EFFECTS OF POMEGRANATE POLYPHENOL SUPPLEMENTATION ON BIOMARKERS OF OXIDATIVE STRESS AND INFLAMMATION IN ADULTS WITH TYPE 2 DIABETES VERSUS HEALTHY CONTROLS

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CHAPTER I

INTRODUCTION

Diet and lifestyle factors continue to be the cornerstone in health and prevention of chronic disease. Fruit and vegetable consumption is associated with the primary prevention of cardiovascular disease as well as improved management of diabetes (1,2). Many of the health benefits associated with diets high in fruits and vegetables are attributed to their phytochemical content (2,3). Pomegranate fruit, high in fiber and antioxidant phytochemicals, exhibits a protective effect against the development of cardiovascular disease (4-6). However, little is known about the effects of pomegranate supplementation on the oxidative stress and inflammation associated with type 2 diabetes. Thus, there exists a need to study the effects of pomegranate polyphenol supplements on biomarkers of oxidative stress and inflammation in adults with type 2 diabetes versus healthy controls.

Phytochemicals

Phytochemicals are nonnutritive, biologically active, secondary plant metabolites that are currently of intense research interest due to their proposed benefits to human health (3). Specific health benefits are related to chronic diseases such as cancer and cardiovascular

disease (7). Phytochemicals provide much of the flavor and color of the edible plants and the beverages that are derived from them. They are not considered essential nutrients like proteins, fats, carbohydrates, vitamins, minerals, and water and for this reason are often referred to as “nonnutritive” compounds (3).

Phytochemicals are found in fruits, vegetables, legumes, grains, herbs, tea and spices (8). They are thought to be produced by plants as a means of protection against dangers such as harmful ultraviolet (UV) radiation, pathogens, and herbivorous predators (3). Phytochemicals are also known to possess numerous structural variations. These variations impart unique chemical and biological properties to phytochemical classes and subclasses. Polyphenolic phytochemicals, which are phenolic rings bearing multiple hydroxyl groups, make up the largest category of phytochemicals in the plant kingdom (8). There are over 8,000 polyphenol compounds that may be further divided into a variety of classes depending on the classification system, which is based on structure. Flavonoids are one of the largest of these classes and can be broken down into several subclasses, listed in **Table 1 (**8,9). Although the flavonoid group includes many of the thousands of phytochemicals, numerous other phytochemicals are found in food. Examples of other phytochemicals are also listed in **Table 1** (8,9). Most plant foods contain multiple phytochemicals. For example, the tomato may contain as many as 10,000 different phytochemicals (8).

Table 1. Phytochemicals and their sources

Phytochemical Class	Phytochemicals	Sources
Flavonoids		
Flavonols	Quercetin, kaempferol, myricetin	Onions, tea, olive, kale, leaf lettuce, cranberry, tomato, apple, turnip, green, endive, ginkgo biloba
Flavanols	Catechins, epicatechins	Green tea, pear, wine, apple
Flavones	Apigenin, luteolin	Parsley, some cereals
Flavanones	Tangeritin, naringenin, hesperitin, hesperedin	Citrus fruits
Anthocyanidins	Cyanidin	Berries, cherries, plums, red wine
Isoflavones	Genistein, daidzein, equol	Legumes, especially soybeans, nuts, milk, cheese, flour, tofu, miso, soy sauce
Carotenoids	β-carotene, α-carotene, lutein, lycopene	Tomato, pumpkin, squash, carrot, watermelon, papaya, guava
Lignans	Secoisolariciresinol, mataresinol	Berries, flaxseed/oils, nuts, rye bran
Glucosinolates	Glucobrassicin, gluconapin, sinigrin, glucoiberin	Cruciferous vegetables: Broccoli, cabbage, Brussel sprouts, mustard, watercress
Isothiocyanates	Allylisothiocyanates, indoles, sulforaphane	Cruciferous vegetables (see above)
Terpenes	Limonene, carvone	Citrus fruits, cherries, ginkgo biloba
Phenolic Acid		
Hydroxycinnamic acids	Caffeic, ferulic, chlorogenic, neochlorogenic, curcumin	Blueberry, cherry, pear, apple, orange, grapefruit, white potato, coffee bean, St. John's Wort, echinacea
Hydroxybenzoic acids	ellagic, gallic	Raspberry, strawberry, grape juice
Phytosterols	β-sitosterol, campesterol, stigmasterol	Vegetable oils (soy, rapeseed, corn, sunflower)
Tannins		
Condensed	Catechin, epicatechin polymers	Lentils, black-eyed peas, dark and light grapes, red wine, white wine, apple juice
Hydrolyzable	ellagic, gallic	Berries, nuts
Resveratrol		Grapes, red wine, purple grape juice, peanuts

Gropper SS, Smith JL, Groff JL. Advanced nutrition and human metabolism. 5th ed. Belmont: Wadsworth; 2009.
King A, Young G. Characteristics and occurrence of phenolic phytochemicals. J Am Diet Assoc. 1999; 99:213-218.

Phytochemicals are believed to play several important roles in the body. Flavonoids are found in the cell membranes between the aqueous and lipid bilayers, where they exhibit antioxidant functions in the body by scavenging free radicals like hydroxyl, peroxyl, alkyl peroxyl and superoxide (8). Flavonoids can also terminate chain reactions. Carotenoids serve as antioxidants and are also thought to play a role in cell proliferation, growth and differentiation as well as enhancing cell-mediated immune responses. Lignans are known for their anti-estrogenic and antioxidant effects. Glucosinolates and isothiocyanates, as well as terpenes and phenolic acids such as hydroxycinnamic acid seem to have some protective effects against tumor formation associated with cancer. Phytosterols and isoflavones have been found to exhibit cholesterol-lowering effects, which may protect against heart disease. In fact, various margarines with added phytosterols are currently being marketed for use in the diets of people who have hypercholesterolemia. Tannins are high molecular weight phenolic compounds that react with mouth proteins to cause the taste sensation known as astringency (9). Tannins can be divided into two groups: condensed and hydrolysable. The condensed tannins typically accumulate in the outer layers of plants. The phytochemical class resveratrol has been associated with estrogenic and anti-esterogenic activity as well as inhibition of vascular smooth muscle cell proliferation.

As mentioned previously, the consumption of a plant based, phytochemical rich diet has been associated with reduced risk of chronic illnesses including cancers, inflammation, cardiovascular and neurodegenerative diseases. For this reason, it is of utmost importance for researchers to explore the health benefits of phytochemicals in human health.

Diabetes, Obesity and Oxidative Damage

Diabetes mellitus is characterized by high blood glucose as a result of impaired insulin secretion, action, or a combination of both (10). Insulin is a hormone produced by the β-cells of the pancreas and is necessary for the uptake of glucose by the cells for energy. As a result of impaired insulin secretion, hyperglycemia occurs and can eventually cause long-term and short-term health complications. Such complications can include: dyslipidemia (high total cholesterol, and/or triglycerides or low high density lipoprotein), hypertension (high blood pressure), nephropathy (kidney disease), retinopathy (damage to the retina of the eye), and neuropathy (loss of sensation from peripheral nerve and vessel damage). According to the National Diabetes Statistics for 2007, a total of 23.6 million people—7.8% of the United States population—have diabetes (11). Approximately 17.9 million people are diagnosed and 5.6 million people are undiagnosed.

Diabetes is classified as either type 1 or type 2 depending on impaired insulin production by the pancreas or impaired insulin utilization by cells, respectively (10). Type 1 diabetes involves the inability of the β-cells of the pancreas to produce insulin. Thus exogenous insulin is required for survival. People who suffer from type 1 diabetes tend to be lean, presenting significant weight loss in addition to other abrupt symptoms. Type 2 diabetes is the most prevalent form of diabetes, as it accounts for approximately 90% to 95% of all diagnosed cases of diabetes. Type 2 diabetes is associated with insulin resistance, regardless of the endogenous insulin level. Therefore, even if the β-cells produce a sufficient amount of insulin, the cells do not respond to the insulin and glucose remains in the blood, causing hyperglycemia. Type 2 diabetes is considered to be a progressive

disease and is often present for many years before it is diagnosed. Because type 2 diabetes develops gradually, symptoms often go unnoticed in the early stages when the condition could be reversed. Family history, obesity, intra-abdominal fat, lack of physical activity, and race or ethnicity are some of the risk factors associated with type 2 diabetes.

The prevalence of obesity and related chronic diseases including type 2 diabetes and cardiovascular disease have been increasing at a rapid rate worldwide. Obesity is characterized by excessive accumulation of adipose tissue particularly around the waistline (12). This excess accumulation of adipose tissue greatly increases the risk of a number of metabolic disorders including dyslipidemia, insulin resistance, chronic inflammation, endothelial dysfunction, and hypertension. Metabolic syndrome, which is considered a prediabetic state, is diagnosed by increased central obesity, elevated serum triglycerides, reduced HDL-cholesterol, raised blood pressure or raised fasting plasma glucose (13).

As mentioned previously, diabetes is related to impaired metabolism of glucose and insulin in the muscle, adipose tissue and liver, resulting in reduced sensitivity and secretion of insulin as well as a higher resistance to the action of insulin (12). The development of obesity-related insulin resistance has been associated with oxidative stress and sub-clinical grade inflammation. Oxidative stress is the result of an imbalance between free radical formation and antioxidant status and oxidative stress has also been shown to stimulate inflammatory responses (14,15). It appears that hyperglycemia may directly contribute to the generation of oxidative stress. Hyperglycemia induces an

inflammatory-immune response as well as oxidative stress reactions, and generation of free radicals, which underlie the etiology for the complications and mortality of obesity and type 2 diabetes (16).

A growing body of evidence supports the role of oxidative stress in the development of atherosclerosis (17), a major degenerative disease of the arteries, involving a series of inflammatory and oxidative modifications within the wall of the artery (18). The established risk factors for atherosclerosis include obesity, diabetes mellitus, hypertension, dislipidemia, smoking, aging, diets rich in saturated fat, and reduced physical activity (17, 19-26). Although the mechanism of the development of atherosclerosis is not yet fully understood, two major components appear to be involved (8):

- Cells of the immune system, mainly monocytes and macrohpages (phagocytic cells), and T lymphocytes
- Lipids and lipoproteins, of which LDL is the most important (oxidized or otherwise)

A major risk factor for cardiovascular disease is high circulating levels of cholesterol (8). Specifically, LDL has been implicated as the prime contributor to the process, as LDL is the major transporter of cholesterol in the serum. Endothelial cell injury typically begins the initiation of the atherogenic process. Injury could be the result of mechanical stress such as hypertension or a high level of oxidized LDL, which is toxic to endothelial cells. Monocytes and T-lymphocytes adhere to the affected area at an increased rate following

injury, along with the infiltration of platelets. These cells are activated as a result of concurrent penetration of the endothelium by LDL. The platelets release growth factors, which stimulate the proliferation of smooth muscle cells in the arterial media. The smooth muscle cells collect the lipid delivered to them as LDL, which transforms them into lipid-laden foam cells. Monocytes or macrophages will also take up the LDL particles and become foam cells.

Additional growth factors and chemotactic factors attract more macrophages to the site to create more foam cells as the macrophages are simulated by the uptake of LDL and release additional growth factors (8). Eventually, a plaque is formed by the proliferation of foam cells and smooth muscle cells. The plaque may enlarge enough to narrow the lumen of the artery, which restricts blood flow. The plaque cap may also rupture at some point or inflammatory stimuli may increase the expression of pro-coagulant tissue factor, which triggers thrombus formation upon the rupture of the plaque. The formation of a thrombus leads to acute coronary syndrome (19). Therefore, oxidative stress and inflammation initiate, participate in and enhance the process of atherosclerosis, and have been the principle targets of therapeutic interventions with dietary phytochemicals (4, 27-33).

A significant body of evidence indicates that acute hyperglycemia leads to oxidative stress, which is an important factor in the pathogenesis of many diabetic complications (34). The acute increases in blood glucose concentrations have been shown to cause an increase in the production of free radicals by non-enzymatic glycation and also by an imbalance in the ratio of NADH to NAD+ induced by the glucose in cells. Studies in both

healthy subjects and subjects with diabetes have provided direct evidence that induced hyperglycemia (35) or hyperglycemia resulting from meal intake increases blood glucose (35,36) and therefore can induce oxidative stress and reduce antioxidant defenses.

In another study, Ceriello et al. (1999) reported that increases in oxidative stress were significantly greater after meals that produced a greater degree of hyperglycemia (37). Hu et al. (2006) reported that chronic consumption of high glycemic index foods may lead to chronically high oxidative stress (38). According to Dembinska-Kiec et al. (2008) the depletion of antioxidants and its contribution to cardiovascular complications in diabetes is well known (12). Many studies have found a significant decrease in plasma antioxidants including: α- and γ- tocopherol, β- and α- carotene, lycopene, β-cryptoxanthin, lutein, zeaxanthin, retinol, and also ascorbic acid in diabetes and its associated complications like endothelial dysfunction and atherosclerosis (39-42). The research supporting the therapeutic use of antioxidants for the treatment and prevention of complications linked to diabetes is strong (12).

A high dietary intake of fruits, vegetables, and whole grains has been associated with a reduced risk of type 2 diabetes (1). Phytochemicals are believed to be responsible for the health benefits associated with a diet that is high in plant based foods. Based on the data from experimental models, flavonoids, carotenoids, ascorbic acid and tocopherols are the major antioxidants recommended as they have demonstrated inhibition of the production of reactive oxygen species (43, 44). However, evidence of the potential benefits of polyphenols in the regulation of cellular processes including redox control and inflammatory responses is increasing as demonstrated in animal models and cultured

cells (12). Kobayashi et al. (2000) reported that tea and several plant polyphenols inhibit α- amylase and sucrase activity, thereby decreasing postprandial glycemia (45). Research has also shown that polyphenolic anthocyanins, present in berries, may prevent type 2 diabetes and obesity (12). Results from in vitro studies suggest that anthocyanins may reduce the intestinal absorption of glucose by delaying the release of glucose during digestion.

Pomegranates

The pomegranate (*Punica granatum L.)* originated in the Middle East and was used extensively in ancient cultures for medicinal purposes for many centuries (46). Pomegranates adorned the vestments of the high priest in the ancient Hebrew tradition and the Babylonians regarded the seeds of the pomegranate as an agent of resurrection. The Persians believed that the pomegranate seeds conferred invincibility on the battlefield and the ancient Chinese believed that the seeds symbolized longevity and immortality.

Many different types of phytochemicals have been identified from various parts of the pomegranate tree, fruit and seeds (3). The major class of phytochemicals present in the pomegranate is polyphenols. The pomegranate polyphenols include flavonoids (flavonols, flavanols, and anthocyanins), condensed tannins (proanthocyanidins), and hydrolysable tannins (ellagitannins and gallotannins). Additional phytochemicals present in pomegranates include organic and phenolic acids, sterols and triterpenoids, and alkaloids.

The fruit is the major source of dietary pomegranate phytochemicals (3). Pomegranates may be consumed in a variety of ways; however, they are most popularly consumed as fresh fruit, in beverages (wine and juice), as food products (jams and jellies), and also as extracts in which they are used as botanical ingredients in herbal medicines and dietary supplements. The edible parts of the pomegranate fruit, which is approximately 50% of total fruit weight, are comprised of 80% juice and 20% seeds (6). The fresh juice contains 85% water, 10% total sugars, and 1.5% pectin, ascorbic acid, and polyphenolic flavonoids. The seeds of the pomegranate are rich in crude fibers, pectin and sugars. Fructose and glucose are present in similar amounts in pomegranate juice (PJ) and 50% of its ash content is calcium. The principle amino acids in pomegranate juice are glutamic and aspartic acids (6).

Table 2 displays the nutritional value for a whole pomegranate (47). The whole fruit weighs approximately 282 grams. Vitamin C is included in the nutritional analysis of the whole fruit; however, 8 oz. of pomegranate juice contains no vitamin C as vitamin C is very unstable and destroyed by the pasteurization process.

