THE EFFECTS OF DIETARY COCOA POWDER ON INFLAMMATORY MARKERS AND CATALASE ACTIVITY IN PARTICIPANTS WITH TYPE-2 DIABETES FOLLOWING A FAST FOOD STYLE MEAL CHALLENGE

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Dietary polyphenols, such as those in cocoa, have been shown to counteract chronic inflammation in animal models as well as in clinical studies. However, limited data exist on the role of dietary cocoa on postprandial inflammation and oxidative stress. Using a randomized placebo-controlled crossover trial, we investigated the postprandial effects of a cocoa beverage (960 mg total polyphenols) vs. placebo in participants with type 2 diabetes (n=18; age (mean±SE): 56±3y; BMI: 35.3±2.0 kg/m^2; 14 women; 4 men) following a high-fat meal challenge. Blood draws were conducted at fasting, 30 minutes, 1, 2, 4, and 6 hours of the postprandial phase. We measured selected markers of inflammation and catalase enzyme activity at these time points using the quantitative sandwich enzyme immunoassay technique, as well as non-enzymatic colorimetric assays. Interleukin-18 (IL-18) was shown to be significantly lower at 1, 4, and 6 hours, while C-reactive protein was lower at 6 hours postprandially after cocoa vs. placebo supplementation. Additionally, catalase activity was lower at 1 hour postprandially (all $p<0.05$). No significant effects were noted in interleukin-6 (IL-6), adiponectin, and nitrite concentrations between the two treatments. Thus, cocoa polyphenols may exert modest effects in reducing postprandial inflammation and in modulating catalase activity in type 2 diabetes.

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CHAPTER I

INTRODUCTION

Diabetes is one of the leading causes of morbidity and mortality in the U.S. (National Diabetes Surveillance System, 2012). The prevalence of diabetes is now 9% in the U.S. population, approximately double what it was 20 years ago. Adults and children with diabetes are at an increased risk of developing cardiovascular disease (CVD) (American Diabetes Association, 2014), which further increases the risk of mortality, as CVD is currently the leading cause of death in the U.S. (Centers for Disease Control and Prevention, 2013).

Many risk factors for developing diabetes such as obesity, poor dietary quality, and lack of physical activity, are the same as those for developing CVD (American Heart Association, 2013). Some of these risk factors can be decreased through healthy diet and exercise. Consuming foods and beverages high in antioxidants has been found to decrease the risk of developing CVD and to delay overall mortality (Bazzano et al. 2002). Polyphenols found in plants, such as apples, berries, coffee, onions, and tea possess antioxidant properties and have been associated with cardiovascular benefits (Haytowitz et al. 2014, Scalbert & Williamson 2000).

Dietary cocoa has gained popularity in nutrition research because of its high polyphenol content (Lee, K. W. et al. 2003). Cocoa powder has been shown to decrease oxidation (Wan et al. 2001, Mathur et al. 2002) and improve blood pressure (Taubert et al. 2003, Grassi et al 2005) in clinical studies of non-diabetic adults. It has also been shown to improve circulation in clinical studies of

non-diabetics adults (Heiss et al 2003, Westphal et al. 2011) as well as diabetic adults (Balzer et al 2008). Antioxidants have been shown to decrease inflammation (Mates et al. 1999, Mangge et al. 2014), and a few studies have shown effects of cocoa powder in reducing inflammation (Rahman et al. 2006, Parsaeyan et al. 2014).

Postprandial metabolism refers to the metabolism that occurs after a meal (American Diabetes Association, 2001). People with type 2 diabetes (T2D) typically have high postprandial plasma glucose levels (Woerle et al. 2005) along with high postprandial lipid levels (Mero et al. 1998). Studies suggest that postprandial lipidemia increases the risk of developing CVD (Hyson et al. 2003). Polyphenols have been shown to reduce postprandial hyperglycemia, hyperinsulinemia, and hyperlipidemia (Torronen et al. 2010, Hlebowicz et al. 2007, Yi et al. 2013, Murase et al. 2012). Since cocoa powder is one of the highest sources of polyphenols (Lee et al., 2003), it may be beneficial in reducing postprandial lipidemia and thus in reducing the risk of developing CVD.

Purpose

The purpose of this study is to examine the effects of dietary cocoa powder on postprandial inflammation markers and catalase activity in participants with type 2 diabetes following a high-fat meal challenge.

Null Hypotheses

1. Consumption of cocoa powder with a high-fat meal will not affect postprandial blood levels of C-reactive protein, Interleukin-6, Interleukin-18, adiponectin, and nitrite when compared to controls.
2. Consumption of cocoa powder with a high-fat meal will not affect postprandial blood levels of antioxidant catalase activity when compared to controls.

CHAPTER II

REVIEW OF LITERATURE

A. Diabetes

The American Diabetes Association defines diabetes as, "a group of metabolic diseases characterized by hyperglycemia resulting from a defect in insulin secretion, insulin action, or both." (American Diabetes Association, 2014). Nearly 29 million people 20 years or older were estimated to have diabetes in the United States in 2012 and diabetes was the seventh leading cause of death in the United States in 2010. In 2003-2006 rates of death among adults with diabetes was 1.5 times higher than those without diabetes (National Diabetes Statistic Report, 2014). Type 2 diabetes (T2D) is the most common form of diabetes. Most people with T2D are either obese or they have increased central body fat. Obesity has been associated with insulin resistance. People with diabetes have an increased risk of developing atherosclerosis and other vascular diseases, as well as hypertension and abnormal lipoprotein metabolism (American Diabetes Association, 2014).

B. Phytochemicals

Phytochemicals are plant-based compounds with various physiological functions and have been shown to reduce risks of chronic diseases (Scalbert et al. 2000). Polyphenols are the largest group of phytochemicals in the human diet (King & Young 1999), with an average intake of about one gram per day in the U.S. (Scalbert et al. 2000). Flavonoids are a sub-type of polyphenols, which have antioxidant properties and subsequent decreases in oxidative stress. Subclasses of flavonoids include the following: flavanols, flavonals, flavanones, flavones, isoflavones, & anthocyanidins. Total flavonoid intake is about 132 mg per day in the U.S., with

black tea, onions, parsley, and blueberries being some of the main sources (Haytowitz et al. 2014).

Most plant foods contain a variety of phytochemicals. The phytochemical composition can vary based on where the plant was grown, its ripeness, storage, and processing. For example, roasting cocoa beans causes a 10-30% reduction in polyphenol content due to exposure to high temperatures and oxygen, which accelerates oxidation (Bordiga et al. 2015). Many foods are not eaten whole, but rather included as a part in another food or dish. The effects of food components on the bioavailability of polyphenols are controversial. It has been suggested that lipid and carbohydrates may increase absorption, while fiber may decrease absorption. The effects of protein on polyphenol bioavailability have shown conflicting results depending on the protein and polyphenols involved (Bohn, 2014). For example, in the case of chocolate, dark chocolate typically has about two-thirds the polyphenol content of pure cocoa powder, while milk chocolate has only about one-third the polyphenols of pure cocoa powder. The polyphenol content of chocolate and cocoa powder vary widely depending on the brand, processing methods that were used, and where the cocoa beans originated (Brcanovic et al. 2013).

Several observational studies reveal significant associations of various types of phytochemical intake, such as flavonoids with the risk reduction of chronic diseases. A prospective study of postmenopausal women found that dietary intake of foods high in flavonoids was associated with a reduced risk of coronary heart disease (CHD), cardiovascular disease (CVD), and death from all causes (Mink et al. 2007). A study of elderly men found that tea consumption high in flavonoids decreased the risk of ischemic heart disease (Arts et al. 2001). An inverse relationship has been found between flavonoid intake and risk of developing lung cancer (Marchand et al. 2000), colon cancer (Kyle et al. 2010), and T2D (Liu et al. 2014). Thus, polyphenols may be suggested to those at risk, as well as to the general population, as a preventative measure against chronic diseases.

C. Antioxidants

Antioxidants can be defined as "any substance that delays, prevents or removes oxidative damage to a target molecule" (Halliwell, 2007). Antioxidants moderate levels of reactive oxygen species (ROS) and are usually reducing agents. Antioxidants can be either exogenous or endogenous (Bouayed et al. 2010). Exogenous antioxidants include compounds such as vitamin E, vitamin C, carotenoids, and polyphenols. Endogenous antioxidant systems include enzymes, such as superoxide dismutase (SOD), glutathione peroxidase (GPx), and catalase (CAT) (Bouayed et al. 2010). Several enzymes in the vasculature including NADPH oxidase, uncoupled endothelial nitric oxide synthase (eNOS), xanthine oxidase, and the mitochondrial electron chain, produce reactive oxygen species (ROS). Increased ROS have been found in CVD and diabetes (Li et al. 2014). Oxidative stress occurs when there are more ROS than antioxidants; and oxidative damage occurs when there is damage caused due to oxidative stress (Halliwell, 2007). Oxidative damage contributes to the progression of chronic diseases including atherosclerosis (Li et al. 2014).

