 of whole pie | 1.00 | sl.
Gelatin, flavored | 0.50 | c.
Pudding or custard | 0.50 | c.
Ice Cream | 0.50 | c.
Ice Milk | 0.50 | c.
Sherbet | 0.50 | c.
Candy bar, chocolate bar (1 bar), M&Ms (1 pkg.) | 1.00 | item
Hard candy, gum drops, Lifesavers | 1.00 | item

### Miscellaneous Foods

<table>
<thead>
<tr>
<th>Food</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fast food - pizza</td>
<td>1.00 sl.</td>
</tr>
<tr>
<td>Fast food - hamburger or cheeseburger</td>
<td>1.00 item</td>
</tr>
<tr>
<td>Fast food - burrito or taco</td>
<td>1.00 item</td>
</tr>
<tr>
<td>Bacon</td>
<td>2.00 sl.</td>
</tr>
<tr>
<td>Popcorn, popped</td>
<td>2.00 c.</td>
</tr>
<tr>
<td>Potato chips, corn chips, tortilla chips</td>
<td>1.00 oz.</td>
</tr>
<tr>
<td>Catsup or chili sauce</td>
<td>1.00 T.</td>
</tr>
<tr>
<td>Tomato based sauce (spaghetti sauce)</td>
<td>0.50 c.</td>
</tr>
<tr>
<td>Pickles or pickle relish (1 Tbsp)</td>
<td>1.00 T.</td>
</tr>
<tr>
<td>Olives</td>
<td>5.00 item</td>
</tr>
<tr>
<td>Sauces: soy sauce, steak sauce, barbeque sauce</td>
<td>1.00 T.</td>
</tr>
<tr>
<td>Brown gravy, giblet gravy, or white sauce</td>
<td>0.25 c.</td>
</tr>
<tr>
<td>Soups, vegetable or noodle type</td>
<td>1.00 c.</td>
</tr>
<tr>
<td>Soups, cream</td>
<td>1.00 c.</td>
</tr>
<tr>
<td>Chewing gum</td>
<td>1.00 item</td>
</tr>
<tr>
<td>Sugar, honey, jam, jelly, syrups</td>
<td>1.00 T.</td>
</tr>
</tbody>
</table>

Can you think of any other food or drink that you had in the past week that was not on this form? If so, what was it? What was the amount? How many times did you have this in the past week?

Food __________________________________________
Amount ___________________________ How often? _______ per day, _______ per week

Food __________________________________________
Amount ___________________________ How often? _______ per day, _______ per week

Food __________________________________________
Amount ___________________________ How often? _______ per day, _______ per week

Food __________________________________________
Amount ___________________________ How often? _______ per day, _______ per week

Food __________________________________________
Amount ___________________________ How often? _______ per day, _______ per week