Table 2. Nutritional content of whole pomegranate (282 grams) based on samples of the California Wonderful ™ variety

Nutrient	Unit	1.00X1 pomegranate (4" dia)---282 grams
Energy	kcal	234
Protein	g	4.71
Fat	g	3.3
Carbohydrate	g	52.7
Fiber	g	11.3
Phosphorus	mg	102
Potassium	mg	666
Vitamin C	mg	28.8

United States Department of Agriculture. Beltsville (MD): Human Nutrition Research Center. c2008—[cited 2009 March 15]. Available from: http://www.nal.usda.gov/fnic/foodcomp/cgi-bin/list_nut_edit.pl.

Pomegranate fruit *(Punica granatum L.)* is cultivated in India, Spain, Israel, and the United States and has been rated to contain the highest antioxidant capacity in its juice form, when compared to the commonly consumed polyphenols-rich beverages in the United States (46). Gil et al. (2000) reported the antioxidant activity of PJ to be three times higher than those of red wine and green tea, based on the evaluation of the free-radical scavenging and iron-reducing capacity of the juices (48). It was also found to have one of the highest antioxidant capacities when compared to other fruit juices, such as grape, cranberry, grapefruit, blueberry or orange juice (3,49,50). Pomegranate juice contains approximately 5 mmol/L of total polyphenols in comparison to other fruit juices which contain approximately 1.3 to 4.0 mmol/L of total polyphenols (3).

Ellagitannins and anthocyanins are the principal antioxidant polyphenols in PJ (48). Anthocyanins, which are the water-soluble pigments that give pomegranate juice its bright red color, are found in the arils of the pomegranate (3). The ellagitannins are present in the peel, the membranes and piths of the fruit and account for approximately 92 % of the total antioxidant activity of pomegranate juice (3,48). The major ellagitannins present in the whole fruit are punicalagins, which can be hydrolyzed to ellagic acid (EA) and other smaller polyphenols in vivo (51). Commercial PJ obtained by pressing whole pomegranate fruit and its peels contains significant amounts of water-soluble compounds, including punicalagins. However punicalagin levels vary widely in PJ and can range from as low as 0.017 to 1.5 g/L of juice depending on the fruit cultivar as well as the processing and storage conditions (48,52).

Phytochemicals are proposed to have health benefits related to the prevention of chronic diseases such as cancer and cardiovascular disease (7). Recently research has focused on the role of antioxidant polyphenols present in the pomegranate, as pomegranate juice has one of the highest antioxidant capacities when compared to other fruit juices (3,49,50). Clinical trials thus far have focused on the role of pomegranate polyphenols in the oxidative stress associated with cardiovascular disease complications (4-6). However, research is now beginning to examine the effect of pomegranate supplementation on diabetic parameters as well (53).

Thus the objective of our study was to examine the effects of pomegranate polyphenol supplementation, in the form of a POMx™ capsule, on clinical variables (fasting blood glucose, blood pressure, and dyslipidemia [high TCs and/or high TGs or low HDL levels]) and on biomarkers of oxidative stress (oxidized LDL [ox-LDL] and malondialdehyde [MDA] and 4-hydroxynonenal [HNE]) and inflammation (C-reactive protein [CRP]) in subjects with abdominal adiposity and type 2 diabetes versus healthy controls.

The null hypotheses were that:

- Pomegranate polyphenol supplementation will not affect glucose, lipids, and biomarkers of oxidative stress and inflammation in subjects with type 2 diabetes and healthy controls.
- Pomegranate polyphenol supplementation will not affect safety parameters, such as, liver, kidney, and thyroid function tests, and complete blood cell count, in subjects with type 2 diabetes and healthy controls.

CHAPTER II

REVIEW OF LITERATURE

The pomegranate (*Punica granatum L.*), high in a variety of polyphenolic phytochemicals, has been found to exert anti-atherosclerotic, anti-hypertensive, and anti-oxidative properties in both human and animal studies (4-6). However, much of the research with pomegranates thus far has been conducted in the Middle East with pomegranate juice and limited research has been conducted in human subjects with type 2 diabetes. Type 2 diabetes is associated with oxidative stress and sub-clinical grade inflammation, thus warranting the need to further investigate the effects of pomegranate polyphenols in subjects with type 2 diabetes, which is the scope of our study.

Pomegranate Juice Clinical Trials

A two week clinical trial was conducted among ten hypertensive, non-smoking patients who were supplemented with PJ (50 ml containing 1.5 mmol total polyphenols per day, for two weeks) (5). The aim of this clinical trial was to determine the effect of PJ, which possesses anti-oxidative properties, on blood pressure and on angiotensin converting enzyme (ACE). Participants were all between the age of 62 and 77 years and had a mean blood pressure of 155 ± 7/83 ± 7 mmHg at baseline. Two participants were diabetic

(serum glucose >126 mg/dl) and two were hyperlipidemic (serum cholesterol >240 mg/dl). Adherence to protocol was confirmed by serum total polyphenols analysis. Researchers also studied the in vitro effects of PJ on serum ACE activity.

In seven of the ten hypertensive patients, ACE activity was significantly decreased by 36% after two weeks PJ consumption (5). The inhibitory effects of PJ consumption on serum ACE activity may be secondary to the antioxidant properties of PJ or a result of the direct effect of the juice's active compounds on serum ACE activity. The possible direct effect of PJ on serum ACE activity was assessed by adding increasing concentrations of PJ to human serum, which was subsequently incubated for 15 minutes at 37 ºC. Pomegranate juice exhibited a dose-dependent inhibitory effect of up to 31% on serum ACE activity. This effect could be secondary to the ability of antioxidants associated with PJ, such as complexed tannins, to inhibit ACE activity. The inhibitory effect of PJ consumption on serum ACE activity may also have been the outcome of a direct interaction of PJ constituent with the serum ACE.

Pomegranate juice was also found to have an effect on blood pressure, as there was a minimal (5%), but a significant ($p < 0.05$) reduction in systolic blood pressure (5). After two weeks of PJ consumption, mean blood pressure was 147 ± 10/82 ± 5 mmHg. These results suggest a significant inhibitory effect of PJ on serum ACE activity and a minor attenuation in blood pressure in hypertensive patients, which suggests that PJ consumption can offer protection against hypertension, a risk factor for cardiovascular disease.

Rosenblat et al. (53) investigated the effects of PJ consumption on parameters of oxidative stress in diabetic patients. Ten healthy male subjects (controls) and 10 non-insulin dependent diabetes mellitus (NIDDM) patients between the ages of 35-71 years were recruited for this study. The controls were non-smokers, history without diabetes (glucose levels below 100 mg/dL and hemoglobin A1c levels in the range of 4.8-6.2%), hypertension or coronary artery disease, and not on any medications. The duration of diabetes mellitus in the patients ranged from 4-10 years. Patients with diabetes mellitus had glucose levels above 160 mg% and hemoglobin A1c levels between 7.5-11.3%. None of the patients presented ischemic heart disease or hypercholesterolemia and none were smokers. However, 50% of the patients were hypertriglyceridemic with serum triglyceride levels ranging from 300-790 mg%. Glucophage (Metformin) was used for treatment in 80% of the patients and (Glybenclamid) Gluben was used by 50%.

The patients consumed 50 ml of pomegranate juice (containing 1.5 mmol of total polyphenols) for a three month period (53). Blood samples were collected from the diabetic patients and the controls before and after three months of PJ consumption for analysis of biochemical parameters. Also, blood samples from two healthy controls and three diabetic patients before and after PJ consumption were used for preparation of monocytes-derived macrophages.

Neither serum glucose, cholesterol, nor triglyceride levels appeared to be affected by consumption of PJ (53). The patients had higher levels of lipid peroxides (by 350%) and TBARS (by 51%) when compared to the controls. However, serum sulfhydryl (SH) group content was as much as 21% lower in patients versus the controls and paraoxonase-

1 (PON1) arylesterase activity was 23% lower. The consumption of PJ resulted in a significant reduction in serum lipid peroxides and TBARS levels by 56% and 28%, while serum SH groups and PON1arylesterase activity significantly increased by 12% and 24%, respectively.

The human monocytes-derived macrophages (HMDM) of the diabetic patients were observed to have an increased level of cellular peroxides (by 36%) and a decreased level of glutathione (by 64%) when compared to the healthy controls (53). Consumption of PJ significantly decreased cellular peroxides by as much as 71%, and increased glutathione levels by 141% in the patients' HMDM. Furthermore, the HMDM of the patients compared to the controls took up oxidized LDL (ox-LDL) at an enhanced rate by 37% and PJ consumption decreased the extent of ox-LDL cellular uptake significantly (by 39%). These results indicated that PJ consumption did not worsen diabetic parameters in the patients, and instead resulted in anti-oxidative effects on both serum and macrophages, which could likely contribute to attenuation of atherosclerosis development in patients with NIDDM.

The effects PJ on LDL atherogenic modifications including its retention, oxidation, and aggregation were examined in healthy male subjects (6). Two ex vivo studies were performed to determine the antioxidant effect of PJ. In the first study, 13 healthy men aged 20-35 years were supplemented with 50 ml PJ/d (1.5 mmol total polyphenols) for two weeks. In the second study, three subjects were given increasing doses of PJ (20-80 mL/d, equivalent to 0.54-2.16 mmol total polyphenols/d). Results from the ex vivo human studies showed that after two weeks of PJ supplementation there was a small but

significant (6%; $p < 0.01$) decrease in susceptibility to 2,2□-azobis-2-amidinopropane hydrochloride (AAPH)-induced lipid peroxidation compared with plasma obtained prior to study entry. Also, a significant (9%; $p < 0.05$) increase was observed in plasma total antioxidant status after two week consumption of PJ. Three subjects were studied further, in order to determine the effects of varying doses of PJ on AAPH-induced plasma lipid peroxidation and to analyze the ability of PJ to maintain its effect after consumption of the juice had stopped. The results indicated the inhibitory effect of PJ intake on plasma lipid peroxidation was maintained for two weeks after the PJ supplementation was discontinued.

After two weeks of PJ consumption, there was a significant (18%: $p<0.01$) increase in serum paraoxonase (6). Due to the fact that serum paraoxonase is bound to HDL, the researchers questioned whether increased serum paraoxonase activity would be associated with increased resistance of HDL to oxidation after PJ supplementation. PJ consumption was found to gradually and significantly ($p < 0.01$) increase the resistance of HDL to copper ion-induced oxidation. HDL-associated paraoxonase activity in serum is linked with the protection of LDL from oxidation. When human serum was incubated with increasing concentrations of PJ for 10 minutes at 37˚C, PJ exerted a dose dependent increase in serum paraoxonase activity by up to 33%. Such results suggest that PJ inhibits plasma LDL lipid peroxidation in vitro and that this effect is related to its capacity to scavenge free radicals and to also increase serum paraoxonase activity.

Furthermore, the susceptibility of LDL to copper-ion induced oxidation was also gradually reduced with consumption of PJ (6). A pattern of reduction in LDL aggregation

was observed in 7 of the 13 subjects; however, the mean value did not change significantly.

The effect of PJ on human platelet aggregation was assessed in vitro and ex vivo (6). Platelet-rich plasma was incubated with increasing concentrations of PJ and aggregation was induced by adding collagen. The PJ inhibited collagen-induced platelet aggregation by as much as 90% in a dose dependent manner. In the ex vivo study, collagen-induced platelet aggregation was significantly reduced by 11% ($p < 0.02$) compared with platelet aggregation before PJ consumption. Based on the results it was concluded that the antiatherogenic properties of PJ are related to its ability to inhibit lipid peroxidation in plasma and lipoproteins. Thus PJ appears to have potent antiatherogenic effects in healthy humans that may be attributable to its antioxidative properties.

Pomegranate juice consumption has become increasingly popular due to its reported benefits on human health (54). Thus research with pomegranates has focused on the effects of PJ on biomarkers of oxidative stress and inflammation. However, pomegranate extracts, which contain the major antioxidants present in pomegranates, have been developed as a botanical dietary supplement to offer a convenient alternative to consuming the bioactive polyphenols in juice form. Despite the fact that pomegranate supplements are commercially available, few studies have analyzed them for their safety and potential benefits to human health. Heber et al. (2007) was the first to analyze the safety and antioxidant activity of a pomegranate ellagitannin-enriched polyphenol extract

(POMx™) in overweight individuals with increased waist size in two pilot studies to demonstrate both the safety and efficacy of POMx™ in humans.

In the first study, the safety of POMx™ was determined in overweight subjects (54). The subjects were instructed to consume either one or two POMx™ capsules per day (1 capsule = 710 mg extract with 435 mg gallic acid equivalents [GAEs]; 2 capsules = 1420 mg extract with 870 mg GAEs). In order to maintain blinding, the one capsule per day group received one bottle of placebo and one bottle of POMx™ capsules. Participants in the two capsules per day group received two bottles of POMx™ capsules. Additionally, 7 of the 64 subjects received only one placebo during the trial to assess the incidence of adverse effects in the group receiving only the placebo. In this study, there was a formal assessment of adverse reactions and a comprehensive series of blood tests for toxicity by comparison to the control group that received a placebo. None of the subjects discontinued the study as the results of an adverse event and no serious adverse events were reported. Of the adverse events reported none were believed to be related to the supplement. Also, there were no apparent treatment-related changes of clinical significance and no laboratory values were outside the normal range in any of the chemistry, hematology, or urinalysis parameters.

In the second study, subjects consumed two POMx™ capsules daily, which provided 1000 mg of extract containing 610 mg of GAEs, in order to determine the antioxidant activity of the supplements (54). Subjects were instructed to avoid foods with strong antioxidant properties for the duration of the trial. Thiobarbituric acid reactive substances

(TBARS) in plasma were measured before and after POMx™ supplementation as a marker of antioxidant activity. There was a significant decrease ($p = 0.044$) in TBARS between baseline and four weeks. No significant differences were detected for glucose, BUN, creatinine, lipids, c-peptide, paraoxonase-1, or electrolytes or liver enzymes (aspartate amino transferase [AST] or alanine amino transferase [ALT]).

Thus these pilot studies demonstrate that a pomegranate ellagitannin-enriched polyphenol (POMx™) dietary supplement is safe for healthy human subjects in amounts up to 1420 mg/day to provide 870 mg of GAEs/day for a duration of 28 days (53). However, future studies with POMx™ capsules are needed to confirm these results as well as the effects of the POMx™ capsules in patients with chronic diseases such as type 2 diabetes.

Epidemiological Findings

Although observational data on pomegranate intake is lacking, several epidemiological studies have examined the effect of dietary patterns that are high in fruit and vegetable intake on mortality from a variety of health outcomes including obesity, hypertension and cardiovascular diseases (1,2). Such studies have found that increased consumption of fruits and vegetables may exert beneficial effects as a result of the antioxidants and other phytochemicals present in these food groups (2).

Bazzano et al. (2008) examined the association between fruit, vegetable, and fruit juice intake and the development of type 2 diabetes (1). This was a large prospective cohort study that included 71,346 female registered nurses between the ages of 38-63 years who did not have cardiovascular disease, cancer, or diabetes in 1984. The participants were

followed for a total of 18 years. A semi-quantitative food frequency questionnaire was administered every four years to obtain dietary information. Data on body mass index (BMI), physical activity, smoking status and other lifestyle factors was also collected. The diagnosis of diabetes was self-reported by the participants.

The results of this study indicate that the women with higher intakes of fruits and vegetables were older, less likely to smoke cigarettes, more likely to exercise on a regular basis and were also more likely to use hormone replacement therapy than those women who did not consume fruits and vegetables as frequently (1). Approximately 4,529 cases of type 2 diabetes were documented over the 18 year time frame.

An increase of three servings per day in total fruit and vegetable intake was not associated with the development of diabetes based on the multivariate-adjusted hazard ratio of 0.99 [95% CI 0.94-1.05] (1). An increase to three servings per day for whole fruit consumption was associated with a lower hazard of diabetes 0.82 [0.72-0.94]. A modestly lower hazard of diabetes (0.91 [0.84-0.98]) was associated with an increase of one serving per day of green leafy vegetables, while an increase of one serving per day in fruit juice intake was associated with an increased hazard of diabetes (1.18 [1.10-1.26]). These results suggest overall fruit and vegetable intake was not associated with the development of type 2 diabetes. However, there was a positive association between intake of fruit juices and incidence of type 2 diabetes, while intake of whole fruits and green leafy vegetables was inversely associated.