Catalase (CAT) is an endogenous antioxidant enzyme (Bouayed et al. 2010) that belongs to the group of monofunctional heme-containing catalases (Kodydkova et al. 2014). The primary function of CAT is to breakdown hydrogen peroxide into water and oxygen. CAT is found in all aerobic organisms mainly in peroxisomes (Kodydkova et al. 2014). CAT activity is most prominent when hydrogen peroxide levels are high while GPx has been found to be the main reducing enzyme for hydrogen peroxide at normal levels (Cohen et al. 1963).

D. Post-Prandial Metabolism

Postprandial metabolism signifies the metabolism that occurs after consumption of a meal (American Diabetes Association, 2001). People with T2D typically have high postprandial plasma glucose levels (Woerle et al. 2005). Postprandial glucose concentrations are associated

with mortality even more so than fasting glucose concentration (Borch-Johnson et al. 1999). Postprandial hyperglycemia is also associated with postprandial lipemia (Mero et al. 1998). Studies have shown the associations between impaired postprandial metabolism and coronary artery disease (Karpe, 1999). Even for people who do not have diabetes, but with CHD, higher postprandial hyperglycemia has been correlated with progression of atherosclerosis (Sasso et al. 2004).

Insulin resistance in diabetes causes an increase in free fatty acids in circulation. Insulin resistance is associated with a failure to suppress hormone sensitive lipase, thus leading to an increase in serum endogenous non-esterified fatty acids (Sanders, 2003); it is also associated with an increase in lipoprotein lipase, which hydrolyzes chylomicrons in the postprandial state, also leading to increased free fatty acids (Hyson et al 2003). These taken together lead to an increase in serum triglycerides and an increase in VLDL production by the liver. Insulin is also needed to inhibit microsomal triglyceride transfer protein, a protein involved in VLDL synthesis in the liver, thus also leading to increased VLDL (Choi et al. 2011). Due to exchange of lipids with HDL, high levels of dietary lipids and VLDL can lead to an decrease in HDL and an increase in LDL, which leads to increased cardiovascular risks (Hyson et al. 2003).

After consuming a high-fat meal, the initial rise in serum triglycerides occurs after 10-30 minutes, and the postprandial spike in serum triglycerides occurs 3-4 hours after a meal (Lambert, 2012). Many studies suggest that postprandial lipidemia increases the risk of developing atherosclerosis and CVD (Hyson et al. 2003). Polyphenols have been shown to reduce postprandial hyperglycemia, hyperinsulinemia, and hyperlipidemia (Torronen et al. 2010, Hlebowicz et al. 2007, Yi et al. 2014, Murase et al. 2012). Polyphenols may decrease hyperglycemia and hyperlipidemia by interacting with digestive enzymes and membrane receptors, activating insulin receptors, stimulating insulin secretion, and decreasing pancreatic lipase activity (Fraga et al. 2010, Marrelli et al. 2013, Hanhineva et al. 2010). Many polyphenols have been found to bind to

digestive enzymes, modulating their activity as well as bind to and decrease the activity of lipases (Bandyopadhyay et al. 2012). In vitro studies have found flavonoids to decrease α-amylase and α-glucoosidase activity, two essential enzymes in carbohydrate digestion. Other polyphenols have been found to decrease SGLT1 and GLUT2 glucose transport and to preserve β-cell function. Collectively these actions reduce blood glucose levels by reducing carbohydrate absorption (Hanhineva et al. 2010). However, few studies have been reported on the role of dietary polyphenols in postprandial metabolism in human subjects with diabetes.

E. Inflammation

Inflammation is part of a non-specific immune response that occurs with injury. Under normal conditions, inflammation is helpful in wound healing, but chronic inflammation can lead to disease (Ferrero-Miliani et al. 2006). Obesity induces a state of low-grade inflammation. Inflammation has also been associated with metabolic syndrome, insulin resistance (Inglesson et al. 2008), T2D, and CVD, as well as many other chronic conditions (Rodrigues-Hernandez et al. 2013).

Obese individuals have higher levels of inflammatory cytokines than normal weight individuals (Rodrigues-Hernandez et al. 2013). Cytokines include a large group of proteins that may promote inflammation, suppress it, or exert a combination of the two processes. Many cytokines are produced in adipose tissue, and thus increased body fat can increase their production (Aldhahi et al. 2003). Interleukin-6 (IL-6) is a cytokine that is often used as a biomarker of inflammation in research studies, especially involving diabetes and CVD. It is secreted linearly with adipose tissue, more so in intra-abdominal fat than in subcutaneous fat. IL-6 interferes with insulin signaling by decreasing expression of the IRβ (insulin receptor β) subunit and IRS-1 (insulin receptor substrate 1) and the GLUT4 glucose transporter (Lagathu et al. 2003). IL-6 increases lipolysis through activation of extracellular signal-related kinases (Yang et al. 2008), and it has been shown to decrease lipoprotein lipase (Greenberg et al. 1992, Aldhahi et al. 2003, Trujillo et

al. 2004). Additionally, IL-6 may increase macrophage uptake of lipids (Yudkin et al. 2000, Keidar et al. 2001). These actions of IL-6 contribute to the development of CVD. Interleukin-18 (Il-18) is another cytokine that is secreted in response to inflammation, and it has also been shown to be associated with several inflammation-associated disorders including atherosclerosis (Alboni et al. 2010).

C-reactive protein (CRP) is an acute-phase reactant that is regulated by cytokines, including IL-6 (Rodrigues-Hernandez et al. 2013). CRP is widely measured in clinics to evaluate inflammation. CRP has also been shown to be elevated in people with high levels of HbA1c, as is found in diabetes (King et al. 2003, Pradhan et al. 2001).

Adiponectin is a protein produced in adipose tissue that can be classified as a hormone because of its effects on other tissues (Guerre-Millo, 2008). Studies have shown adiponectin to be low in patients with heart disease, diabetes, hypertension, and metabolic syndrome. Adiponectin has also been shown to be inversely associated with BMI, as well as with inflammation markers such as TNF-α, IL-6, and CRP. Roles of adiponectin include stimulating nitric oxide (NO) production, suppressing superoxide generation within cells, and decreasing uptake of oxidized LDL, thus leading to decreased foam cell formation. Adiponectin has also been shown to be antithrombotic and to increase insulin sensitivity (Nishida et al. 2007).

Nitric oxide (NO) is a cellular signaling molecule produced in the endothelium of healthy blood vessels. NO is also known as "endothelium-relaxing factor". However, there are two main forms of nitric oxide synthases: endothelium nitric oxide synthase (eNOS) and inducible nitric oxide synthase (iNOS). NO from eNOS is associated with endothelium dilation and platelet anti-aggregation, while NO from iNOS is associated with inflammation (Forstermann & Sessa 2011, Vallance, 1994). Endothelium dysfunction and insulin resistance often coexist (Yki-Jarvinen, 1998). Hyperglycemia in diabetes has been found to increase reactive oxygen species (ROS)

leading to endothelium dysfunction. Endothelium dysfunction and increased ROS lead to reduced NO, which leads to vasoconstriction and increased risk of CVD (Pitocco, 2010). Also, if the pathway of synthesis of NO is disturbed, then NOS may also produce reactive oxygen species (ROS). This is because NOS uses NADPH, and in the presence of oxygen a reactive superoxyferrous-peroxy ferric complex is formed. This NADPH and oxygen complex is referred to as NADPH oxidase. If the NOS cofactor or substrate, tetrahydrobiopterin and L-arginine respectively, are missing, then this complex can form superoxide anions or hydrogen peroxide. NO may then react with superoxide anions to form peroxynitrite, which is highly toxic. High rates of NOS pathway disturbances are often seen with risk factors for atherosclerosis, such as smoking, obesity, hypertension, and hypercholesterolemia (Rassaf et al. 2008, Forstermann & Li 2011). Insulin plays a part in activating NOS, while NO signaling enhances insulin sensitivity (Levine et al. 2012). Thus, NO has a significant role in the development and progression of T2D.