The positive association between the consumption of fruit juice and diabetes risk is related to the lack of fiber and other phytochemicals in juice, as well as the high sugar load (1). An important mechanism by which fruit juices could possibly contribute to the development of diabetes is related to the fast delivery of concentrated sugar without the components that are part of the whole fruit. The authors report that the observed associations between fruits and vegetables and diabetes in their study are weaker than those for cardiovascular disease. Yet if fruits and vegetables replace refined grains and white potatoes, which are associated with the increased risk of developing diabetes, the benefits of consuming fruits and vegetables regularly could be considerable.

In a cross-sectional study, the association of fruit and vegetable intake with blood C-reactive protein (CRP) concentrations and the prevalence of metabolic syndrome among female teachers between the ages of 40 and 60 years was assessed (2). All participants with a history of cardiovascular disease, diabetes, cancer, or stroke were excluded due to possible changes in diet associated with such conditions. Other exclusions included subjects who left > 70 items blank of the food-frequency questionnaire (FFQ), who reported a total daily energy intake outside the range of 800-4200kcal, and also subjects who were taking medications that would affect serum lipoprotein, blood pressure, and carbohydrate metabolism. Such exclusions resulted in a total 486 subjects eligible for participation. A validated 168-item semiquantitative FFQ was used to assess the usual dietary intake. Assessment parameters included weight, height, body mass index (BMI), waist circumference, blood samples, blood pressure, and physical activity. The mean daily intakes of fruit and vegetables were reported as 228 ±79 and 186 ± 88 g/d, respectively.

Participants in the highest quintile of fruit and vegetable intake were more physically active, less likely to be obese, and had lower anthropometric measures and lower prevalence of metabolic syndrome (2). In this study, higher intakes of fruit and vegetables were associated with an overall healthier diet; subjects with high fruit and vegetable intake also consumed less cholesterol, meat, and refined grains and more dietary fiber and whole grains. Fruit and vegetable intakes were both inversely associated with plasma CRP concentrations. After statistical control was performed for age, BMI, and waist circumference, the mean plasma CRP concentrations across increasing quintile categories for fruit were 1.94, 1.79, 1.65, 1.61, and 1.56 mg/L (P for trend < 0.01) and of vegetables were 2.03, 1.82, 1.58, 1.52, and 1.47 mg/L (P for trend < 0.01). After controlling for any potential confounders, it was determined that those subjects in the highest quintile of fruit intake had a 34% lower and those in the highest quintile of vegetable intake had a 30% lower risk of having metabolic syndrome.

The results suggested that higher intakes of fruits and vegetables were associated with a lower risk of metabolic syndrome, and that the lower risk may be attributed to lower CRP concentrations (2). Such results provide additional evidence for the hypothesis that a high intake of fruits and vegetables is associated with reduced plasma concentrations of inflammatory markers. The beneficial effect of fruits and vegetables may be related to a combination of antioxidants, fiber, potassium, magnesium, and other phytochemicals. Thus, the findings from this study are in confirmation with those of other studies which have shown protective effects of fruit and vegetable intake against mortality, cardiovascular disease, and diabetes.

The phytochemicals present in PJ and in fruits and vegetables have shown significant anti-atherosclerotic, anti-hypertensive, and anti-inflammatory effects in human subjects (1-2,5-6,53-54). Pomegranate juice appears to exhibit anti-oxidant effects on both serum and macrophages, which could likely contribute to attenuation of atherosclerosis development in patients with NIDDM (53). Pomegranate juice also demonstrated a dose-dependent inhibitory effect on serum ACE activity, which is believed to result from ability of antioxidants associated with PJ to inhibit ACE activity (5). The recent research on pomegranate polyphenols and prevention of oxidative stress is promising. However, additional research on pomegranate polyphenol supplementation in patients with type 2 diabetes is necessary to strengthen support for its cardio-protective effects.

Mechanistic Studies

Due to its potent antioxidant capacity and potential to improve chronic disease states associated with oxidative stress and inflammation the pomegranate has become of intense research interest. In mechanistic studies, PJ demonstrates potent anti-oxidative, anti-atherosclerotic, and anti-hypertensive effects indicating a potential role for pomegranate polyphenols in reducing the risk of cardiovascular disease (4,6).

Previous studies in both humans and animals afflicted with atherosclerosis indicate that regular administration of PJ produces a significant protective effect (4). These positive effects have been extended to mice treated with pomegranate fruit extract (PFE). Pomegranate fruit extract, like PJ, is rich in polyphenolic antioxidants that are known to be potent against low density lipoprotein (LDL) oxidation and atherosclerosis. In fact, the

polyphenols present in PFE have the ability to reduce the expression of oxidation-sensitive genes at the sites of perturbed shear-stress. Metabolic syndrome, which includes diabetes, dislipidemia, hypertension, and obesity, has become a clinically widespread condition associated with oxidative stress.

De Nigris et al. (2007) conducted a study to compare the influence of PFE to PJ and seed oil on the biological actions of nitric oxide (NO) and arterial function in obese Zucker rats, an animal model of metabolic syndrome (4). The rats were matched for blood pressure and serum cholesterol and received an atherogenic diet (ATH), or an ATH diet supplemented with regular PJ (ATH + PJ), pomegranate fruit extract (ATH + PFE), or seed oil (ATH + seed oil).

Acetylcholine (Ach)-induced relaxation responses were significantly increased by supplementation of PJ and PFE ($p < 0.01$) and also by seed oil supplementation ($p < 0.05$), but, to a lesser extent (4). The lipid profile was found to be similar among all groups in that PFE supplementation had no significant effect on total cholesterol and LDL cholesterol. However, data did indicate that supplementation with either PFE or PJ significantly reduced both thrombospondin (TSP-1) and cytokine TGFβ1 expression ($p < 0.05$), while seed oil supplementation only had a significant effect on expression of TSP-1 ($p < 0.05$). Interestingly, the effect of PFE was comparable to that of PJ in increasing vascular endothelial NO synthase (eNOS) expression. PJ and PFE supplementation also significantly increased plasma NO_x levels ($p < 0.05$), whereas the seed oil

supplementation produced no effect. Analysis of plasma insulin and glucose levels did not reveal any significant differences.

These results demonstrate that beneficial effects related to vascular function and inflammation in obese Zucker rats can be achieved when an atherogenic diet is supplemented with PFE. Pomegranate fruit extract is expected to possess beneficial effects similar to those exerted by PJ as a result of its high content of polyphenolic antioxidants. The chronic administration of PJ, and more recently of PFE, has been shown to minimize the proatherogenic effects induced by perturbed shear-stress. This study provides evidence that PFE supplementation may positively affect arterial reactivity, vascular expression of eNOS and NO_x levels by increasing NO production and preventing its degradation. Thus these data suggest potential clinical applications in patients with metabolic syndrome.

The effect of pomegranate juice on NO has become a topic of interest because when NO is exposed to oxidative stress, rapid destruction occurs (50). This rapid destruction is typically associated with the initiation and development of a variety of cardiovascular diseases. Nitric oxide is known to be a potent inhibitor of oxidative stress and inflammation as a result of its ability to react with and subsequently remove certain reactive oxygen species. If endothelial NO levels are decreased there is further oxidation and destruction of NO. This cycle of oxidative stress and reduced NO action can lead to atherosclerosis.

Pomegranate juice is known to be a rich source of antioxidants, thus Ignarro et al. (2006) conducted a study to compare PJ with other products (concord grape juice, red wine, white wine, blueberry juice, alcohol, vitamin C and vitamin E) that are commonly promoted as having antioxidant properties to determine their protective effects on NO when subjected to conditions of oxidative stress (50). For this study, a chemiluminescence headspace assay was developed to measure the antioxidant activity by monitoring the destruction or disappearance of NO when exposed to superoxide anions generated by pyrogallol. The results indicated that the inclusion of PJ clearly protected against the pyrogallol-elicited disappearance of NO. Pomegranate juice produced significant anti-oxidative activity with concentrations ranging from as little as 3 to 100 µl of 30-fold dilution of PJ concentrate.

Of the other juices tested, blueberry juice displayed appreciable antioxidant activity in the system observed (50). Yet the quantity required to produce the significant antioxidant activity (300 µl of undiluted juice) was greater than that required with PJ. Thus, PJ was determined to be more potent than blueberry juice in protecting NO from oxidative destruction as determined by this particular assay. Of the other juices tested at quantities of 300 µl, only the Concord grape juice and red wine exhibited significant antioxidant activity.

In the second experiment, PJ was studied to determine its ability to enhance the biological activity of NO (50). When PJ was added to cell cultures there was a noticeable increase in the proliferative action of NO in rat aortic smooth muscle cells (RASMC).

Pomegranate juice alone, in absence of NO, produced no effect on cell proliferation at concentrations ranging from 3 to 100 µl. Such results indicate the marked protective effect of PJ on the anti-proliferative action of NO in the cell medium. Of the other juices tested, the grape juice showed significant augmentation of the anti-proliferative action of NO, but only at the highest concentration tested (100 µl). The blueberry juice was effective at 30 and 100 µl; however, the effects were only approximately 50% of the effect of PJ.

Additionally, PJ was analyzed for its affect on endothelial NO synthase (eNOS) protein expression, eNOS catalytic activity, and eNOS gene promoter activity (50). It was determined that PJ did not have appreciable influence on eNOS protein expression in bovine pulmonary artery endothelial cells (BPAEC) based on results of the Western blot analysis.

The results indicated that the antioxidants present in PJ are capable of protecting NO against superoxide anion mediated destruction, and also has the ability to augment certain biological actions of NO including inhibition of vascular smooth muscle cell proliferation (50). Thus the authors conclude that PJ is rich in potent antioxidants that are associated with anti-atherosclerotic effects in both animals and humans.

In a study conducted by Aviram et al. (2001) atherosclerotic apolipoprotein E-deficient (E^0) mice were analyzed to determine the effect of PJ consumption on lipoprotein oxidation, aggregation and retention; macrophage atherogenicity; platelet aggregation;

and atherosclerosis. Thirty, six week old E^0 mice were divided in to three groups of 10 for this study (6). The three groups were given 0, 6.25, or 12.5 µL PJ (equivalent to 0, 0.175, and 0.350 µmol total polyphenols) in their drinking water per mouse per day. Blood was drawn at 6, 9, and 14 weeks of age for the plasma and LDL analysis and peritoneal macrophages and aortas were obtained at the end of the study. In order to evaluate the effect of PJ supplementation on atherosclerotic lesion progression, three week old E^0 mice were supplemented for 11 weeks with 31 µL PJ (equivalent to 0.875 µmol total polyphenols) per mouse per day. Control mice received only water.

PJ supplementation was found to have antioxidant effects when administered to E^0 mice. Plasma lipid peroxidation was markedly lower in mice that were fed PJ (6). A concentration dependent relationship was determined for this effect. Additionally, serum total antioxidant status was higher in the E^0 mice that consumed PJ than in the control mice. This effect was also concentration dependent. Susceptibility of LDL from E^0 mice to copper ion-induced oxidation was also reduced and the susceptibility of LDL to oxidation associated with the progressive increase in age was significantly attenuated by PJ consumption.

Due to the fact that LDL oxidation by the macrophages is thought to be a major event in the initial development of atherosclerosis and is associated with cellular uptake of modified lipoproteins, which ultimately contributes to macrophage accumulation and foam cell formation, macrophage atherogenicity was examined in the E^0 mice (6). Lipid peroxidation of the mouse peritoneal macrophages (MPMs) after supplementation with

PJ was 53% less than lipid peroxidation of MPMs isolated from the control mice. The atherosclerotic lesions in the control mice were found to be larger than those in the mice treated with PJ and the control mice had many more lipid-laden macrophage foam cells.

This study showed the antiatherogenic properties of PJ as related to its inhibitory effect on lipid peroxidation in plasma, in lipoproteins, and in macrophages. Also of great importance, PJ treatment significantly inhibited the progression of atherosclerotic lesions. Thus PJ has several antiatherogenic capabilities related to its potent antioxidant capacity against lipid peroxidation, which is believed to be the central link for PJs effects on lipoproteins and macrophages.

Research in animal models of obesity and atherosclerosis show the potential for beneficial cardio-protective health effects with administration of PJ or PFE (4,6,50). Epidemiological studies emphasize the importance of consumption of fruits and vegetables known for the phytochemical content and clinical studies have examined the effects of the antioxidant polyphenols present in PJ. However, initial clinical trials have focused on the role of pomegranate polyphenols on the oxidative stress and inflammation associated with cardiovascular disease.

Relevance of Present Study

Phytochemicals are currently of intense research interest due to their proposed benefits to human health. The pomegranate, rich in potent antioxidant polyphenols, is believed to inhibit the oxidative stress and inflammation that contributes to a variety of chronic

disease states. However, most research with pomegranates examines the effects of PJ on biomarkers of oxidative stress, inflammation, and atherogenic modifications. Few studies have examined the effects of pomegranate polyphenol capsules and the available studies have been conducted with overweight individuals who were otherwise healthy and free of chronic disease. Thus the present pilot study was conducted to determine the effects of pomegranate polyphenol supplementation, in the form of a POMx™ capsule, on safety parameters as well as biomarkers of oxidative stress and inflammation in adults with abdominal adiposity and type 2 diabetes versus healthy controls.

CHAPTER III

METHODOLOGY

Institutional Review Board Approval

The pomegranate supplementation study was designed as a four week clinical trial that was conducted to examine the effects of pomegranate polyphenol supplementation, in the form of a POMx™ capsule, on biomarkers of oxidative stress and inflammation associated with type 2 diabetes. This study was conducted according to the guidelines presented in the Declaration of Helsinki and approval was obtained from the Oklahoma State University Institutional Review Board (IRB) for all procedures. Prior to involvement in the study, all investigators and graduate research assistants (GRA) completed the IRB training for human subjects research practices through the Collaborative Institutional Training Initiative (CITI) and also received training on the process of consenting and explaining the study, as well as subject follow-up and data collection. All participants provided a signed informed consent before their enrollment in the study.

Subjects

Subjects were recruited at the Oklahoma State University campus. Flyers were posted at the Department of Nutritional Sciences and the Seretean Wellness Center. Advertisements were also sent through e-mail as well as posted on the Oklahoma

State University Headlines page and the College of Human and Environmental Sciences homepage. Telephone questionnaires were used for the initial screening purposes and all potential participants were scheduled for a screening visit at the Clinical Assessment Unit at the Department of Nutritional Sciences (NSCI), Oklahoma State University, to ensure that they met study inclusion criteria.

This study required participation of subjects with diabetes as well as healthy controls. Adults over the age of twenty-one years were recruited for both study groups.

Inclusion criteria: Participants in the diabetic group were required to meet the following criteria for study inclusion: abdominal adiposity (waist circumference > 35 inches in women and > 40 inches in men), diabetes mellitus, stable on medications, normal hemoglobin (Hb), white blood cells (WBC), platelet count, and liver, kidney, and thyroid function tests. In order to be included in the study, healthy controls had to present a waist measurement of < 35 inches for women and < 40 inches for men and be free of any chronic disease such as diabetes, cancer, or any form of cardiovascular disease. Subjects were included if they were on stable medications.

Exclusion criteria: Individuals were excluded if they had any form of pre-existing disease including cancer, heart disease, liver or renal disorders, or anemia. Subjects were also excluded if they were pregnant, nursing, taking mega doses of antioxidants/fish oil supplements (> 1g/day), had an abnormal Hb (normal range: 4.0-11.0 K/mm^3), WBC (normal range: 12.0-18.0 g/dL), or platelets (normal range: 140-440 K/mm^3), abnormal liver enzymes (normal range for AST: 7-40 units/L; ALT: 10-45 units/L). Individuals

who smoke or used any other form of tobacco were excluded as well as those who consumed > 1 oz. of alcohol per day.

The initial screening visit consisted of a blood draw and blood pressure, waist circumference, weight and height measurements. Prior to the blood draws and measurements participants were informed of the purpose of the study, the procedures and any risks or potential benefits associated with the study. Participants also read and signed the informed consent participation agreement before anthropometric and blood pressure measurements and blood draws were conducted. If participants were unable to read the informed consent, the GRAs would read the consent to the participant. Based on the initial screening results and the inclusion criteria, individuals were included or excluded from the study and were notified of their participation status by telephone.

Study Design

This was pilot study with a convenience sample and a pre-test/post-test intervention with no specific control or placebo, all participants received the intervention. The intervention period consisted of daily supplementation of POMx™ capsules (2 capsules/day; 1 capsule = 753 mg polyphenols) for a period of four weeks. The POMx™ capsules were purchased from Pom Wonderful LLC (Los Angeles, CA). Participants were asked to restrict intake of strawberries, or other berries, and green tea as intake of such foods could interfere with the effect of the pomegranate polyphenol supplement.
Blood draws, blood pressure and anthropometrics (height, weight, and waist circumference) were obtained at the initial screening visit for both diabetic patients and

healthy controls. At the end of the four week intervention period subjects returned to Clinical Assessment Unit at NSCI for final anthropometric and blood pressure measurements and blood samples. A certified phlebotomist performed all blood draw samples from participants. Subjects were asked to maintain their typical diet, physical activity, and lifestyle during the study. For study participation subjects were compensated $30 at screen, week two and week four of the study or received $90 for the entire study.

Subjects were instructed to complete detailed three day food records during the first and the final week of the supplementation period of the study. Participants were instructed on how to use the food records and also how accurately record food portions consumed. Participants recorded/described the type of food and amount of food consumed, as well as location (home, restaurant, with friends, or at work) and type of meal or snack during which the food was eaten (breakfast, morning snack, lunch, afternoon snack, dinner, or evening snack). Participants were also instructed to record the names of specific restaurants where food was consumed and names of menu items eaten. Submission of recipes and nutrition labels for foods prepared at participant's home were requested in order to ensure accurate dietary analysis was performed.

Measures:

Blood Pressure: Systolic and diastolic blood pressure measurements were obtained using a portable blood pressure device with arm cuff, Spot Vital Signs Device (Welch Allyn, Skaneateles Falls, NY)

Anthropometrics: The Health-o-Meter weight tracking scale (2008 Sunbeam Products, Inc., Maitland, FL) were used to determine the participant's body weight and the Gulick II tape measure (Vital Signs, Gay Mills, WI) was used to measure waist circumference at the super iliac crest in inches. Height was determined in centimeters with the standiometer in HES 307.

Blood Draws: Serum separator tubes (SST) and tubes containing the anticoagulant EDTA were used in collecting 45-60 mL of blood from the participants after a period of fasting. Serum and plasma samples were stored at -80 ˚C until removed for analysis. In order to separate plasma and serum, centrifugation at 1464 g was performed for 10 minutes at 4˚C using the Centrifuge 5810 R (Eppendorf, Hamburg, Germany) in Human Nutrition Laboratory at NSCI. Stillwater Medical Center (Stillwater, OK) performed analysis of each participant's serum glucose, lipids, hemoglobin and hematocrit, platelets, and liver, renal, and thyroid function tests. Plasma not used for laboratory tests continued to be stored at -80˚C for later analysis of biomarkers of oxidative stress and inflammation.

Polyphenol Content Analysis: Total phenolics and total ellagic acid content of individual capsules were determined by Brunswick Laboratories, Norton, MA.

Macronutrient Content Analysis: Percent moisture, ash, protein, fat and carbohydrate was determined by the Robert M. Kerr Food and Agriculture Products Center, OSU, Stillwater, OK.

Analysis of Biomarkers of Oxidative Stress

Blood samples from baseline and week four of the study were analyzed for biomarkers of oxidative stress. Biomarkers of oxidative stress present in the participant's blood samples that were measured for this study included oxidized LDL and malondialdehyde and 4-hydroxynonenal (MDA and HNE). Serum oxidized LDL was measured in triplicate using an oxidized LDL competitive ELISA (Mercodia, Uppsala, Sweden) which is based on the monoclonal antibody 4E6 (mAb - 4E6). In this procedure, the oxidized LDL in the participant's blood sample competes with a pre-determined amount of oxidized LDL in the microtiter well for binding with biotin-labeled specific antibodies. Following a washing to remove un-reactive sample components, the biotin-labeled antibody is identified with streptavidin. In the final steps, the bound conjugate is detected through its reaction with 3, 3́, 5, 5́ - tetramethylbenzidine (TMB), stop solution is added, and the sample is read spectrophotometrically using the Synergy HT plate reader (BioTek Instruments, Inc., Winooski, VT) at 450 nm. The following flow diagram illustrates the principle of the procedure for oxidized LDL:

25 µl of each sample diluted with 1,000 µl sample buffer

↓

50 µl of individually diluted samples placed in well plate in triplicate with calibrators

↓

50 µl antibody (mAb) added to all wells except the blank

↓

Samples incubated on shaker for 2 hours at room temperature

↓

Samples washed 6 times manually with 350 µl wash buffer

↓

100 µl enzyme conjugate added to all wells

↓

Samples incubated on shaker for 1 hour at room temperature

↓

Samples washed 6 times manually with 350 µl wash buffer

↓

200 µl TMB added to all samples

↓

Samples incubated for 15 minutes, with no shaking

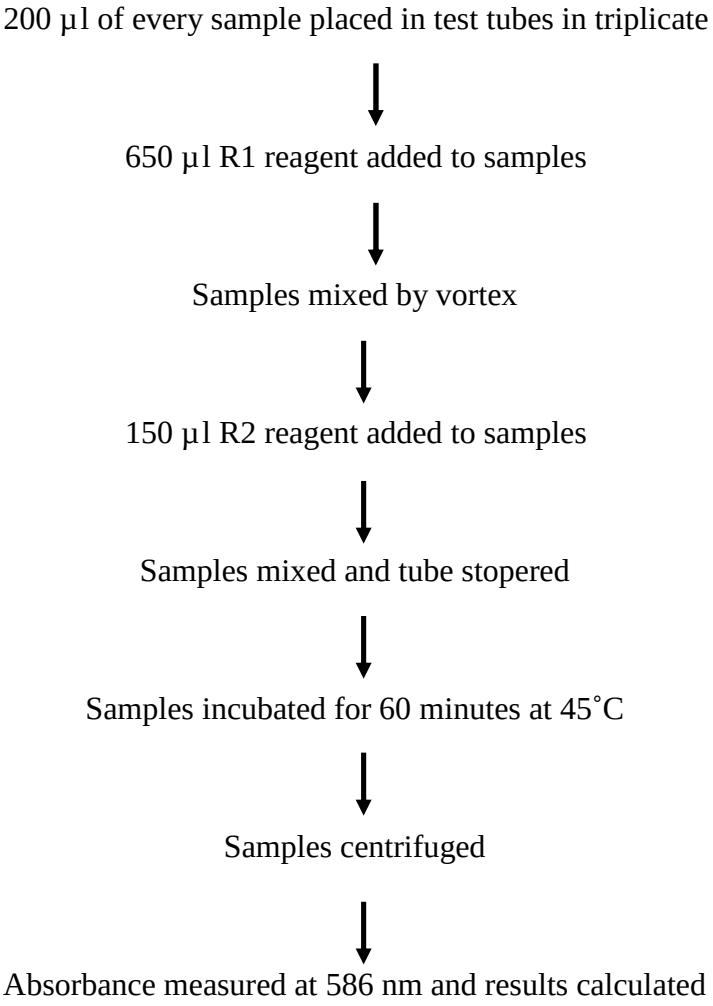
↓

50 µl stop solution added to samples

↓

Absorbance measured at 450 nm and results calculated

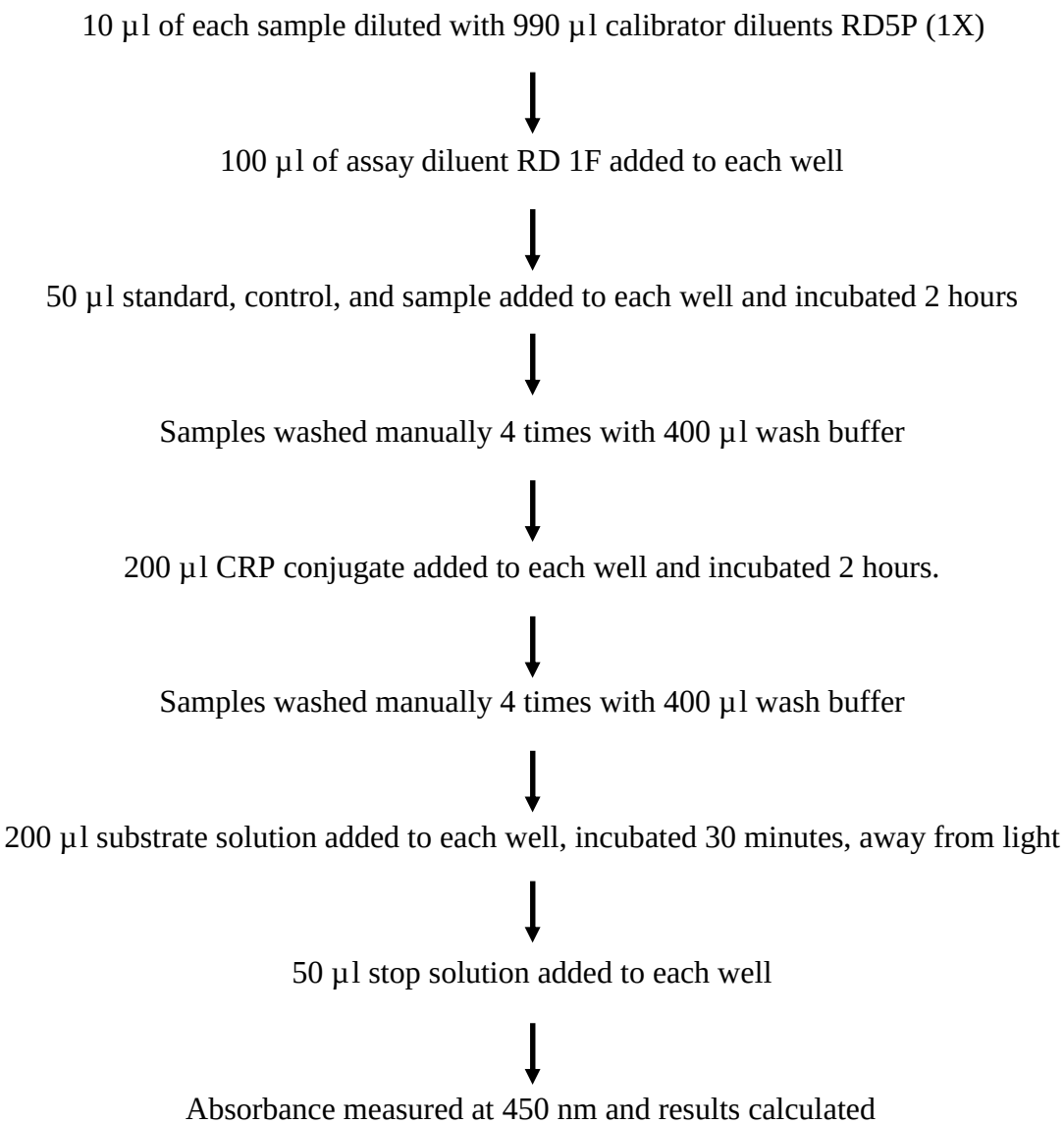
Serum levels of MDA and HNE were determined with the Bioxytech® LPO – 586™ assay (OxisResearch™ Inc., Foster City, CA). This assay for detecting lipid peroxidation is based on the reaction of MDA and 4-hydroxyalkenals with the chromogenic reagent, N-methyl-2-phenylindole at 45˚C. The acid solvent for this reaction is methanesulfonic acid. The sample is read spectrophotometrically using the Synergy HT plate reader (BioTek Instruments, Inc., Winooski, VT) at 586 nm. The following flow diagram illustrates the principle of the procedure for MDA and HNE:



Analysis of Biomarkers of Inflammation

Blood samples from baseline and week four of the study were analyzed for biomarkers of inflammation. Concentration of human C-reactive protein (CRP) present in the participant's blood samples were measured for this study as a biomarker of inflammation. The procedure for determining serum levels of human CRP utilizes the quantitative sandwich enzyme immunoassay technique with a monoclonal antibody that is specific for CRP. Standards and samples are added to the wells and CRP present in the plasma becomes bound by the immobilized antibody. Subsequent washing removes any unbound

substances and an enzyme-linked monoclonal antibody specific for CRP is then added to the wells. After a second wash, addition of a substrate solution causes color to develop in proportion to the amount of CRP that was bound in the first step of the procedure. A stop solution stops the color development and the intensity of the color will be measured spectrophotometrically with Synergy HT plate reader at 450 nm. The following flow diagram illustrates the principle of the procedure for CRP:



Dietary Analysis

Participants' detailed dietary food records were used to account for any dietary habits and dietary changes that could influence the results of the study. At completion of the study, three day food records from baseline and week four were entered and analyzed by ESHA Food Processor 9.1.0 (ESHA Research Inc., Salem, OR). The following dietary components were included in the analysis: energy (kcal), protein (g), fiber (g), total fat (g), vitamins A (mg), C (mg), and E (mg), copper (mg), iron (mg), & zinc (mg).

Statistical Analysis

Tests for biomarkers of oxidative stress and inflammation were performed in triplicate. Paired t-tests were performed to determine differences between pre- and post-intervention anthropometric and blood pressure measures as well as blood glucose, lipid profile, and liver, kidney, and thyroid function tests. Independent sample t-tests were used as appropriate to compare healthy controls to subjects with type 2 diabetes. Multiple regression was conducted for MDA and HNE. Changes in body weight, waist circumference, blood pressure, blood glucose, lipid profile, and liver, kidney, and thyroid function tests were analyzed as outcome measures of the study. Statistical significance was set at $p < 0.05$ (two-sided test) and all data analysis was performed with SPSS® version 16.0 (SPSS Inc., Chicago, IL).

CHAPTER IV

FINDINGS

Nutrient Content of POMx Capsules

Composition analysis was conducted to determine the macronutrient and polyphenol content of the POMx™ capsules (**Table 3**). Analysis revealed that two POMx™ capsules taken by participants each day added less than eight calories to the participants' dietary intake. The two capsules are equivalent to 16 ounces of POM™ juice and provide the same polyphenol content without the additional calories (55; personal communications, POM™ Wonderful). Sixteen ounces of pomegranate juice or two POMx™ capsules are equivalent to approximately four to six whole fresh pomegranates. The two capsules provide 1801 mg carbohydrate (86.0 %), 6 mg fat (0.28 %), and 73 mg protein (3.5%) and have an ash content of 112.6 mg and a moisture content of 116 mg. The pomegranate supplements were also found to be high in phenolics as two POMx capsules provide 1506 mg total phenolics and 372 mg total ellagic acid. Additionally POMx™ is comprised of a vegan capsule and vegetable stearate and silica as fillers.

Baseline Characteristics

Due to the fact that baseline characteristics between the healthy controls and the type 2 diabetic subjects differed greatly in terms of pathophysiological characteristics, the primary objective of our study was not to compare between group differences before and after POMx™ supplementation. Rather, the primary objective was to compare differences from baseline to week four of the study within each individual group. However, an independent sample t-test and multiple regression was conducted as a secondary analysis between groups for MDA and HNE.

The baseline characteristics for the subjects are outlined in **Table 4**. A total of 17 subjects, 9 healthy controls and 8 subjects with type 2 diabetes completed the study. The subjects were predominantly female with only two male subjects in the diabetic study group and no male subjects in the healthy control group. The duration of diabetes among the eight diabetic subjects ranged from four months to three years. Six of the diabetic subjects were on daily prescription medications for diabetes management. Medications included Metformin, Byetta, Actos, and Avandia. None of the subjects discontinued the study due to any adverse events. Only minor adverse events were reported and such events included one report of increased appetite after taking the capsules and one report of dizziness and nausea on two occasions. However, these effects subsided early in the study. One healthy control was excluded due to having high triglyceride levels (420 mg/dL) at the end of the fourth week of the study. These changes were most likely due to dietary changes that had taken place between week one and week four of the study.