Table 1. Correlation of inflammation with cardiovascular disease (CVD) and type 2 diabetes (T2D)

Author (year)	**Study design**	**Population characteristics**	**Inflammatory Exposure**	**Outcome (CVD & diabetes)**
Yudkin et al. (1999)	Cross-sectional clinical follow-up of CVD risk factors	Nondiabetic middle-aged men (n= 59) and women (n=48) subjects, age 40-75y	↑ CRP and IL-6	↑ likelihood for insulin resistance
Duncan et al. (2003)	Prospective, case-cohort study lasting 9y	Atherosclerosis Risk in Communities Study Participants (n=10275), age 45-64y	↑ IL-6	↑ risk of T2D (except in African Americans and smokers)
Pradhan et al.	Prospective,	Women over	↑ CRP and IL-6	↑ risk of T2D

(2001)	nested case-controlled, cohort study lasting 4y	age 45y who developed T2D (n=188) and controls who did not (n=362)		
King et al. (2003)	Cross-sectional analysis of population data	NHANES III subjects (n=1,018) over age 17y with diabetes (or who previously had diabetes, excluding gestational)	↑ CRP	↑ likelihood of HbA1c > 9.0%
Mendall et al. (1996)	Population based cross-sectional study	Men (n=388) age 50-69y	↑ CRP	↑ risk of CHD
Daimon et al. (2003)	Prospective, cohort study lasting 5y	Men (n=769) and women (n=1,023) age 46-71y	↓Adiponectin	↑ risk of T2D

Footnote: CRP – C- reactive protein; IL-6 – Interleukin – 6; T2D – Type 2 Diabetes; CHD – Coronary Heart Disease; NHANES – National Health and Nutrition Examination Survey

F. Cocoa

The cocoa bean is a seed of the *Theobroma cocoa* tree, largely found in Mesoamerica and South America. Cocoa drink was popular among the Mayan and Aztec populations. The Mayans believed that the gods discovered cocoa. During the 16th century, cocoa drinks were often used as a medicine to treat stomach problems in Mesoamerica. Over the decades, cocoa has been believed to cure multiple ailments including colds, insomnia, obesity, scurvy, and stomach cancer (Dillinger et al. 2000).

The antioxidant capacity of cocoa powder is attributed to its phenolic compounds, especially the flavonoids. The flavonoids in cocoa powder have been identified as catechins, epicatechins, dimers, and trimers. Methylxanthines, including caffeine, theobromine, and theophylline, are

also present in cocoa powder but they all either have a low antioxidant capacity or could reduce the antioxidant capacity of flavonoids (Maleyki et al. 2010).

It has been found that cocoa powder has more phenolic phytochemicals and a higher antioxidant capacity than tea or red wine (Bhagwat et al. 2013, Lee et al. 2003). In a study by Lee et al., cocoa was reported to contain 611 mg of gallic acid equivalents (GAE) and 564 mg of epicatechin equivalents (ECE), which was about twice the flavonoid content and antioxidant capacity found in red wine, about three times that of green tea, and about four times that of black tea (Lee et al. 2003).

A study by Schroeter and colleagues showed that (-)-epicatichin is likely the flavanol metabolite responsible for the cardiovascular effects seen after cocoa consumption. Pure (-)-epicatechin administered to volunteers mimicked the effects of flavanol-rich cocoa. Long-term intake of high-flavanol diets has been shown to promote nitric oxide synthesis (Schroeter et al. 2005). This may be explained by the role of (-)-epicatechins in inhibiting NADPH oxidase, which as explained previously, is shown to decrease NO (Rassaf et al. 2008). Specifically, (-)-epicatechin has been shown to scavenge the $O_2^{\bullet-}$ formed from NADPH oxidases that binds to NO to form peroxynitrite, while *O*-methyl ethers of (-)-epicatechin inhibit NADPH oxidase activity directly (Steffen et al. 2007).

Flavanol intake correlates with decreased risks of CVD, cancer, and diabetes in observational studies (Bazzano et al. 2002, Mink et al. 2007, Buijsse et al. 2006, Djoussé et al. 2011, Larsson et al. 2012, Mostofsky et al. 2010). This has also been supported by the traditional dietary cocoa consumption among the Kuna Indians living in the San Blas islands off the coast of Panama. The Kuna who live on the islands are habitual cocoa drinkers, a source of approximately 900 mg flavanols a day. This has been shown to be associated with a significantly lower risk of CVD in

this island population, compared to those living on the mainland (Bayard et al. 2007). Many other studies have shown benefits of cocoa as illustrated in Tables 2 and 3 below.

Table 2. Benefits of cocoa on cardiovascular disease (CVD) risk factors in otherwise healthy subjects.

Author, Year	Study Design and Duration	Subject Characteristics	Intervention	Effect
Wan et al. (2001)	Randomized, cross-over; 10 weeks (including a 2 week washout period)	Healthy men (n=10) and women (n=13) volunteers, age 21-62y	An AAD with or without 20 g cocoa powder & 16 g DC /d	↓LDL-C oxidation; ↑ serum antioxidant capacity; ↑ HDL-C
Mursu et al. (2004)	Non-randomized, 3 weeks	Healthy men (n=12) and women (n=33) adult volunteers, age 19-49y	75 g of white chocolate, DC, or DC enriched with polyphenols /d	↑HDL-C in DC and even more so in enriched DC
Mathur et al. (2002)	Pre and post test, 6 weeks (followed by a 6 week washout period)	Healthy men (n=12) and women (n=13), age 20-60y	36.9 g DC and 30.95 g cocoa powder drink	↓LDL-C oxidation; no effect on CRP or IL-6
Westphal et al. (2011)	Randomized, placebo-controlled, double-blind, crossover, measured over 6 hrs. postprandially on two separate days	Healthy adult men (n=2) and women (n=16) volunteers, age 22-28y	3 mL whipping cream per kg body weight plus 9.18 g cocoa powder, or whipping cream only	↑FMD; no effect on TG or FFA

	(with at least a week long washout period)			
Vlachopoulos et al. (2005)	Randomized, single-blind, crossover, on two separate days	Healthy adult men (n=12) and women (n=5), age 24-32y	100 g DC or sham eating (pretending to chew)	↑FMD
Sansone, et al. (2015)	Randomized, placebo-controlled, double blind, one month	Healthy adult men (n=55) and women (n=50), age 35-60y	Cocoa beverage (450 mg flavanol) or control twice a day	↑FMD

Footnote: AAD – Average American Diet; DC – dark chocolate; CRP – C- reactive protein; IL-6 – Interleukin – 6; FMD – Flow mediated dilation; TG – triglycerides; FFA – free fatty acids

Many additional studies examining the effect of cocoa powder or chocolate on health outcomes have been done in populations at risk for CVD.

Table 3. Benefits of cocoa in subjects with cardiovascular disease (CVD) risk factors

Author, Year	**Study Design and Duration**	**Subject Characteristics**	**Intervention**	**Effect**
Mellor et al. (2010)	Randomized, placebo-controlled, double-blind, cross-over; 20 weeks (including a 4 week washout period)	T2D men (n=7) and women (n=5), age 41-72y	45 g high polyphenol DC vs. placebo	↑HDL-C; ↓total cholesterol/ HDL-C ratio
Grassi et al. (2005)	Randomized, cross-over, 37 days (including a 7 day washout period)	Men (n=10) and women (n=10) volunteers with essential hypertension, mean age= 43y	100 g DC or 90 g flavanol-free white chocolate	↓ BP & LDL-C; ↑FMD & insulin sensitivity