Daily aspirin use, commonly recommended to lower risk of heart attack, was reported by only one subject in the diabetic group. Seven healthy controls and four subjects with type 2 diabetes reported using supplements, mainly a multivitamin or mineral, at the screening visit and continued throughout the duration of the study. Three total subjects reported antioxidant supplement intake in the past. However, none of the subjects took such supplements during the course of the study. One healthy control and one diabetic subject reported taking a fish oil supplement at the screening visit and continued throughout the duration of the study. Blood pressure medication use was reported by two diabetic subjects during the study.

All diabetic subjects were obese as determined by their body mass index (BMI) at the initial screen. The mean BMI for the diabetic group was 35.3 kg/m^2. Diabetic males had waist circumferences > 40 inches and diabetic females had waist circumferences > 35 inches. The mean waist circumference of the diabetic group was 42.4 inches. The healthy controls were required to have a normal BMI and waist circumference < 40 inches for males and < 35 inches for females at the screening visit to qualify for the study. The mean BMI for the control group was 23.3 kg/m^2 and the mean waist circumference was 31.06 inches.

A significant decrease ($p < 0.05$) in BMI (35.26 ± 10.28 kg/m^2 to 34.81 ± 9.98 kg/m^2) and bodyweight (99.32 ± 10.28 kg to 98.05 ± 9.98 kg) was observed at week four in the diabetic group. Such decreases were not supported by corresponding differences in

dietary data for macronutrients. Significant weight and BMI changes were not observed in the healthy controls from baseline to week four.

Blood Glucose, Blood Pressure, and Lipid Profile

Supplementation with POMx™ capsules did not worsen diabetic parameters such as glucose, mean blood glucose, hemoglobin A1c, or glycosylated hemoglobin in the diabetic subjects or healthy controls (**Table 5**). Significant differences were not observed in the mean blood glucose values of the healthy controls (85.11 ± 4.48 mg/dL to 85.67 ± 8.03 mg/dL; $p > 0.05$) after POMx™ supplementation. Normal fasting blood glucose level is less than 100 mg/dL (56). Likewise, the mean blood glucose for the diabetic group (105.88 ± 29.57 mg/dL to 114.25 ± 33.36 mg/dL; $p > 0.05$) did not significantly differ after the supplementation period. Blood glucose levels greater than 126 mg/dL are used to diagnose diabetes (56).

The LDL-cholesterol levels of the healthy controls increased significantly by 7% from baseline to week four of the study (109.22 ± 27.87 mg/dL to 117.22 ± 30.05 mg/dL; $p < 0.05$). The reference value for LDL-cholesterol is less than 100 mg/dL (57). The mean LDL-cholesterol level of the diabetic group did not significantly increase during the study. At baseline the mean LDL-cholesterol level of the diabetic subjects was 87.50 mg/dL ± 43.09, which met reference value and at week four the mean LDL-cholesterol level was 103.75 ± 27.42 mg/dL. Supplementation with POMx™ did not significantly affect blood pressure or other parameters of the lipid profile such as total cholesterol, triglycerides, HDL-cholesterol, VLDL-cholesterol, LDL/HDL ratio, or chol/HDL ratio

(**Table 5**). Also, the mean values for these parameters in both the diabetic group and the healthy controls remained within what was considered the normal range at both baseline and week four (total cholesterol: < 200 mg/dL, triglycerides: < 150 mg/dL, HDL-cholesterol: 40-60 mg/dL) (57).

Safety Parameters

Aspartate aminotransferase (AST) levels were significantly increased (21.78 ± 4.29 U/L to 25.33 ± 6.82 U/L; $p < 0.05$) by 16% in healthy controls from screen to week four and there was a significant decrease of 10% (36.88 ± 13.47 U/L to 33.13 ± 10.05 U/L; $p < 0.05$) in alanine aminotransferase (ALT) levels from screen to week four in the diabetic group (**Table 6**). All liver parameters were within what is considered the normal range, including the significant differences in AST in healthy controls and ALT in diabetic subjects (AST: 14-36 U/L; ALT: 7-56 U/L). Electrolyte levels, renal function, and hematology were unaffected by POMx™ supplementation as all laboratory values were within normal limits and no significant differences were detected.

Biomarkers of Oxidative Stress

Two measures of oxidative stress were analyzed to determine the effectiveness of POMx™ as an antioxidant in both diabetic subjects and healthy controls. Oxidized LDL levels did not significantly change in the diabetic subjects after supplementation with POMx™ (201.22 ± 34.87 U/L to 183 ± 49.02 U/L). Likewise, ox-LDL levels of the healthy controls did not differ significantly (182.91 ± 32.34 U/L to 173.59 ± 21.83 U/L). Baseline levels of the lipid peroxidation by-products malondialdehyde (MDA) and 4-hydroxynonenal (HNE) were significantly ($p < 0.05$) higher, by 45.5%, than that of the

healthy controls (**Figure 1**). Mean values of MDA and HNE were not significantly reduced in the healthy controls after supplementation with POMx™ (0.96 ± 1.02 µM to 1.02 ± 0.15 µM; $p > 0.05$). However mean levels were significantly reduced by as much as 40% in the diabetic group (1.76 ± 0.02 µM to 1.06 ± 0.26 µM; $p < 0.05$) after supplementation. The mean difference from baseline to week four in the diabetic group was significantly ($p < 0.05$) different from the mean difference observed in the healthy controls. At week four a significant difference was no longer observed between the raw mean scores of the diabetic group and the healthy controls.

Biomarkers of Inflammation

C-reactive protein (CRP) was used to determine the effect of POMx™ supplementation on inflammation. Results indicated that CRP levels were unaffected by POMx™ supplementation in both the healthy control and the diabetic group.

Dietary Analysis

The average caloric intake of both the healthy controls and diabetic participants did not change significantly from baseline to week four of the study (**Table 7**). Dietary data was not collected for week two and week three of the study. The POMx™ capsule was not included in the dietary analysis, as the supplement was not considered part of the participants' normal dietary pattern. Also, contribution of multivitamins and mineral supplements to the participant's dietary intake was not included in the dietary analysis. At baseline, the macronutrient composition for healthy controls was 50.7% carbohydrate, 16.8% protein, and 35.3% fat of total calories on average. The macronutrient composition for diabetic subjects was 44.4% carbohydrate, 19.2% protein, and 38.1% fat of total

calories on average. Macronutrient intake was not significantly different at baseline and week four among healthy controls or diabetic subjects. The mean cholesterol intake of the healthy controls was 180.57 ± 69.85 mg/day at baseline and 144.51 ± 65.79 mg/day at week four. The mean cholesterol intake of the diabetic participants was 256.11 ± 179.52 mg/day at baseline and 236.83 ± 164.64 mg/day at week four of the intervention. The diabetic group had a lower mean intake of fiber than the healthy controls at both baseline and week four (healthy control: 22.53 ± 5.33 g/day to 19.75 ± 7.11 g/day; diabetic: 13.46 ± 4.83 g/day to 12.11 ± 3.64 g/day).

Intake of micronutrients also did not significantly differ from baseline to week four. The healthy controls appear to have higher mean intakes of vitamin C than the diabetic subjects (healthy control: 123.30 ± 55.90 mg to 278.77 ± 453.17 mg; diabetic subjects: 69.25 ± 44.85 mg to 51.56 ± 36.33 mg; $p > 0.05$). However, between group comparisons for significance were not conducted for the purpose of this study. The mean vitamin E intake for the healthy controls was 6.66 ± 4.97 mg at baseline and 3.63 ± 2.36 mg at week four. The mean vitamin E intake in the diabetic group was 5.05 ± 4.60 mg at baseline and 4.21 ± 5.46 mg at week four.

Table 3: Composition of POMx™ capsules[1]

Component (weight)	**Weight (mg)**
Carbohydrates (mg)	1801
Hexane Extract (mg)	5.86
Protein (mg)	72.5
Ash (mg)	112.6
Moisture (mg)	116.08
Total Ellagic Acid (mg)	372
Total Phenolics (mg)*	1505.28

[1] Data presented per 2101 mg (2 capsules). Source: Pom Wonderful (LA,CA,USA)

* Expressed as mg gallic acid equivalents

Table 4. Baseline characteristics of healthy controls and diabetic subjects

Characteristics	**Healthy Controls**	**Diabetic Subjects**
Gender		
Male/Female (n/n)	0 / 9	2 / 6
Age (mean, SD)	47.1, 6.3	52.4, 13.3
Weight kg (mean, SD)	62.6, 10.3	99.3, 29.1
Height cm (mean, SD)	163.7, 6.0	167.6, 7.2
BMI kg/m2	23.3, 3.0	35.3, 10.3
Waist Circumference in (mean, SD)	31.06, 2.3	42.4, 7.9
Supplement Use (%)	7 (78%)	4 (50%)
Vitamin/Mineral (%)	7 (78%)	4 (50%)
Herb or Botanical (%)	0 (0%)	0 (0%)
Antioxidant in the past(%)	2(22%)	1 (13%)
Fish Oil (%)	1 (11%)	1 (13%)
Blood Pressure Medication (%)	0 (0%)	2(25%)
Diabetic Medication (%)	0 (0%)	6 (75%)
Aspirin Use (%)	0 (0%)	1 (13%)
Other Medications (%)	1 (11%)	6 (75%)

Table 5. Effects of POMx™ supplementation on blood glucose, blood pressure, and lipid levels[1]

	Healthy Controls (n=9)		Diabetic Subjects (n=8)	
Variables	**Screen**	**Week 4**	**Screen**	**Week 4**
Glucose (mg/dL)	85.11 ± 4.48	85.67 ± 8.03	105.88 ± 29.57	114.25 ± 33.36
Mean blood glucose (mg/dL)	97.33 ± 16.93	97.33 ± 19.01	121.25 ± 16.73	116.38 ± 11.56
Hemoglobin A1C (%)	5.51 ± 0.51	5.51 ± 0.57	6.22 ± 0.50	6.08 ± 0.35
Glycosylated hemoglobin (%)	6.45 ± 0.76	6.45 ± 0.85	7.51 ± 0.75	7.31 ± 0.53
Insulin (U/L)	7.77 ± 2.44	10.52 ± 12.06	18.61 ± 7.30	19.06 ± 6.03
Systolic blood pressure (mmHg)	114.67 ± 6.18	115.33 ± 5.81	129.00 ± 18.19	134.25 ± 23.81
Diastolic blood pressure (mmHg)	71.33 ± 7.37	72.67 ± 9.25	81.63 ± 8.25	79.88 ± 5.17
Cholesterol (mg/dL)	187.89 ± 30.16	195.56 ± 30.53	182.13 ± 40.47	181.50 ± 36.73
Triglycerides (mg/dL)	77.11 ± 18.43	77.56 ± 26.88	149.75 ± 94.73	135.63 ± 66.06
LDL-cholesterol (mg/dL)	109.22 ± 27.87	117.22 ± 30.05*	87.50 ± 43.09	103.75 ± 27.42
HDL-cholesterol (mg/dL)	63.11 ± 7.56	62.89 ± 9.35	51.75 ± 11.45	50.38 ± 11.03
VLDL-cholesterol (mg/dL)	15.33 ± 3.67	15.44 ± 5.29	30.00 ± 19.04	27.25 ± 13.32
LDL/HDL ratio	1.76 ± 0.52	1.93 ± 0.66	1.98 ± 0.68	2.15 ± 0.75
Chol/HDL ratio	2.89 ± 0.78	3.05 ± 0.88	3.63 ± 0.92	3.58 ± 0.96

[1] Data are mean ± SD

*significantly different from baseline ($p < 0.05$)

Table 6. Effects of POMx™ supplementation on safety parameters[1]

Variables	Healthy Controls (n=9)		Diabetic Subjects (n=8)	
	Screen	Week 4	Screen	Week 4
Aspartate aminotransferase (U/L)	21.78 ± 4.29	25.33 ± 6.82*	30.00 ± 9.71	30.75 ± 9.42
Alanine aminotransferase (U/L)	25.33 ± 8.23	27.22 ± 11.94	36.88 ± 13.47	33.13 ± 10.05*
Alkaline Phosphatase (U/L)	72.89 ± 8.23	74.22 ± 24.79	91.50 ± 17.96	87.50 ± 11.07
Bilirubin, Total (mg/dL)	0.43 ± 0.26	0.39 ± 0.24	0.33 ± 0.10	0.43 ± 0.18
Total Protein (g/dL)	6.94 ± 0.32	6.96 ± 0.38	7.15 ± 0.33	7.23 ± 0.44
Albumin (g/dL)	4.18 ± 0.31	4.20 ± 0.25	4.14 ± 0.32	4.19 ± 0.32
Globulin (g/dL)	2.78 ± 0.29	2.76 ± 0.22	3.00 ± 0.21	3.05 ± 0.21
Albumin/Globulin ratio	1.51 ± 0.20	1.53 ± 0.14	1.40 ± 0.17	1.38 ± 0.12
Sodium (mEq/L)	139.78 ± 1.79	139.89 ± 2.67	140.38 ± 3.02	140.50 ± 2.73
Potassium (mEq/L)	4.28 ± 0.42	4.21 ± 0.31	4.25 ± 0.18	4.25 ± 0.37
Chloride (mEq/L)	106.56 ± 2.60	107.56 ± 1.67	105.00 ± 1.20	105.50 ± 1.31
Calcium (mg/dL)	9.60 ± 0.46	9.40 ± 0.44	9.51 ± 0.52	9.49 ± 0.39
Thyroxine (T4) (ug/dL)	6.91 ± 1.55	6.42 ± 1.47	8.14 ± 2.20	7.70 ± 1.55
T3 Uptake (%)	31.22 ± 3.31	31.56 ± 3.48	29.10 ± 1.70	30.79 ± 2.75
Blood urea nitrogen (mg/dL)	12.11 ± 2.67	13.00 4.12	15.25 ± 3.92	15.50 ± 2.93
Creatinine (mg/dL)	0.82 ± 0.20	0.80 ± 0.15	0.83 ± 0.18	0.86 ± 0.18
Blood urea nitrogen/Creatinine ratio	14.93 ± 2.72	16.28 ± 5.01	18.39 ± 2.71	18.64 ± 5.40
White blood cell (K/mm3)	5.17 ± 0.77	5.09 ± 1.18	7.89 ± 2.01	7.40 ± 1.80
Red blood cell (M/mm3)	4.45 ± 0.32	4.49 ± 0.33	4.78 ± 0.25	4.74 ± 0.28
Hemoglobin (gm/dL)	13.74 ± 0.90	13.86 ± 0.91	13.69 ± 0.69	13.58 ± 0.71
Hematocrit (%)	40.87 ± 2.82	40.92 ± 2.51	41.23 ± 2.30	40.84 ± 2.28
Platelet Count	271.22 ± 59.16	269.78 ± 63.44	259.00 ± 70.07	276.13 ± 55.24

[1] Data are mean ± SD

* significantly different from baseline ($p < 0.05$)