Balzer et al. (2008)	Randomized, placebo-controlled, 30 days	Diabetic patients (n=41), age 50-80y	Cocoa powder beverage (321 mg flavanol) or placebo three times/d	↑serum flavanols & FMD
Taubert et al. (2003)	Randomized, cross-over trial; 5 weeks (including 1 week washout period)	Adult men (n=6) and women (n=7) with stage 1 hypertension; age 55-64y	100 g DC or 90 g white chocolate	↓ BP
Hermann et al. (2006)	Randomized, parallel study; single dose measured over 8 hrs.	Male smokers (n=20), mean age = 26y	40 g DC or 40 g white chocolate	↑FMD
Heiss et al. (2003)	Randomized, double-blind, cross-over, placebo controlled, measured at 2 hrs.	Outpatients with at least 1 cardiovascular risk factor (n=20), mean age= 41y	100 mL flavanol-rich cocoa drink or placebo	↑circulating NO and ↑FMD
Heiss et al. (2005)	Randomized, double-blind, cross-over, placebo controlled, measured at baseline & 2 hrs. on two separate days	Smoker men (n=6) and women (n=5) volunteers, mean age= 31y	100 mL high flavanol drink (176 to 185 mg flavanols) or placebo	↑circulating NO and ↑FMD
Baba et al. (2007)	Randomized, controlled, parallel; 12 weeks	Normo-cholesterolemic or mildly hyper-cholesterolemic volunteers (n=25), mean age= 38y	26 g cocoa powder with 12 g sugar beverage or 12 g sugar beverage twice/d	↑HDL-C, & 9% LDL oxidation prolongation
Flammer et al. (2007)	Randomized, double-blind, controlled trial, measured at baseline & 2 hrs. on a	Heart transplant recipient men (n=18) and women (n=4), mean age= 54y	40 g DC or cocoa-free chocolate	↑coronary vasodilation, ↓platelet aggregation

	single day			
Njike et al. (2011)	Randomized, double-blind, cross-over, 6 weeks (including a 4 week washout period)	Overweight men (n=18) and women (n=98), mean age= 52y	Cocoa beverage sweetened with sugar or sugar-free sweetener, or cocoa-free placebo	↑FMD
Stote et al. (2012)	Randomized, cross-over, 5 5-day treatment periods each, separated by 10 days	Men (n=10) and women (n=10), age 25-55y, BMI > 27kg/m^2	Control; low, medium, or high flavanol cocoa beverage; or green tea	↓ CRP linearly with flavanol content
Parsaeyan, et al. (2014)	Randomized, controlled, 6 weeks	T2D men and women (n=100 total, equal ratio between the groups), mean age= 54y	10 g cocoa powder + 10 g milk powder, or 10 g milk powder only, mixed in hot water	↓ TNF-α, CRP, & IL-6
Rostami, et al. (2015)	Randomized, placebo-controlled, double blind, 8 weeks	T2D men (n=24) and women (n=36), age 35-70y	25 g DC (450 mg flavonoids) or white chocolate	↓CRP, BP, FBG, & HbA1c

Footnote: DC- dark chocolate; BP – blood pressure; FMD – flow mediated dilation; NO – nitric oxide; T2D – type 2 diabetes; CRP – C-reactive protein; TNF-α – tumor necrosis factor α; IL-6 – Interleukin-6; FBG – fasting blood glucose; HbA1c – hemoglobin A1c

People with T2D are at an increased risk of developing CVD (American Diabetes Association, 2014). This is in part because obesity and T2D are associated with an increase in inflammation (Rodrigues-Hernandez et al. 2013) and oxidation of biomolecules (Li et al., 2014). Phytochemicals have been shown to decrease inflammation and oxidative stress (Haytowitz et al., 2014). Cocoa powder is a rich source of phytochemicals and many studies have shown its beneficial effects in reducing inflammation markers (Parsaeyan et al. 2014), improving FMD

(Njike et al. 2011), and decreasing oxidation of biomolecules (Baba et al. 2007). Very few studies have examined the effects of cocoa powder postprandially. Postprandial hyperglycemia and hyperlipidemia are common in people with diabetes (Mero et al. 1998). Recurring postprandial hyperlipidemia increases the risks of CVD (Karpe, 1999). Polyphenols found in plant foods and beverages, such as berries, cinnamon, tea, and coffee, have been found to reduce hyperglycemia and hyperlipidemia in human studies, (Torronen et al. 2010, Hlebowicz et al. 2007, Yi et al. 2013), as well as in animal models (Benn et al. 2015, Qin et al. 2009, Murase et al. 2012, Takahashi et al. 2014).

Keeping in view the role of cocoa in reducing CVD risks in reported long-term clinical trials, there exists a strong rationale to examine its effects on postprandial metabolism, specifically on inflammatory and antioxidant biomarkers in diabetes. This remains the focus of the present study.

CHAPTER III

METHODOLOGY

3.1 Participants and study design

Participants were recruited at the Harold Hamm Diabetes Center at OU Health Sciences Center and at the Nutritional Sciences Clinical Assessment Unit at Oklahoma State University through campus wide email advertisements and physicians referral at Harold Hamm Oklahoma Diabetes Center. Adult type 2 diabetic patients not on insulin therapy were included in this study. Patients with clinical diagnosis of coronary heart disease (CHD), cancer, or neurodegenerative disorders were excluded. Patients on stable blood pressure medications, enlarged waist circumference (men>40 inches, women>35 inches), mild to moderate hyperlipidemia (total cholesterol <240mg/dL, triglycerides<200 mg/dL) not on hyperlipidemic agents were included.

Participants came in three different days for this study. On the first day (screen) patients were screened for abdominal adiposity and type 2 diabetes; they were also screened for safety parameters, such as liver and renal functions. On the second visit (Day 1) the patients were randomly assigned to consume either a cocoa beverage or control beverage with a high-fat, low polyphenol breakfast that was prepared at the clinic. Blood draws and vascular measurements were taken. On the next visit (Day 2) the patients were crossed over to the other treatment option, either the cocoa beverage or control beverage, consumed with a high-fat, low polyphenol breakfast that was provided. The two intervention visits were separated by a one-week washout

phase. On each day of the two-day postprandial phases, patients arrived at the clinic around 7:30 AM for a fasting blood draw, after which they consumed the breakfast with or without cocoa within 20-25 minutes, followed by postprandial blood draws. At each visit blood pressure and blood draws were taken at fasting, 1, 2, 4, & 6 hours postprandial. Subjects were monitored throughout the 6-hour postprandial phase at the clinic. Subjects were not allowed to eat or drink any other food or beverage, except water that was provided, during that time. Lunch was provided at the end of the six hours.

3.2 Meal and beverage composition

The breakfast meal consisted of one egg yolk, two slices of white bread with 13 grams of butter, one banana, and one pork sausage patty. The breakfast provided a total of 766 kcals and 50 grams of fat. As shown in Table 4, the cocoa and control beverages were similar in calories and fat, and while carbohydrates and protein were higher in the cocoa beverage, the ratio of carbohydrate to protein was nearly identical between the two beverages. The cocoa beverage contained significantly more polyphenols than the control beverage (960 vs 110 mg). The cocoa beverage contained flavanols, proanthocyanidins, epicatechins, and catechins (480 mg, 201 mg, 40 mg, and 18 mg respectively), while the control contained none or insignificant amounts of these compounds (<0.1 mg).

3.3 CRP, IL-6, IL-18, adiponectin, nitrite, and catalase

CRP values were previously measured using freshly drawn blood samples from participants in this study at the Oklahoma University Medical Center (OUMC) using standard clinical chemistry.

Using stored samples, IL-6, IL-18, and adiponectin were measured using ELISA kits (R&D Systems, Minneapolis, MN) based on the manufacturer's protocol. For the ELISA assay, 100 µL of Assay Diluent, then 100 µL of sample was added to each well in use. The plate was then covered with a sealer and incubated at room temperature for two hours. After incubation the plate was aspirated and washed four times before 200 µL Conjugate was added to each well in use. The plate was then covered with a plate sealer and incubated at room temperature for an additional two hours. After incubation the plate was again aspirated and washed four times. Next, 200 µL Substrate Solution was added to each well in use and the plate was incubated at room temperature for 20 minutes. Finally, 50 µL of Stop solution was added to each well and absorbance was read on a plate reader within 30 minutes at 540 or 570 nm.

Nitrite was measured using the Griess Reagent System (Promega BioSciences, LLC. San Luis Obispo, CA) based on the manufacturer's protocol. First, 50 µL of each sample was added in duplicate. Using a multichannel pipettor, 50 µL of Sulfanilamide Solution was added to each well in use followed by eight minutes of incubation protected from light. Using a multichannel pipettor, 50 µL of *N*-1-napthylethylenediame dihydrochloride (NED) was added to each well in use followed by eight minutes of incubation protected from light. Absorbance was immediately measured on a plate reader at 550 nm.

Catalase enzyme activity was measured using the Cayman Chemical Company Catalase Assay Kit (Ann Arbor, Michigan) based on the manufacturer's protocol. Each sample was diluted in Phosphate Buffer Solution 1:10. For each sample 100 µL of diluted Assay Buffer, 30µL methanol, and 20 µL of sample were pipetted into wells in duplicate. The reaction was started by adding 20 µL diluted hydrogen peroxide to all wells being used and then the plate was incubated on a plate shaker for 20 minutes. Then 30 µL of potassium hydroxide, followed by 30 µL of catalase purpald, was added to each well and the plate was returned to the plate shaker 10 minutes of incubation. Following the addition of 10 µL of catalase potassium periodate to each well being used, the plate was incubated on the plate shaker for an additional five minutes. Absorbance was immediately measured on a plate reader at 540 nm.