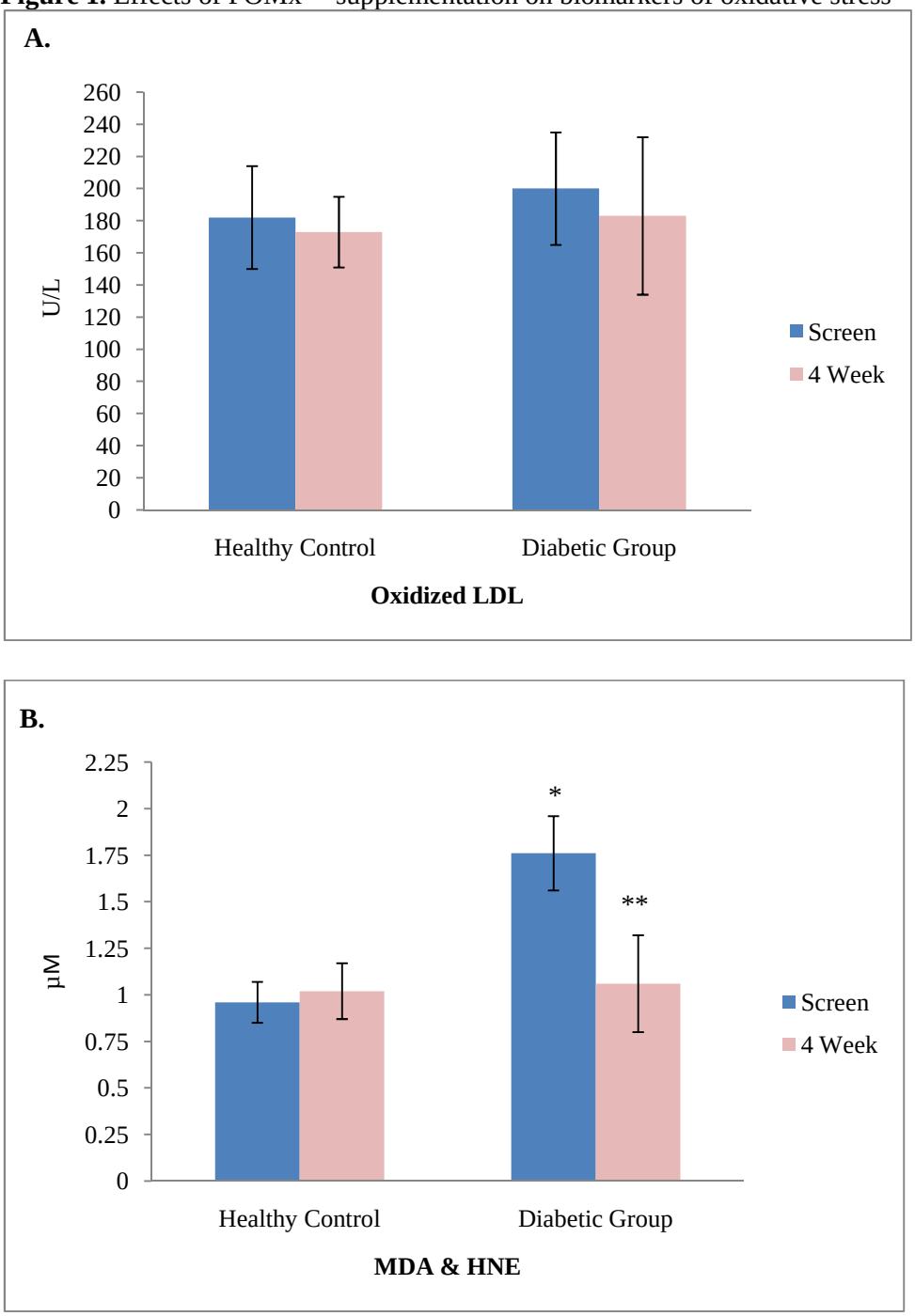
Table 7. Dietary analysis of participant's intake from week 1 and week 4 of intervention[1,2]

	Healthy Controls (n=9)		Diabetic Subjects (n=8)	
Nutrient	**Screen**	**Week 4**	**Screen**	**Week 4**
Energy (kcal)	1679.42 ± 285.13	1739.05 ± 462.52	1266.32 ± 413.65	1273.38 ± 412.62
Protein (g)	70.71 ± 10.48	62.14 ± 15.29	60.74 ± 11.70	70.95 ± 23.45
Carbohydrate (g)	213.00 ± 45.42	254.90 ± 113.23	140.59 ± 50.55	129.42 ± 51.92
Fiber (g)	22.53 ± 5.33	19.75 ± 7.11	13.46 ± 4.83	12.11 ± 3.64
Total fat (g)	65.94 ± 16.21	55.62 ± 21.71	53.64 ± 22.81	54.98 ± 20.18
Saturated fat (g)	19.07 ± 6.40	15.74 ± 8.85	15.09 ±7.05	18.91 ± 6.53
Monounsaturated fat (g)	17.18 ± 6.37	14.90 ± 7.80	13.71 ± 8.25	14.54 ± 7.11
Polyunsaturated fat (g)	12.36 ± 5.32	9.00 ± 5.03	9.91 ± 6.68	6.90 ± 5.07
Cholesterol (mg)	180.57 ± 69.85	144.51 ± 65.79	256.11 ± 179.52	236.83 ± 164.64
Carotenoids (RE)	773.01 ± 443.40	434.37 ± 240.67	486.79 ± 294.38	428.45 ± 207.05
Vitamin C (mg)	123.30 ± 55.90	278.77 ± 453.17	69.25 ± 44.85	51.61 ± 36.33
Vitamin E (mg)	6.66 ± 4.97	3.63 ± 2.36	5.05 ± 4.60	4.21 ± 5.46
Copper (mg)	0.99 ± 0.32	0.78 ± 0.40	0.60 ± 0.23	0.59 ± 0.23
Iron (mg)	13.82 ± 3.87	12.95 ± 3.06	9.85 ± 4.24	12.11 ± 6.02
Zinc (mg)	8.39 ± 2.24	7.30 ± 2.43	4.93 ± 1.85	7.43 ± 2.99

[1] Data are mean ± SD

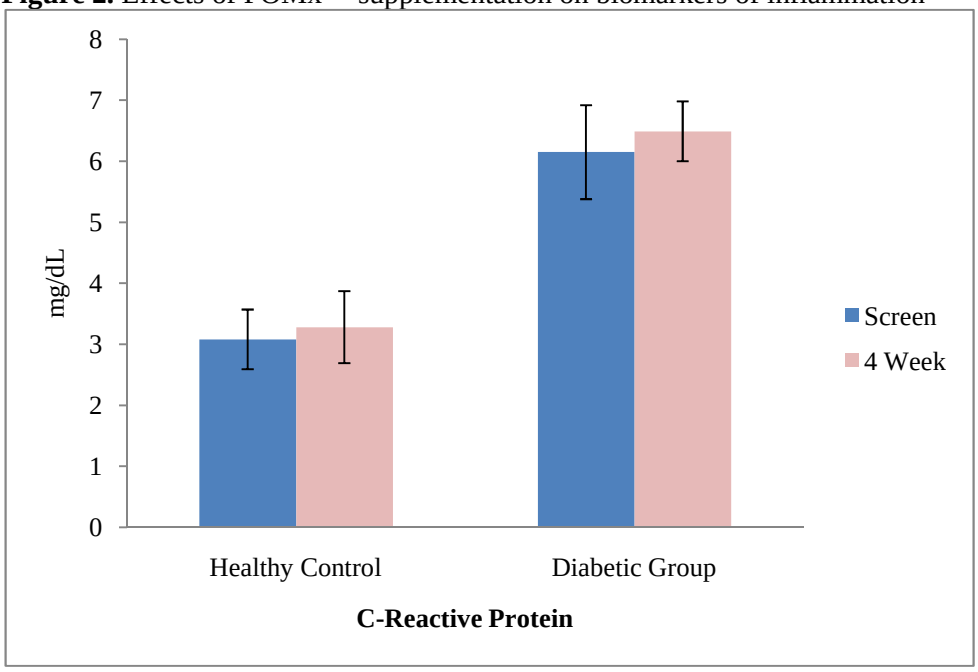
[2] Data was collected from 3 day food records

Figure 1. Effects of POMx™ supplementation on biomarkers of oxidative stress[1]



(A) Oxidized LDL, (B) Malondialdehyde and 4-hydroxynonenal at screen and 4 weeks of POMx™ supplementation (healthy controls, n=9; diabetic group, n= 8)
[1]Presented as mean ± SD
* Significantly different from healthy controls at $p < 0.05$
**Significantly different from baseline at $p < 0.05$

Figure 2. Effects of POMx™ supplementation on biomarkers of inflammation[1]



C-Reactive protein at screen and 4 weeks of POMx™ supplementation (healthy controls, n=9; diabetic subjects, n=8)
[1]Presented as mean ± SD

CHAPTER V

CONCLUSION

In the present study, we have shown the effects of pomegranate polyphenol supplementation on biomarkers of oxidative stress and inflammation in subjects with abdominal adiposity and type 2 diabetes versus healthy controls. To our knowledge this is the first study with both type 2 diabetic subjects and healthy controls reporting the impact of POMx™ capsules on safety parameters as well as biomarkers of oxidative stress and inflammation. Based on our findings, it appears that the POMx™ capsules are safe and well tolerated in subjects with type 2 diabetes and healthy controls. Though the capsules did not contribute to significant changes in ox-LDL or CRP, they did demonstrate the potential to improve lipid peroxidation in the diabetic subjects as determined by the significant decrease in mean MDA and HNE levels. Confirmation of our findings regarding the beneficial effects of the POMx™ capsules on lipid peroxidation could be important in reducing diabetic complications associated with cardiovascular disease.

Diabetes has been reported to be a powerful independent risk factor for the development of coronary artery disease, stroke and peripheral arterial disease, as well as atherosclerosis, which is responsible for eighty percent of all deaths among patients with diabetes (53). Three major mechanisms exist to explain the pathological alterations observed in diabetic vasculature as determined by human and animal studies (53).

These mechanisms include non-enzymatic glycosylation of proteins and lipids, cellular protein kinase C (PKC) activation of oxidative stress pathways, and depletion of natural antioxidants and production of free radicals as a result of prolonged exposure to hyperglycemia. The inflammatory-immune response as well as oxidative stress reactions and generation of free radicals induced by hyperglycemia underlie the etiology for the cardiovascular complications and mortality associated with type 2 diabetes (16).

Diabetic patients are at an increased risk of developing heart disease. The hyperglycemia in type 2 diabetes stimulates oxidative stress reactions and inflammatory-immune responses that play a key role in the development of diabetes related cardiovascular complications, such as atherosclerosis (16). As a result of the increased oxidative stress, diabetic patients have been found to have increased levels of lipid peroxidation, an indicator of oxidative stress, and high concentrations of the lipid peroxidation by-product malondialdehyde (MDA) (53,58). In a previous study, Aviram et al. (2000) showed that under conditions of oxidative stress macrophages can undergo lipid peroxidation and subsequently oxidize LDL (6). The oxidation of LDL by macrophages is considered to be a key event in the early stages of atherogenesis and is also associated with the further uptake of oxidized LDL which leads to cholesterol accumulation in the macrophage and thus formation of a foam cells.

In our study, ox-LDL levels did not significantly differ in either the healthy controls or the diabetic subjects after four weeks of supplementation with POMx™. Likewise significant changes were not observed in levels of CRP. These null results could be due

to the fact that the study duration of four weeks was not long enough to detect significant changes in ox-LDL or CRP.

However, supplementation with POMx did significantly impact lipid peroxidation in the diabetic group as determined by analysis of MDA and HNE. The diabetic subjects presented significantly higher mean levels of the lipid peroxidation by-products MDA and HNE at baseline. Higher levels at baseline can best be explained by the fact that diabetic patients experience increased levels of oxidative stress as a result of hyperglycemia, which contributes to lipid peroxidation (53,58). The decrease in MDA and HNE from baseline to week four in the diabetic group was found to be statistically significant and the mean difference in MDA and HNE was also significantly greater in the diabetic subjects than in the healthy controls. Interestingly, at week four a significant difference was not detected between the healthy controls and the diabetic subjects, indicating that the MDA and HNE levels of the diabetic subjects had been lowered to a level comparable to that of the healthy controls. Thus POMx™ did exert potential beneficial cardiovascular health effects by reducing lipid peroxidation in our study.

The cardiovascular health effects of pomegranates related to impact on oxidative stress and lipid peroxidation have been well investigated in animal models of atherosclerosis. In atherosclerotic apolipoprotein E-deficient (E^0) mice PJ (6.25 or 12.5 µL/d [equivalent to 0.175 and 0.350 µmol total polyphenols]) reduced plasma lipid peroxidation and increased total antioxidant status in comparison to control E^0 mice supplemented with water (6). Pomegranate juice also reduced susceptibility of LDL from E^0 mice to copper

ion-induced oxidation and attenuated susceptibility of LDL to the oxidation associated with the progressive increase in age. Supplementation with PJ reduced lipid peroxidation of mouse peritoneal macrophages (MPMs) by as much as 53% in comparison to MPMs isolated from control mice. Additionally, PJ reduced atherosclerotic lesions and decreased the number of lipid-laden foam cells in E^0 mice.

The beneficial health effects of pomegranates on biomarkers of oxidative stress were also demonstrated in humans (6). Supplementation with 50 ml PJ/d (1.5 mmol total polyphenols) significantly reduced susceptibility to lipid peroxidation by 6% in healthy male subjects (6). The inhibitory effect of PJ on lipid peroxidation was maintained for an additional two weeks after the PJ supplementation ended. A significant increase was also observed in plasma total antioxidant status after two weeks of PJ consumption. Pomegranate juice was found to reduce LDL aggregation in seven of the thirteen subjects, although the mean value was not statistically significant. Decreases in platelet aggregation are important because the atherogenecity of LDL is also attributed to aggregation in addition to oxidative modification. Once LDL becomes oxidized it is more prone to aggregation.

The results from both human and animal studies suggest antiatherogenic properties of PJ as related to its inhibitory effect on lipid peroxidation in plasma, in lipoproteins, and in macrophages (6). PJ supplementation significantly inhibited the progression of atherosclerotic lesions and inhibited atherogenic modifications to LDL. The strong antioxidant capacity of PJ to protect against lipid peroxidation is believed to be the

central link for the antiatherogenic actions of PJ on lipoproteins, macrophages, and platelets. It seems that similar results may be obtained with POMx™ supplementation. However, our results must be confirmed in future clinical trials.

Our study was the first human study reporting the effects of POMx™ supplementation on biomarkers of oxidative stress and inflammation in subjects with type 2 diabetes as well as healthy controls. Thus it was important for us to assess a variety of safety parameters including liver and kidney function tests in order to identify any possible negative health outcomes. Overall our results indicate that kidney and liver function as well as hematology parameters were unaffected by supplementation with POMx™. Although, AST levels in the healthy controls significantly ($p < 0.05$) increased and ALT levels significantly ($p < 0.05$) decreased, neither result was clinically abnormal. Even though these values were found to be statistically significant, they still fall within what is considered the normal healthy range for adults. Thus, these significant differences are not a cause for safety concerns. However our findings need to be confirmed in further studies.

In a similar four week study, Heber et al. (2007) assessed the safety and antioxidant activity of pomegranate polyphenol extracts (POMx™ capsules) in non-diabetic overweight (BMI of 25 – 32 kg/m^2) individuals with increased waist size (> 40 inches in males and > 35 inches in females) (54). A formal assessment of adverse reactions and a comprehensive series of blood tests for toxicity were conducted for comparison between supplement and placebo groups. None of the subjects discontinued the study due to

adverse events and there were no apparent treatment-related changes of clinical significance. None of the laboratory results were outside of the normal range in any of the chemistry, hematology, or urinalysis tests. Their results were similar to the ones obtained in our study in that all of the safety parameters were within the normal range. Although AST levels in healthy controls and ALT levels in diabetic subjects in our study were significantly different ($p < 0.05$) from screen to week four these values were still within the normal safe range. Interfering factors such as change in medications, exercise, or intramuscular injections could contribute to increases in AST levels (59).

Clearly, hyperglycemia is an established risk factor for cardiovascular disease (CVD) and increased oxidative stress and inflammation. In our clinical investigation, four week supplementation with two POMx™ capsules daily did not significantly affect diabetic parameters. Although we detected a decrease of 4% in mean blood glucose and decrease of 2% in hemoglobin A1c these results were not statistically significant. Rosenblat et al. (2006) examined the effects of three month consumption of PJ (50 mL/day; 1.5 mmol total polyphenols) by diabetic subjects and healthy controls and found that PJ consumption resulted in a non-significant reduction of 8% in serum glucose levels with no significant effects on blood hemoglobin A1c (53). Likewise, a non-significant decrease in serum insulin levels was also reported. Thus despite the presence of sugars in PJ, serum diabetic parameters were not worsened.

These results were similar to the results in our present study as both the POMx™ capsules and PJ resulted non-significant decreases in glucose levels and hemoglobin A1c

in diabetic subjects and diabetic parameters were not worsened. It is possible that the percent decrease was larger for the subjects in Rosenblat's study because our subjects had mean blood glucose levels of 105.88 ± 29.57 mg/dL at the screening visit, whereas the subjects in Roseblat's study had blood glucose levels of 160 mg%. Additionally, differences between their study and ours could result from differences in type of supplementation given, as they used a specially formulated pomegranate juice developed in their laboratory and we used commercially formulated capsules by PomWonderful LLC.