Statistical analysis

Data were analyzed by repeated measures ANOVA using SPSS software with a significance level set at $p < 0.05$. All data were expressed as means ± standard error. The objective was to identify significant differences in the means of inflammation markers and catalase at each time point within each group, as well as to identify differences in means between the cocoa and placebo group.

CHAPTER IV

FINDINGS

Baseline Characteristics

Among the participants screened, 18 met the inclusion and exclusion criteria and were invited to participate in the study. Tables 5a & b show the baseline characteristics of the participants who completed the study. All participants had T2D and were not on insulin therapy, but the majority (83%) were on oral hypoglycemic agents. Over half of the participants (56%) were on Angiotensin-Converting Enzyme Inhibitors or Angiotensin II Receptor Blockers. Five participants were on statins or fibrates. Additionally, a few participants were on diuretics, calcium channel blockers, or regularly took aspirin or multivitamins. All participants were overweight and had enlarged waist circumference. Overall, the participants consumed a high-fat diet (~37% fat). Fruit and vegetable intakes were low among these participants, averaging 0.6 and 1.6 cups per day respectively, in comparison to the USDA recommendations for this age group (1.5-2 cups per day fruits and 2-2.5 cup per day vegetables) (United States Department of Agriculture, 2010).

Interleukin-18 (IL-18)

As shown in Figure 1, serum levels of IL-18 were significantly different between cocoa and placebo at one, four and six hours postprandial ($p<0.05$). At these time points, cocoa intervention

resulted in lower levels of IL-18 compared to the placebo treatment. These results persisted after adjusting for BMI and HbA1c as covariates.

Catalase activity

As shown in Figure 2, serum catalase activity was significantly different between cocoa and placebo at one hour postprandial ($p<0.05$). At this time point, cocoa intervention resulted in a lower catalase activity compared to the placebo treatment. These results persisted after adjusting for BMI and HbA1c as covariates.

C-reactive protein (CRP)

As shown in Figure 3, serum levels of CRP were significantly different between cocoa and placebo only at six hours postprandial ($p<0.05$). At this time point, cocoa intervention resulted in lower levels of CRP compared to the placebo treatment. These results persisted after adjusting for BMI and HbA1c as covariates.

Adiponectin, Interleukin-6 (IL-6), Nitrite

As shown in Figures 4, 5, and 6, serum IL-6, adiponectin, and nitrite did not show any significant differences between cocoa and the placebo groups at any postprandial time points.

Table 4. Composition of the cocoa and placebo powder.

	Cocoa	Placebo
Serving Size, g	20	12
Calories, kcal	67	66
Fat, g	4.7	5
Proein, g	2.7	1.1
Carbohydrates, g	9.6	4
Carbohydrates: Protein	3.06:01	3.06:01
Total Polyphenols, mg	960	110
Total Flavanols, mg	480	<0.1
Proanthocyanidins (PAC) 1-10, mg	201	<0.001
Epicatichins, mg	40	0
Catechins, mg	18	0

Source: The Hershey Company

Table 5a. Baseline Characteristics: Biochemical Variables and Blood Pressure, Mean ±SEM

Variable	Value
N (Gender, M/F)	18 (4/14)
Age, y	56±3.2
Weight, kg	100±12
BMI, kg/m^2	35.3±2.0
Waist Circumference, inches	45±1.8
Glucose, mg/dL	136±16
Insulin, mU/L	14.8±2.6
Insulin resistance (HOMA-IR)	3.5±0.9
HbA$_{1c}$, %	8.2±0.6
Total Cholesterol, mg/dL	188±11
LDL-cholesterol, mg/dL	112±11
HDL-cholesterol, mg/dL	46±2.5
LDL:HDL	2.54±0.24
Triglycerides, mg/dL	140±13
hs CRP, mg/dL	5.3±1.2
Systolic blood pressure, mm Hg	144±5.5
Diastolic blood pressure, mm Hg	85±3.0
Small artery elasticity index, mL/mmHg x 100	5.6±3.6
Large artery elasticity index, mL/mmHg x 10	17±7.4

Footnote: BMI - body mass index; HOMA-IR – homeostatic model assessment-insulin resistance; HbA$_{1c}$ – hemoglobin A$_{1c;}$ LDL – low-density lipoprotein; HDL – high-density lipoprotein; hs CRP – high sensitivity c-reactive protein

Table 5b. Baseline Characteristics: Medication/Supplement Use

Medication/supplement use, n (%)	
Insulin	0 (0)
Oral hypoglycemic agents	15 (83)
Statins/fibrates	5 (28)
Calcium Channel Blockers	3 (17)
Angiotensin-converting enzyme inhibitors or angiotensin II receptor blockers	10 (56)
Diuretics	2 (11)
Aspirin	6 (33)
Multivitamin/minerals	6 (33)
Botanical supplements	2 (11)

Table 5c. Baseline Characteristics: Food Intake

Macronutrient and food intake, mean ± SEM	
Energy, kcal	2216±198
Carbohydrates, g	263±38
Total fats, g	92±8
Protein, g	94±9
Fiber, g	30±9
Fruit servings, g/day	144±52
Vegetable servings, g/day	160±35

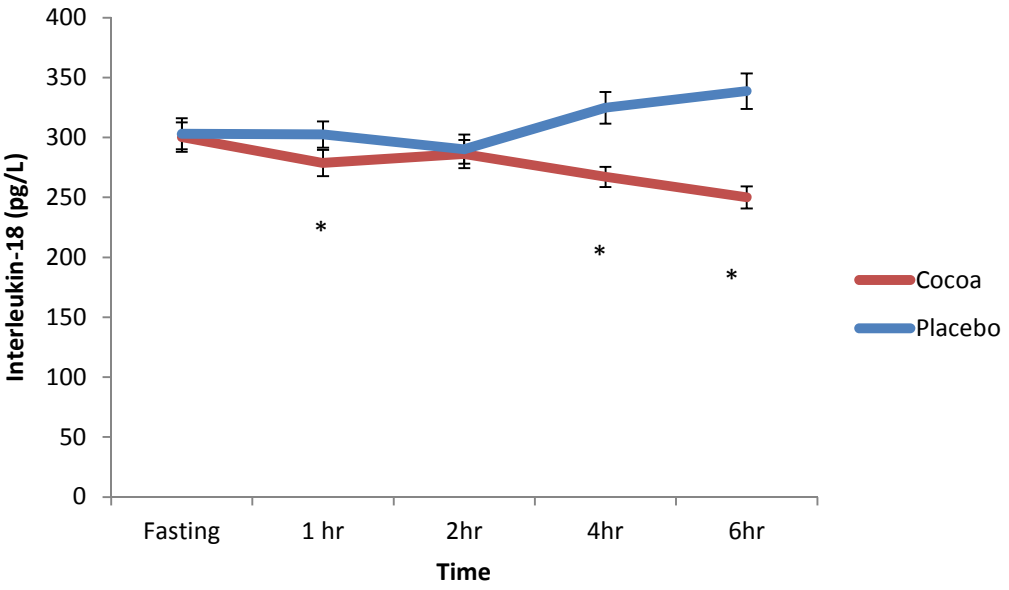

Figure 1. Postprandial serum concentrations of Interleukin-18 (pg/L) following a high-fat meal challenge with or without cocoa beverage. Data shown as means±SE. *p<0.05 at one, four, and six hours.

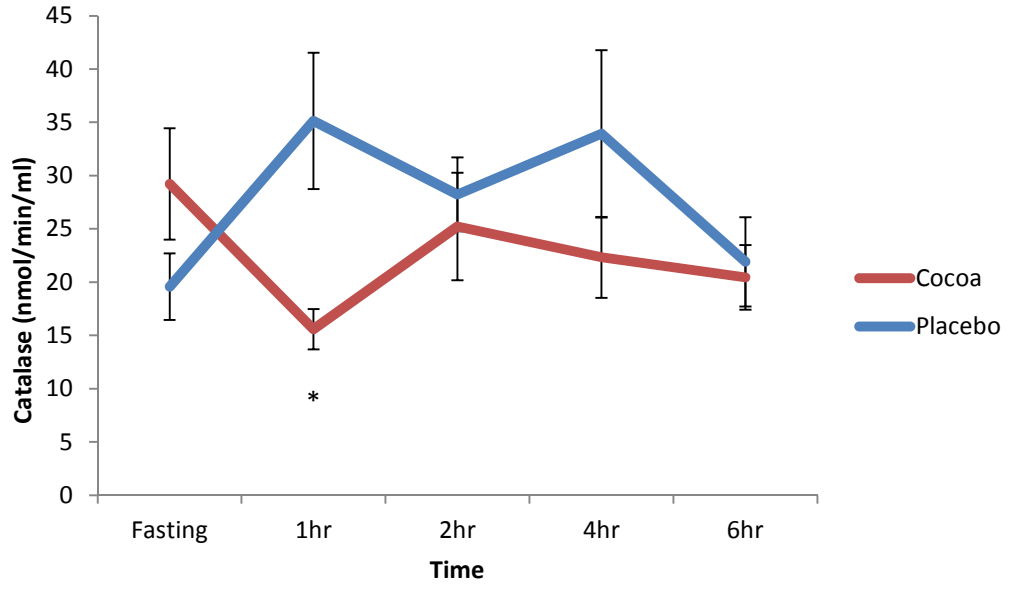

Figure 2. Postprandial serum catalase activity (nmol/min/ml) following a high-fat meal challenge with or without cocoa beverage. Data shown as means±SE. *p<0.05 at one hour.

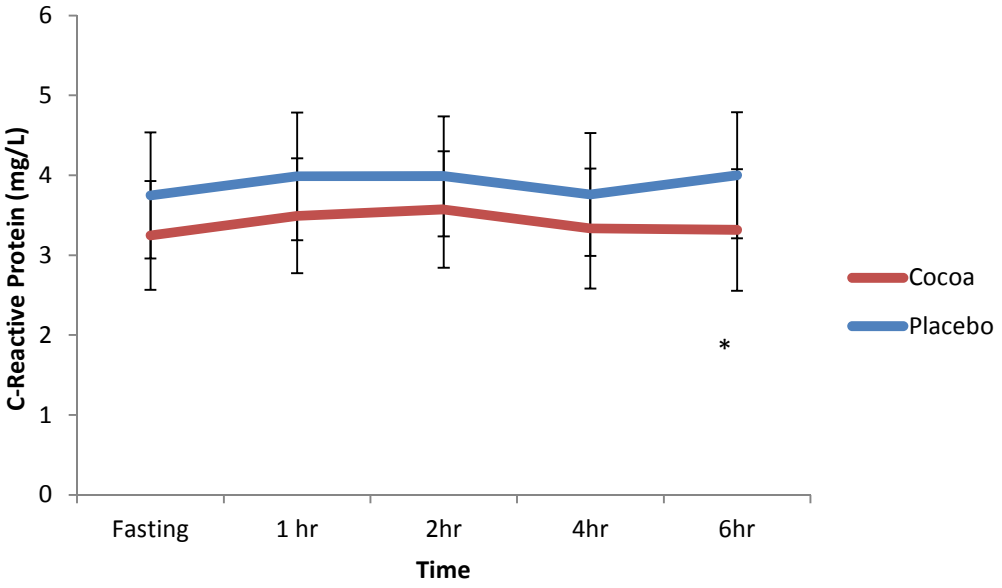

Figure 3. Postprandial serum concentrations of C-Reactive Protein (mg/L) following a high-fat meal challenge with or without cocoa powder beverage. Data shown as means±SE. $P<0.05$ at 6 hours.

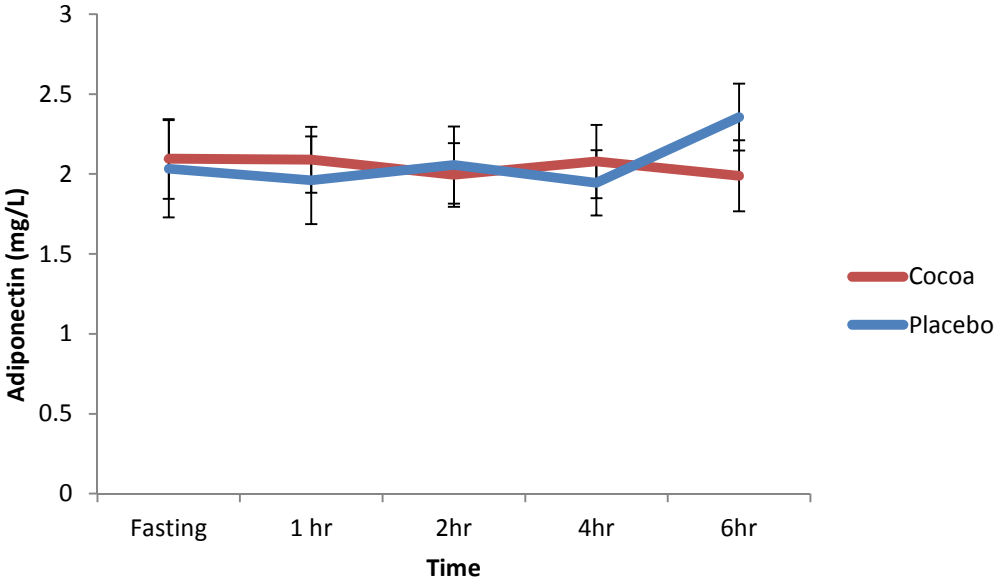

Figure 4. Postprandial serum concentrations of adiponectin (mg/L) following a high-fat meal challenge with or without cocoa powder beverage. Data shown as means±SE.

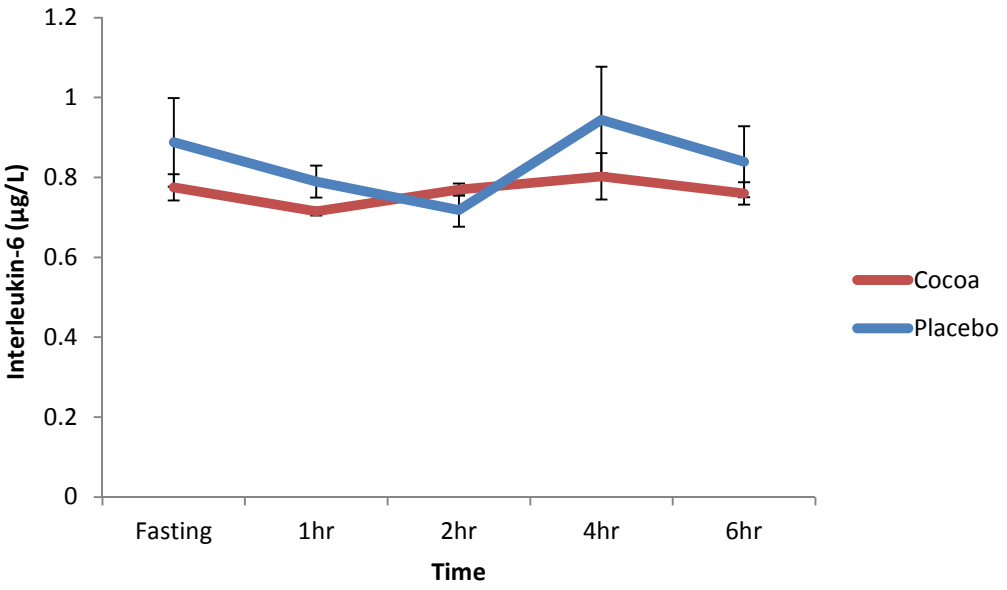

Figure 5. Postprandial serum concentrations of Interleukin-6 (µg/L) following a high-fat meal challenge with or without cocoa powder beverage. Data shown as means±SE.