In addition to hyperglycemia being a risk factor for CVD, elevated blood pressure levels also increase the risk of developing CVD, especially in subjects with a history of diabetes (60). The increased oxidative stress from hyperglycemia may play a role in elevating blood pressure by a direct vasoconstrictor effect or by indirectly reducing the activity of vasodilators such as nitric oxide. Hypertension is of critical concern in patients with diabetes because it can accelerate both macrovascular and microvascular complications. Although between group analysis was not performed for significance in our current study for blood pressure measures, it is interesting to note that the diabetic subjects began the study with blood pressure values that were 12% higher than the healthy controls. The blood pressure values of the diabetic subjects were within what is considered the pre-hypertensive range.

The results of our study indicate that systolic blood pressure was unaffected by POMx™ supplementation in both the control and the diabetic subject groups. However, in a

mechanistic study with diabetic hypertensive rats, administration of PJ (100 mg/kg/day or 300 mg/kg/day) resulted in a significant reduction in mean arterial blood pressure (60). However, the mean arterial blood pressure of rats with diabetes only, remained unchanged after supplementation with PJ.

Although it is difficult to make adequate comparisons between the results of our human study and this mechanistic study in animals, a two week clinical trial with ten hypertensive patients, two of which were also diabetic (serum glucose >126 mg/dL), found PJ (50 ml, 1.5 mmol total polyphenols per day) to have an effect on blood pressure, as there was a significant reduction in systolic blood pressure (5). The difference in findings between our study and their study may be related to the fact that the subjects in our study did not have a clinical diagnosis of hypertension. The hypertensive patients in the aforementioned study presented mean blood pressure levels of 155 ± 7/ 83 ± 7 mmHg, whereas the subjects in our study had a lower mean blood pressure levels at baseline (controls: 114.67 ± 6.18/ 71.33 ± 7.37 mmHg; diabetic: 129.00 ± 18.19/ 81.63 ± 8.25 mmHg). Based on the results of both the clinical study and the mechanistic study, it appears that PJ has a greater impact on blood pressure in the presence of more highly elevated blood pressure values and also in the presence of a combination of diabetes and hypertension. It is important to mention that in both of the comparison studies PJ, specially formulated in the researcher's laboratory, was used versus our commercially produced POMx™ capsules, which could account for some of the variation in outcomes.

Elevated lipid levels, often present in patients with diabetes, can lead to subsequent cardiovascular complications. In fact, high circulating levels of cholesterol, specifically LDL, has been implicated as a major contributor in the atherosclerotic process. In our study, supplementation with POMx™ capsules for four weeks did not result in a significant decrease in lipid levels in any of the subjects. The only statistically significant result was the increase in mean LDL-cholesterol from screen to week four in the healthy subjects. The mean LDL-cholesterol levels of the healthy controls exceeded the reference value (LDL-cholesterol: < 100 mg/dL) at baseline and increased significantly at week four. Such an increase may be the result of dietary changes among the healthy controls or from interfering factors such as drug or supplement intake, stress, or unknown atherosclerosis (59). However, it is important to note that our dietary analysis did not reveal any significant differences in intake of total fat or saturated fat from baseline to week four.

Aviram et al. (2000) also found administration of 50 ml PJ/d (1.5 mmol total polyphenols) to have no significant effects on plasma lipid levels, including total cholesterol, LDL-cholesterol, VLDL-cholesterol, HDL-cholesterol and triglyceride concentrations in healthy male subjects (6). Although similar results were obtained it should be noted that pomegranates (‘Wonderful’ cultivar) were handpicked and used to make specially formulated PJ in their laboratory for their study.

Despite the fact that lipid profile was not improved in our study, PJ has demonstrated the potential to improve aspects of the lipid profile of diabetic hyperlipidemic subjects.

Esmaillzadeh et al. (2006) assessed the effects of 40 g/day concentrated pomegranate juice (CPJ) (875 mg/ 100g total polyphenols) consumption for eight weeks on lipid profiles in type 2 diabetic patients (fasting blood sugar ≥ 126 mg/dL) with hyperlipidemia (total cholesterol or triglycerides of ≥ 200 mg/dL) and significant reductions in total cholesterol ($p < 0.006$), LDL-cholesterol ($p < 0.006$), LDL/HDL-cholesterol ($p < 0.001$), and total cholesterol/HDL-cholesterol were observed (61). Differences between their findings and findings of our study may be related to differential effects of the intervention, CPJ used in their study for eight weeks versus POMx™ supplement used in our study for four weeks. Furthermore, we did not include subjects with hyperlipidemia and thus the normal triglycerides and lower glucose levels in our subjects may have contributed to the null effects of POMx™ on lipids profiles in our study.

The POMx™ supplements (POM Wonderful LLC, LA, CA) used in are study were prepared from partially juice-pressed whole fruit, arils, and seeds (54). The capsules contain the same monomeric and oligomeric ellagitannins as the POM Wonderful™ PJ, but lack the sugars and calories that are naturally present in the juice form. POM Wonderful LLC claims that one POMx™ capsule contains 1,000 mg total natural polyphenol extract on their website http://www.pompills.com/pills/product_pills.aspx. Yet, the composition analysis conducted by Brunswick Laboratories, Norton, MA in our study revealed that one POMx™ capsule provides 753 mg total pomegranate polyphenols or 1506 mg total phenolics per two capsules.

Differences in polyphenol content could be related to temperature, humidity, soil conditions or some step of the collection and manufacturing process (62). Additionally labeling claims may not accurately reflect the true polyphenol content of the capsule (63). For example, in a previous study by Seeram et al. (2006), labeling information for total tea polyphenols in green tea dietary supplements failed to contain the claimed amount (63). Reliable labeling information as well as standardized manufacturing practices are needed to ensure quality control of dietary supplements. However, despite the discrepancies between the labeling claims made by POM Wonderful™ and our analysis as determined by Brunswick laboratories, 1506 mg total phenolics per day is a substantial amount for supplementation.

The POMx™ capsules used by Heber et al. (2007) were also obtained from PomWonderful LLC (54). However analysis of their POMx™ capsules indicated that each capsule containing 1,000 mg extract provided 610 mg gallic acid equivalents (GAEs) while our analysis, conducted by Brunswick Laboratories, found each capsule to contain 753 mg GAEs. Although in both studies capsules were purchased from the same distributor, it is possible that cultivar differences could have influenced the total phenol content of the capsules as well as differences in storage conditions and analytical techniques. Cultivation changes such as variations in plantation season have been found to influence total phenolic content in strawberries according to light and temperature (64). Also, harvest time affects the content of ellagic acid in strawberries, presumably as a result of difference in temperature and rainfall (65). Phenolic content may change from year to year and it is quite possible that the pomegranates used for our capsules were

from a different batch than those used in the study conducted by Heber et al. (2007). Despite the variation in GAEs, results from their study and ours are comparable in terms of pomegranate polyphenol supplement used and composition. The subject population studied was also similar in that both studies included subjects with increased waist size and BMI.

Overall, mean caloric intake did not significantly differ from baseline to week four in either the diabetic group or the healthy controls. It is possible that underreporting of dietary intake occurred in our study as the mean caloric intake of the diabetic subjects seemed exceptionally low (1266.32 ± 413.65 kcal to 1273 ± 412.62 kcal). Previous studies investigating the accuracy of self-reported dietary intake have found that underreporting of total caloric intake and high fat foods occurs frequently, especially among obese participants (66,67). The macronutrient composition of both groups was similar to the recommended range outlined in Dietary Guidelines for Americans (DGA) 2005, which states that 45-65% of total calories should come from carbohydrate sources, 10-35% from protein, and 20-35% from fat (68). It appeared that the diabetic group had a lower mean intake of carbohydrate, a higher mean intake of protein, and a higher intake of fat as compared to the healthy controls. However, this data may have been skewed due to the fact that the mean reported caloric intake was low among the diabetic subjects.

All of the participants had mean LDL-cholesterol levels below 130 mg/dL, thus the mean dietary cholesterol intake in both groups should be less than 300 mg/dL (68). Both groups met this recommendation. However, the diabetic subjects did report a higher mean

dietary cholesterol intake than the healthy controls. The mean intake of dietary fiber was higher among healthy controls and more closely met the recommendations of the DGA 2005, which is 14 grams of fiber per every 1,000 calories (68).The healthy controls also had a higher mean intake of vitamin C than the diabetic subjects and intake exceeded the recommended dietary allowance (RDA) of 75 mg/day for women (healthy controls were all female) (69).The diabetic group did not meet the RDA. The RDA for vitamin E is 15 mg/day for adults and neither group met this recommendation (70).

Although not presented in the tables, significant decreases were observed in the mean weight and BMI of our diabetic subjects. However these decreases were not supported by changes in dietary intake as determined by our analysis of the week one and week four dietary data. The BMI of the diabetic subjects was 35.30 ± 10.3 kg/m^2 at baseline and 34.81 ± 9.98 kg/m^2 at week four of the study. The weight of the diabetic subjects was 99.3 ± 29.1 kg at baseline and 98.05 ± 28.45 at the final visit. Though these changes were statistically significant they did not appear to be clinically relevant and were not likely related to POMx™ supplementation. Some of the participants began this study around the holidays and they may have decreased their intake of high fat food items as a result of New Year's resolutions or dietary changes that typically occur after the holidays. Also, participants could have become more health conscious after being enrolled in our research study. The recording of dietary food intake and pomegranate supplementation may have motivated participants to begin exercising or making other dietary changes. However, subjects were encouraged to keep a detailed record of their food intake and not to make lifestyle changes such as increasing physical activity. Yet, information related to

physical activity was not obtained for the purpose of our pilot study. Overall, it seems that the changes in weight and BMI may have been more related to some unreported change in diet or lifestyle factors than the POMx™ supplementation.

The primary objective of this study was to examine the effects of pomegranate polyphenol supplementation, in the form of a POMx™ capsule, on clinical variables (fasting blood glucose, blood pressure, waist circumference, and dyslipidemia [high TCs and/or high TGs or low HDL levels]) and on biomarkers of oxidative stress (ox-LDL and MDA and HNE) and inflammation (CRP) in subjects with abdominal adiposity and type 2 diabetes versus healthy controls.

The intervention using pomegranate polyphenol supplements (POMx™ capsules) was based on the fact that they contain the major antioxidant phytochemicals present in the pomegranate and provide a convenient alternative to pomegranate juice (54). Additionally, few studies have been performed to evaluate the safety and efficacy of pomegranate supplements in human subjects.

Our null hypothesis was that:

- Pomegranate polyphenol supplementation will not affect glucose, lipids, and biomarkers of oxidative stress and inflammation in subjects with type 2 diabetes and healthy controls.
- Pomegranate polyphenol supplementation will not affect safety parameters, such as, liver, kidney, and thyroid function tests, and complete blood cell count, in subjects with type 2 diabetes and healthy controls.

Based on our results we reject the null hypothesis that pomegranate polyphenol supplementation will not affect glucose, lipids, and biomarkers of oxidative stress and inflammation in subjects with type 2 diabetes and healthy controls because the biomarkers of lipid peroxidation, MDA and HNE were significantly decreased in subjects with type 2 diabetes. We fail to reject the null hypothesis that pomegranate polyphenol supplementation will not affect safety parameters, such as, liver, kidney, and thyroid function tests, and complete blood cell count, in subjects with type 2 diabetes and healthy controls. All safety parameters were within the normal range for healthy adults after the four week supplementation period, despite the significant increase.

Certain limitations of our study need to be taken into consideration. This was a non-randomized clinical pilot study with a convenience sample and a pre- and post- test intervention. Use of a convenience sample recruited at one site limits generalizability of the study results. Thus our results need further confirmation in larger placebo-controlled trials. This pilot study was non-randomized, thus in future studies a randomized crossover trial design would be beneficial as such a design reduces the influence of confounding variables with each subject serving as their own control. Use of a placebo capsule is also recommended in future research to ensure that positive findings are actually the result of the POMx™ capsule and not the “placebo effect.”

Other caveats include the nature of the intervention and human subject compliance. Fresh, whole pomegranates are expensive as are the POMx™ capsules and pomegranate juice ($ 29.95 = 1month supply, Pom Wonderful LLC, Los Angelos, CA), thus limiting

their usefulness as a preventative/therapeutic measure by the general public. Also, human compliance was difficult to determine in this study. Participants were not closely monitored for compliance to supplement intake or accurate completion of dietary food records. In the future, the subjects should be more closely monitored to determine compliance to the therapy and to assess the validity of the food records, especially in terms of what is known about underreporting and inaccuracies in self-reported dietary assessment. It may also be beneficial to include more extensive food records, have participants complete food records for every week of the study, and also to include a pre-intervention food frequency questionnaire (FFQ) that includes flavonoid food products to examine the amount/type of polyphenolic compounds typically consumed by the participants. Additionally, a 24-hour urine analysis would be useful in assessing compliance by detecting polyphenolic urinary metabolites. It may also be beneficial to measure levels of ellagic acid or total polyphenols in the plasma of the participants.

Future studies should also include a more comprehensive analysis of biomarkers of oxidative stress and inflammation, such as, advanced glycation end products (AGEs) or F_2-isoprostanes, as these markers are also elevated in patients with type 2 diabetes. Additionally, the four week trial phase may not be long enough to assess changes accurately in the selected biomarkers. In the future, a clinical trial of a longer duration should be implemented to assess the effects of long-term supplementation. Furthermore, oxidative stress and pro-inflammatory responses may be affected by a variety of stimuli including stress, increased activity, a high-fat diet, or bacterial infection (59,71). Thus it may be difficult to determine whether the selected markers, such as CRP, were elevated as a result of diabetes versus some other stimuli. Although, CRP values have been

reported to be a strong predictor in cardiovascular disease, it is important to note that consensus has not been reached on the usefulness/specificity of CRP as a predictive biomarker of diabetes. Thus in future studies it may be beneficial to assess additional biomarkers of inflammation to verify the results obtained from the CRP analysis. Such biomarkers could include interleukin-6 (IL-6) or tumor necrosis factor (TNF-α).

Though somewhat expensive, the capsules are commercially available thus providing an alternative therapy to prevent diabetic complications. Due to the fact that most individuals in the United States do not consume the recommended servings of fruits and vegetables each day, it may be advised that the general population increase their intake of all fruits and vegetables and include whole fresh pomegranates and pomegranate juice as part of a healthful diet. Increased consumption of functional foods rich in natural polyphenols may be effective in reducing the oxidative stress and inflammation associated with diabetes which can lead to life-threatening cardiovascular disease complications.

The use of dietary supplements among adults in the United States has increased since the early 1970's when the National Health and Nutrition Examination Survey (NHANES I) reported that 27.5% of males and 37.9% of females used dietary supplements (72). Prevalence of supplement intake is now closer to 47.1% in males and 56.7% in females, according to data from NHANES 1999-2000. Over the past decade sales of dietary supplements has increased. In 2003, sales of dietary supplements reached approximately 18.8 billion dollars. Therefore, further studies are needed to address the safety and

efficacy of supplements in healthy individuals as well as those with chronic diseases such as type 2 diabetes. Our pilot study addresses this gap by assessing a supplement that is relatively new in the market, though results warrant further investigation in larger controlled studies.

In conclusion, this pilot study demonstrates the safety of the POMx™ capsules in both healthy controls and subjects with type 2 diabetes, and their efficacy in reducing lipid peroxidation in type 2 diabetics. However, future studies should be conducted to confirm the effects of these pomegranate polyphenol supplements on biomarkers of oxidative stress and inflammation in humans.

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APPPENDICES

Appendix A. Institutional Review Board Approval

Appendix B. Screening Questionnaire

Appendix C. Informed Consent Form

Appendix D. Food Diary Form

Appendix E. Participant Recruitment Flyer

Appendix A. Institutional Review Board Approval

Oklahoma State University Institutional Review Board

Date	Friday, October 09, 2009	**Protocol Expires:**	10/8/2010
IRB Application No:	HE0869		
Proposal Title:	Effects of Pomegranate Polyphenol Supplementation on Biomarkers of Oxidative Stress and Inflammation in Adults With Type 2 Diabetes Versus Healthy Controls		
Reviewed and Processed as:	Expedited **Continuation**		
Status Recommended by Reviewer(s)	**Approved**		

Principal Investigator(s)

Arpita Basu 301 HES Stillwater, OK 74078	Nancy Betts 301 HES Stillwater, OK 74078	Lenka Humenikova Shriver 311 HES Stillwater, OK 74078

Approvals are valid for one calendar year, after which time a request for continuation must be submitted. Any modifications to the research project approved by the IRB must be submitted for approval with the advisor's signature. The IRB office MUST be notified in writing when a project is complete. Approved projects are subject to monitoring by the IRB. Expedited and exempt projects may be reviewed by the full Institutional Review Board.