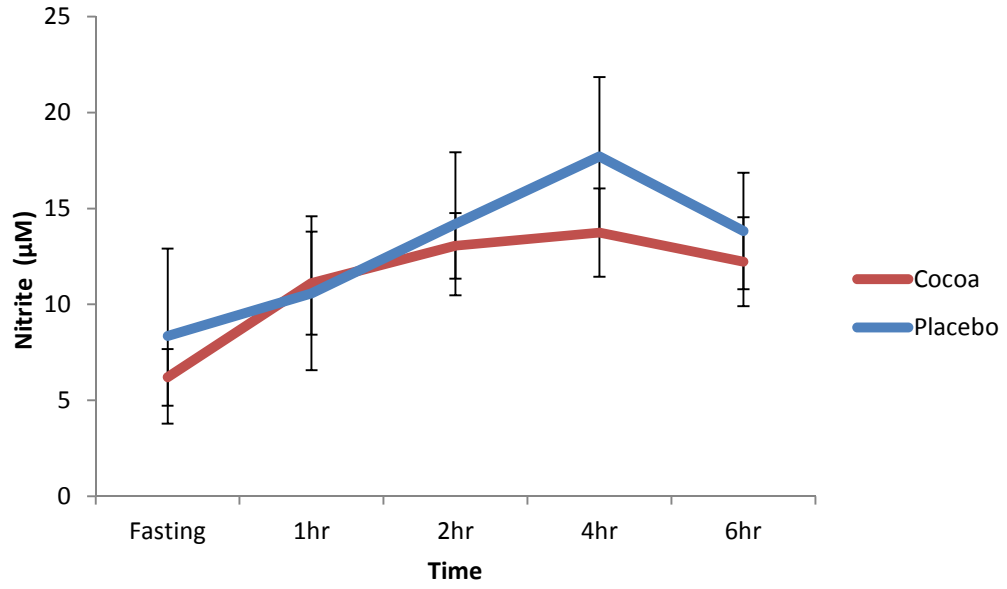

Figure 6. Postprandial concentrations of nitrite (µM) following a high-fat meal challenge with or without cocoa powder beverage. Data shown as means±SE.

Table 6. The effects of cocoa powder vs. placebo on inflammation markers and catalase activity in participants with type 2 diabetes following a high fat meal challenge. Data presented as mean ± standard error of the mean.

	PLACEBO	COCOA	*P value*
C-Reactive Protein (mg/L)			
Fasting	3.75±0.79	3.25±0.68	0.110
1 hr	3.99±0.80	3.27±0.70	0.143
2 hr	3.99±0.74	3.35±0.71	0.187
4 hr	3.76±0.76	3.33±0.76	0.186
6 hr	4.00±0.79	3.11±0.73	0.040
Interleukin-6 (µg/L)			
Fasting	0.89±0.11	0.77±0.03	0.355
1 hr	0.79±0.04	0.72±0.01	0.163
2 hr	0.72±0.04	0.77±0.02	0.258
4 hr	0.94±0.13	0.80±0.06	0.353
6 hr	0.84±0.09	0.76±0.03	0.265
Adiponectin (mg/L)			
Fasting	2.03±0.31	2.09±0.25	0.729
1 hr	1.96±0.27	2.09±0.21	0.459
2 hr	2.06±0.24	1.99±0.20	0.721
4 hr	1.94±0.20	2.08±0.23	0.518
6 hr	2.36±0.21	1.99±22	0.139
Interleukin-18 (pg/L)			
Fasting	303.06±12.98	300.33±12.29	0.342
1 hr	302.44±11.03	278.78±10.96	0.032
2 hr	290.28±12.02	286.11±11.76	0.741
4 hr	324.72±13.22	267.06±8.48	0.001
6 hr	338.61±14.89	250.00±9.16	0.000
Nitrite (µg/L)			
Fasting	8.35±4.56	6.19±1.48	0.542
1 hr	10.58±4.01	11.11±2.68	0.890
2 hr	14.20±3.73	13.06±1.71	0.666
4 hr	17.71±4.15	13.74±2.30	0.192
6 hr	13.83±3.04	12.23±2.32	0.311
Catalase (nmol/min/ml)			
Fasting	19.58±3.13	29.22±5.23	0.066
1 hr	35.14±6.40	15.58±1.90	0.008
2 hr	28.24±3.47	25.23±5.04	0.320
4 hr	33.92±7.86	22.34±3.80	0.091
6 hr	21.91±4.20	20.45±3.03	0.754

CHAPTER V

CONCLUSION

To our knowledge, this is the first randomized placebo-controlled double-blind clinical trial to examine the postprandial effects of dietary cocoa powder on inflammation markers and catalase activity in participants with T2D following a high-fat meal challenge. In this study, we examined effects of cocoa on inflammatory markers that have been shown to be modulated by high-fat meals, mostly in non-diabetic adults in other studies. Interestingly, our study revealed cocoa supplementation following a fast food style high-fat meal challenge to significantly lower CRP later in the postprandial phase (only at six hours), while IL-18 levels were lower throughout the postprandial phase (one, four and six hours). No significant effects were found in levels of IL-6, adiponectin, and nitrite between the cocoa and placebo groups at any time point. Finally, as a biomarker of antioxidant capacity, catalase activity was lower, though only in the early postprandial phase (one hour) following cocoa vs. placebo supplementation.

Inflammation is part of an immune response that occurs with injury (Ferrero-Miliani et al. 2006). Obesity induces a state of chronic low-grade inflammation that can lead to metabolic syndrome, insulin resistance, T2D, and CVD, as well as many other chronic conditions (Inglesson et al. 2008, Rodrigues-Hernandez et al. 2013). Obesity and inflammation lead to higher levels of inflammatory cytokines (Rodrigues-Hernandez et al. 2013, Amato et al. 2014), including CRP (Visser et al. 1999), IL-18 (Alboni et al. 2010), and IL-6 (Aldhahi et al. 2003), and lower levels of adiponectin (Nishida et al. 2007). Phytochemicals, such as those found in cocoa, have been

shown to reduce inflammation, and thus may be a useful treatment in prevention and management of chronic diseases (Rahman et al. 2006, Wang et al. 2014). In previously reported studies, dietary cocoa powder has been shown to reduce blood pressure (Rostami et al. 2015), total cholesterol (Mellor et al. 2010), and inflammation markers (Parsaeyan et al. 2014) in subjects with T2D. Cocoa powder has also been shown to increase HDL cholesterol (Baba et al. 2007) and flow-mediated dilation (Grassi et al. 2005) in subjects with CVD risk factors. Additionally, epidemiological studies have found cocoa consumption to be inversely correlated with the development of T2D (Zamora-Ros et al. 2013, Matsumoto et al. 2015) and CVD (Buijsse et al. 2010, Djousse et al. 2011).

Elevated serum CRP has been correlated with increased risk of developing insulin resistance (Ydkin et al. 1999), T2D (Pradham et al. 2001), and CHD (Mendall et al. 1996, Kaptoge et al. 2010). It is a commonly measured biomarker of inflammation in clinical studies (Salazar et al. 2014). Our study found that consuming cocoa beverage with a fast food style high-fat meal significantly lowered serum CRP, but only at six hours postprandial. Other studies have found interventions with cocoa to decrease CRP in overweight subjects (Stote et al. 2012) or those with T2D (Parseayan et al. 2014), while some studies have shown no effects of cocoa supplementation on CRP (Mathur et al. 2002, Monagas et al. 2009). However, none of these were postprandial studies, and keeping in view the role of impaired postprandial metabolism and inflammation in T2D, our study reveals some protective effects of cocoa, and this needs further investigation in larger trials of T2D.

Elevated serum IL-18 has also been correlated with obesity and T2D (Herder et al. 2011, Amato et al. 2014). We observed lower levels of IL-18 at one, four, and six hours postprandial in

the cocoa group compared to placebo. In observational studies, high flavonoid intake has been correlated with lower levels of IL-18 in the U.S. Women's Health Study (Landberg et al. 2011). Other clinical studies examining effects of diets high in polyphenols, as well as individual polyphenol-containing foods and beverages have also shown a decrease in IL-18 in adults with CVD risk factors. The Mediterranean Diet, high in fruits and vegetables, olive oil and other sources of polyphenols, has been shown to reduce levels of IL-18 in adults with metabolic syndrome (Richard et al. 2013, Espisito et al. 2004). Similar effects were also noted with red wine, bing cherry, or soy consumption (Marfella et a. 2006, Kelley et al. 2012, Azadbakht et al. 2007). Furthermore, resveratrol has been shown to decrease IL-18 levels in human aortic smooth muscle cells (Venkatessan et al. 2009) and in animal liver cancer cells (Salado et al. 2011). Thus, our study findings are supported by previously reported studies on the effects of polyphenols on inflammation, though further studies are needed in diabetes.

Interleukin-6 (IL-6) has been shown to increase linearly with adiposity (Aldhahi et al. 2003, Amato et al. 2014) and elevated levels have been correlated with insulin resistance and T2D (Yudkin et al. 1999, Duncan et al. 2003, Pradhan et al. 2003). Our study revealed no significant difference in IL-6 between cocoa and placebo treatment at any time point. However, other studies have shown cocoa supplementation to decrease IL-6 levels, such as in obese adults and in those with T2D (Stote et al. 2012, Parseayan et al. 2014) at doses equal to or slightly higher than our current study. Cocoa supplementation has also been shown to decrease IL-6 in male C57BL/6J mice fed a high-fat diet for ten weeks (Gu et al. 2014), and in healthy female Wistar rats after seven weeks (Perez-Berezo et al. 2012). Thus, our null findings may be

explained by the postprandial design, small sample size and the high-fat meal challenge as a 'primary' intervention followed by assessing the 'secondary' effects of cocoa supplementation.