☒ The final versions of any printed recruitment, consent and assent documents bearing the IRB approval stamp are attached to this letter. These are the versions that must be used during the study.

The reviewer(s) had these comments:

Protocol continuation approved for continuation data analysis of identifiable data only. Should additional data collection be necessary or desired a modification request must be submitted to the IRB for review and approval prior to initiation.

Signature:

Sheila Kennison, Chair, Institutional Review Board

Friday, October 09, 2009
Date

Appendix B. Screening Questionnaire

Day/ Date of Appointment: _____ *Time:* _____

SCREENING QUESTIONNAIRE FOR POMEGRANATE STUDY

NAME: _____

ADDRESS: _____

_____

PHONE (WORK): _____

PHONE (HOME): _____

AGE: _____ DATE OF BIRTH: _____ GENDER: _____

SCREENING QUESTIONS:

Do you currently take any cholesterol/triglyceride lowering medications? YES NO

Are you pregnant or lactating? YES NO N/A

Do you smoke? YES NO

Do you currently take vitamins or nutritional supplements? YES NO
What are they? _____
_____

Have you taken antioxidant **supplements** regularly in the past 3-6 months? YES NO

Do you take more than 1 g/day of fish oil capsules? YES NO

Do you exercise ≥ 60 min/day? YES NO

Do you drink more than 1 oz of alcohol/day? YES NO
(1 oz alcohol = 2 beers or 10 oz of wine or 2 ½ oz liquor)

Do you have diabetes? YES NO UNSURE
We will confirm with fasting blood glucose

Do you have hypo/hyperthyroidism? YES NO UNSURE
We will check TSH

Day/ Date of Appointment: _____ ***Time:*** _____

Do you have any gastrointestinal problems? YES NO

Do you have anemia? YES NO

Are you suffering from any other disorder or illness? YES NO
(Cardiovascular disease, rheumatoid arthritis, etc.)

Do you have high blood pressure? YES NO
If controlled, what medications does the patient take?
_____

Are you taking any other medications on a regular basis? YES NO

If you are taking medications, what are they? And, how long have you been taking them?
_____
_____

Do you take aspirin? How often? Dose? _____
_____

Is the subject <u>ELIGIBLE</u> based on the questionnaire? YES NO

<u>ELIGIBILITY REQUIRES THE FOLLOWING FEATURES:</u>

(Check all that apply):

1. _____ **Waist circumference** **(Male ≥ 40 inches)** (Value:_____)
(Female ≥ 35 inches)

2. _____ **Blood glucose** **(≥ 126mg/dL or controlled by medications)** (Value:_____)

3. (For healthy controls)-

_____ **Waist circumference** **(Male ≤40 inches)** (Value:_____)
(Female ≤ 35 inches)

_____ **Blood glucose** **(≤100mg/dL)** (Value:_____)

_____ **Total cholesterol** **(< 200 mg/dL)** (Value:_____)

_____ **Triglycerides** **(<150 mg/dL)** (Value:_____)

Appendix C. Informed Consent Form

INFORMED CONSENT DOCUMENT

Project Title: Effects of pomegranate polyphenol supplementation on biomarkers of oxidative stress and inflammation in adults with type 2 diabetes versus healthy controls.

Investigators: Arpita Basu, PhD, RD
Nancy Betts, PhD, RD
Lenka Shriver, PhD

Purpose: This is a research study to find out the health effects of pomegranates. You are being asked to participate because you have high blood sugar levels and have a high risk of heart disease, or because you are healthy and we will study the effects of pomegranates in healthy people versus those with high blood glucose levels. Pomegranates have been shown in other studies to protect the cells against damage that occurs as a result of being overweight, or having high blood sugar. In this research study we will find out whether pomegranate supplementation will reduce those damage or risks.

Procedures: You may qualify for the study if you have the following features-

- Your waist measures greater than 35 inches (if you are a female)or 40 inches (if you are a male), and
- You have high blood sugar, or
- You take medicines for high blood sugar
- For healthy individuals- Your waist measures less than 35 inches (if you are a female) or 40 inches (if you are a male), and
- You do not have high blood sugar or blood lipids or take any medications for the same, and
- You are not suffering from any other disease

In order to qualify you should not have any other serious health problems like heart disease. If you qualify, you will be asked to take 2 capsules of pomegranate extract daily. This is a 4-week study and you will be coming to the Department of Nutritional Sciences or Seretean Wellness Center to get your supply of capsules, for blood draws, as well as for screening and biweekly visits, each for about half an hour, as stated below-

1. Screening- You will be asked to sign the consent form, and we will measure your height, weight, blood pressure, and waist. You will be asked to fast the previous night and about 3-4 tablespoons of blood will be taken for measuring your blood sugar, lipids, blood cell counts, and do some tests to find out how well your liver and kidney are working. If you qualify, we will let you know over the telephone and ask you to come back for the capsules. You will also be asked to record your food intake before you start the study and will also be told how to do it.

2. 2 weeks- You will return at 2 weeks to turn in 3-day food records and for a brief talk on how you are doing in the study.

3. 4 weeks- This will be your final visit and you will give us your 3-day food records. You will be asked to fast the previous night and about 3-4 tablespoons of blood will be taken for measuring

your blood sugar, lipids, and do some tests to find out how well the cells in your body are working. We will also measure your waist, body weight and blood pressure.

If you do not qualify for the study after the screening visit, you will be informed over the telephone and you will also receive a copy of your blood reports. However, if you do not qualify, all other information we have collected will be immediately destroyed.

Risks of Participation: You may experience slight pain during the blood draw. But, a trained nurse will be there for any help. Also, you will not be allowed to participate in the study if you have any blood-related health problems. You may experience slight stomach problems like flatulence if you are not used to taking pomegranate juice or extracts. But, these symptoms may soon go away.

Benefits: You may benefit from the pomegranate capsule, which may reduce some cell damage in your body. Since, this is a research study, we cannot guarantee benefit before we get study results. But, the results of this study will greatly benefit research by providing information on the health benefits of pomegranates.

Confidentiality: The records of this study will be kept private. Any written results will discuss group findings and will not include information that will identify you. Research records will be stored securely and only researchers and individuals responsible for research oversight will have access to the records. It is possible that the consent process and data collection will be observed by research oversight staff responsible for safeguarding the rights and wellbeing of people who participate in research.

Compensation: You will be compensated for $30 at screen, 2 & 4 weeks of the study.. This payment will be made to you in cash and you will need to sign a form as a receipt. No funds have been set aside by Oklahoma State University to compensate you in the event of illness or injury resulting from this study.

Contacts: If you have any questions about the research and the subject's rights, please contact Arpita Basu, PhD, at 405-744-4437 (9AM-5PM, Monday –Friday) or at 916-607-4143 (any time).If you have questions about your rights as a research volunteer, you may contact Dr. Shelia Kennison, IRB Chair, 219 Cordell North, Stillwater, OK 74078, 405-744-1676 or irb@okstate.edu.

Participant Rights: Your participation in this research is voluntary and you may discontinue the research activity at any time without reprisal or penalty. No risks will be involved due to your withdrawal from the study. Your participation may be terminated if you develop any allergy towards the pomegranate capsule or if you fail to make the visits as per schedule.

Signatures:

I have read and fully understand the consent form. I sign it freely and voluntarily. A copy of this form has been given to me.

Signature of Participant Date

I certify that I have personally explained this document before requesting that the participant sign it.

Signature of Researcher Date

Appendix D. Food Diary Form

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As a part of this study, you will be asked to keep a Diary of *everything* you eat and drink for **3 days**. These 3 days should include 2 weekdays and 1 weekend day, *example*: Thursday, Friday and Saturday.
Begin with the first food or beverage in the morning and write down what you eat as you go through the day.
The Nutritionist will review your completed Food dairy.

GENERAL INSTRUCTIONS FOR RECORDING FOOD INTAKE

1. Please record on the Food Diary Form the place (home, home of a friend, restaurant) of each meal and snack.

2. Record one food item per line on the Food Diary Form. Space is provided on both sides of the form. Be sure to include gum, candy and beverages.

3. Record the amount and food item on the Food Diary Form using common household measurements, for example: Tablespoons, cups, package size etc.

4. Remember to record everything you possibly can about a food. The more detail you include the better.

5. When you record an item, please note if it was baked, boiled, broiled, fried, or roasted. This is extremely important, especially for meats.

6. Record any additions to a food item. This **would** include sugar, relish, margarine, butter, catsup, pickles, mayonnaise, mustard, gravies, cream, etc., which were served with the food.

7. When eating out, record the menu item and amount eaten. Refer to Hints for Eating Out.

8. List the method of mixing a package mix if it is different form the directions given on the package. You may record this on a Recipe Form.

9. Use the Recipe Form to record any homemade items you have prepared. Measure each ingredient and record the method of preparation on the bottom of that form.

10. If you have any questions, please call Arpita Basu at 405-744-4437.

HINTS FOR EATING OUT

1. Record the name of the restaurant.

2. Quiz the wait staff regarding portion sizes.

3. Record amounts in standard household measurements, ie: teaspoons (ts), tablespoons (Tb), ounces, cups, etc.

4. For items such as bacon, rolls, and cucumbers, record the number of each item eaten.
   For example: 3 small white rolls
   4 cucumber slices
   2 medium bacon slices

5. For meats, record the dimensions of the cooked meat. Do not include the bone.
   For example: 2 slices of roast beef 4" x 3" x ¼". State the weight of the meat if it is mentioned on the menu.

6. Refer to the Food Description Flow Charts to describe your food.

7. For national fast food restaurants, (i.e. McDonald's, Arby's, Burger King), record the name of the sandwich/item you ate (i.e. Big Mac, Whopper).

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Food Diary

Name: _____ ID#_____ Protocol No:_____

Date of Record:_____ Day of Week:_____

Please record everything you eat today. Please include descriptions, brand names, and weighed and measured amounts (Please save labels). In the first column under meal and place, please put what meal you ate and where you ate it. You may use the codes at the bottom of the page for convenience. Thank you.

Meal* Place*	Amount	Food & Beverage Description	Office Use Only

*Meal Codes:
Breakfast - BR
Morning Snack – MS
Lunch – LU
Afternoon Snack –AS
Supper – SU
Evening Snack – ES

*Place Codes:
Home – HO
Restaurant – RE (Please Specify name of Restaurant)
Friends –FR
Work- W

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Food Diary Continued-Page 2

Name: _____ **ID#**_____ **Protocol No:**_____

Meal* Place*	Amount	Food & Beverage Description	Office Use Only

Appendix E. Participant Recruitment Flyer

Are you overweight? Do you have high blood sugar? Are you healthy?

All assessments will be free of charge. Study participants will receive a compensation every two weeks.

* Find out if you qualify for this 4 week study which will test the health effects of pomegranates.

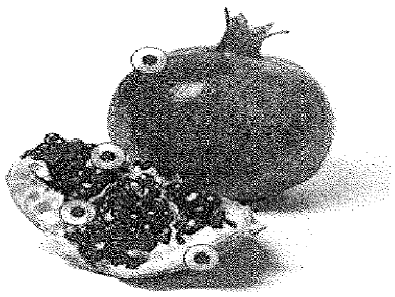
You may qualify for this study if you are a male or female with the following features:

- A waist circumference of greater than **40 in.** in men or **35 in.** in women, and
- fasting blood glucose ≥ 126 or on medications to lower blood Glucose, or
- You are healthy with normal body weight and no disease

CONTACT DR. ARPITA BASU

405-744-4437

All assessments will be free of charge. Study participants will receive a compensation every two weeks.



Oklahoma State University ... Nutritional Sciences... Stillwater...Oklahoma

VITA

Emily Dawn Newman

Candidate for the Degree of

Master of Science

Thesis: EFFECTS OF POMEGRANATE POLYPHENOL SUPPLEMENTATION ON BIOMARKERS OF OXIDATIVE STRESS AND INFLAMMATION IN ADULTS WITH TYPE 2 DIABETES VERSUS HEALTHY CONTROLS

Major Field: Nutritional Sciences

Biographical:

Personal Data: Born in Maysville, Kentucky, on October 19, 1985, the daughter of Danny and Sharon Grooms.

Education: Graduated from West Union High School, West Union, Ohio in 2004; received Bachelor of Science in Dietetics from Bowling Green State University, Bowling Green, Ohio in May of 2008. Completed the requirements for the Master of Science in Nutritional Sciences at Oklahoma State University, Stillwater, Oklahoma in July, 2010.

Experience: Dietetic Intern with Oklahoma State University, 2009. Employed by Oklahoma State University, Department of Nutritional Sciences as a graduate research assistant, 2009 to present. Employed by INTEGRIS Bass Baptist, Enid, Oklahoma, as per-diem registered dietitian, 2009 to present.

Professional Memberships: American Dietetic Association, Oklahoma Dietetic Association, American Society for Nutrition.

Name: Emily Dawn Newman Date of Degree: July, 2010

Institution: Oklahoma State University Location: Stillwater, Oklahoma

Title of Study: EFFECTS OF POMEGRANATE POLYPHENOL SUPPLEMENTATION ON BIOMARKERS OF OXIDATIVE STRESS AND INFLAMMATION IN ADULTS WITH TYPE 2 DIABETES VERSUS HEALTHY CONTROLS

Pages in Study: 93 Candidate for the Degree of Master of Science

Major Field: Nutritional Sciences

Scope and Method of Study: Pomegranates have significant anti-atherosclerotic, anti-hypertensive, anti-oxidative, and anti-inflammatory effects. We tested the hypothesis that pomegranate polyphenols will not affect fasting blood glucose, insulin, or lipid profile, while decreasing biomarkers of oxidative stress and inflammation in subjects with abdominal adiposity and type 2 diabetes versus healthy controls. Nine healthy controls and 8 subjects with type 2 diabetes were assigned to receive 2 capsules of pomegranate polyphenols (POMx™, 1 capsule =753 mg polyphenols) daily for 4 weeks. Blood draws and anthropometrics were performed at baseline and at week 4.

Findings and Conclusions: The POMx™ capsule was well tolerated by all participants and safety parameters remained within normal limits. Pomegranate polyphenol supplementation in healthy controls or type 2 diabetics did not significantly ($p > 0.05$) affect plasma glucose (85.11 ± 4.48 to 85.67 ± 8.03 mg/dL, or 105.88 ± 29.57 to 114.25 ± 33.36 mg/dL, respectively) or total cholesterol (187.89 ± 30.16 to 195.56 ± 30.5 mg/dL, or 182.13 ± 40.47 to 181.50 ± 36.73 mg/dL, respectively) or plasma triglycerides (77.11 ± 18.43 to 77.56 ± 26.88 mg/dL, or 149.75 ± 94.73 to 135.63 ± 66.06 mg/dL, respectively). Baseline levels of malondialdehyde (MDA) and 4-hydroxynonenal (HNE) were significantly higher ($p < 0.05$) than that of the healthy controls at baseline (1.76 ± 0.02 µM to 1.06 ± 0.26 µM versus 0.96 ± 1.02 µM to 1.02 ± 0.15 µM, respectively). The diabetic subjects experienced a significant ($p < 0.05$) decrease in levels of MDA and HNE after supplementation with POMx™; however, no significant difference was detected in the healthy controls. At week 4 mean MDA and HNE levels were similar to that of the healthy controls. Significant differences were not observed in oxidized LDL or C-reactive protein in either group. Our findings suggest that POMx™ reduces lipid peroxidation in diabetic subjects, which could be beneficial in reducing risk of developing cardiovascular complications. Larger clinical trials should be conducted to confirm our results.

ADVISER'S APPROVAL: Dr. Arpita Basu