Adiponectin, an anti-inflammatory cytokine, has been shown to be reduced with elevated BMI (Amato et al. 2014), and low levels have been correlated with increased risks of CHD, T2D, hypertension and metabolic syndrome (Nishida et al. 2007, Herder et al. 2011). Adiponectin has also been found to be inversely correlated with IL-6 and CRP levels in cross- sectional studies (Engeli et al. 2003, Krakoff et al. 2003). Our study revealed no significant difference in postprandial levels of adiponectin between cocoa and placebo. Other trials have revealed no effect on adiponectin levels after a higher dose of cocoa powder than in the present study for two weeks in hypertensive adults (Muniyappa et al. 2008), or after four weeks of a high dose of freeze-dried strawberry powder in women with metabolic syndrome (Basu et al. 2009). Studies have shown polyphenol supplementation to increase adiponectin levels and expression of adiponectin gene in male C57BL/6J mice (Gu et al. 2014, Heber et al. 2015), or have no effect in obese Sprague Dawley female rats (Shen et al. 2012) and human adipose tissue cells (Derdemezis et al. 2011). Thus, effects of polyphenols on adiponectin are conflicting and need further investigation.

Nitric oxide (NO) is a cellular signaling molecule important in endothelium dilation (Vallance, 1994). There are two main forms of nitric oxide synthases; these are endothelium nitric oxide synthase (eNOS) and inducible nitric oxide synthase (iNOS). NO from eNOS is associated with endothelium dilation, while NO from iNOS is associated with inflammation (Forstermann & Sessa 2011). Excess NO synthesis, such as under conditions of elevated lipids, hypertension, hyperglycemia and other CVD risk factors, has been associated with the formation

of the peroxynitrite radical which is an oxidizing agent, and also been shown to upregulate inflammation and decrease endothelial function (Valco, et al. 2007).

NO has a short half-life of only a few seconds, therefore typically measured as nitrite, one of two more stable breakdown components of NO (Liu et al. 1998, Shiva 2013, Lundberg et al. 2008). Our study revealed no significant difference between cocoa and placebo groups for levels of nitrite. However, diets high in polyphenols have been correlated with increased circulating NO in a Spanish cohort study (Medina-Ramon et al. 2015). In addition, clinical trials have shown cocoa supplementation to increase circulating NO levels after two hours (Heiss et al. 2003), and to increase flow-mediated dilation after six weeks at a similar dose to the present study (Njike et al. 2011) and after 15 days at a lower dose (Grassi et al. 2005). None of these were postprandial studies involving a high-fat meal. Again, our null findings may have been due to our small sample size or due to the co-administration of a high-fat meal along with cocoa intervention.

Catalase is an endogenous antioxidant enzyme important in preventing oxidative stress (Valko et al. 2007). Its main function is to reduce hydrogen peroxide into water and oxygen (Kodydkova et al. 2014). We observed lower levels of catalase activity at one hour postprandial in the cocoa group compared to the placebo. Reduced catalase activity may be due to a 'sparing effect' of cocoa polyphenols on antioxidant enzymes. Cocoa supplementation in patients with T2D resulted in reduced glutathione and catalase levels after three months of treatment (Ramirez-Sanchez et al. 2013). On the other hand, cocoa supplementation has shown to increase catalase levels in arthritic female Wister rats (Ramos-Romero et al 2012), or have no effect in male diabetic Zucker rats (Cordero-Herrera et al. 2015). Keeping in view the known synergistic effects

of polyphenols with antioxidant factors *in vivo* (Scalbert et al. 2005, Kang et al. 2013), future studies must examine these associations in diabetes.

Our study has some limitations, which must be considered when interpreting our results. One limitation is the small sample size of only eighteen participants. Having a larger sample size would have increased our power and generalizability. We only had 85 out of 180 samples for IL-6 analysis. Having a complete set of data for this biomarker would have increased our power for detecting differences. Another limitation of our study is that we did not compare the findings in our diabetic subjects with non-diabetic controls. We only used one dose of cocoa powder, and thus a dose-response effect would be of great interest in future studies. We also measured selected biomarkers of inflammation, and did not examine changes in other markers, such as interleukin-1β, interleukin-8, interleuking-10, TNF-α, or E-selectin, and this remains to be explored in larger trials in diabetes

Overall, we reject the null hypothesis that cocoa powder would have no affect on inflammation markers, as we have demonstrated that cocoa powder supplementation with a high-fat meal lowers selected biomarkers of inflammation when compared to the placebo treatment. We also reject the null hypothesis that cocoa powder would have no effect on catalase activity, as we saw a decrease in catalase activity with cocoa supplementation in the presence of a high-fat meal vs. placebo.

Applications and Conclusions

Overall, our study findings show modest anti-inflammatory effects of cocoa in lowering serum CRP and IL-18 at one or more time points during the six-hour postprandial phase in adults

with type 2 diabetes. We also observed selected modulation of catalase activity in early postprandial phase following cocoa supplementation vs. placebo. Thus, based on our findings cocoa supplementation in the presence of a high-fat meal challenge may offer some protective effects against postprandial inflammation. Further research is needed to confirm these findings in larger trials of participants with diabetes and its advanced vascular complications.

REFERENCES

Alboni, S., Cervia, D., Sugama, S., et al. Interleukin 18 in the CNS. *J Neuroinflamm.* 2010;7(9):1-12.

Aldhahi, W. & Hamdy, O. Adipokines, inflammation, and the endothelium in diabetes. *Curr Diab Rep.* 2003;3:293-298.

Amato, M. C., Pizzolanti, G., & Torregrossa, V. et al. Visceral Adiposity Index (VAI) is predictive of an altered adipokine profile in patients with type 2 diabetes. *PLOS.* 2014;9(3):1-10.

American Diabetes Association. Diagnosis and Classification of Diabetes Mellitus. *Diabetes Care*. 2014;37(1):S81-S90.

American Diabetes Association. Postprandial Blood Glucose. *Diabetes Care.* 2001;24(4):775-778.

American Heart Association (2013). Cardiovascular disease and diabetes. Retrieved from http://www.heart.org/HEARTORG/Conditions/Diabetes/WhyDiabetesMatters/Cardiovascular-Disease-Diabetes_UCM_313865_Article.jsp. Accessed August 4, 2015.

Art, I.C.W., Hollman, P.C.H., Feskens, E.J.M., et al. Catechin intake might explain the inverse relation between tea consumption and ischemic heart disease: the Zutphen Elderly Study. *Am J Clin Nutr.* 2001;74:227-232.

Arts, I.C.W., Jacobs, D.R., Harnack, L.J., Gross, M., & Folsom, A.R. Dietary catechins in relation to coronary heart disease death among postmenopausal women. *Epidemiol.* 2001;12(6):668-675.

Azadbakht, L., Kimiagar, M., & Mehrabi, Y. et al. Soy consumption, markers of inflammation, and endothelial function. *Diabetes Care.* 2007;30(4):967-973.

Baba, S., Osakabe, N., Kato, Y., et al. Continuous intake of polyphenolic compounds containing cocoa powder reduces LDL oxidation susceptibility and has beneficial effects on plasma HDL-cholesterol concentrations in humans. *Am J Clin Nutr,* 2007;85:709-717.

Balzer, J., Rassaf, T., Heis, C., et al. Sustained benefits in vascular function through flavanol-containing cocoa in medicated diabetic patients. *JACC.* 2008;51(22):2141-2149.

Bandyopadhyay, P., Ghosh, A. K., & Chosh, C. Recent developments on polyphenol-protein interactions:effects on tea and coffee taste, antioxidant properties and the digestive system. *Food Funct.* 2012;3:592-605.`

Basu, A., Wilkinson, M., Penugonda, K., Simmons, B., Betts, N. M., & Lyons, T. J. Freeze-dried strawberry powder improves lipid profile and lipid peroxidation in women with metabolic syndrome: baseline and post intervention effects. *Nutr J.* 2009;8(43):1-7.

Bayard, V., Chamarro, F., Motta, J., & Hollenberg, N.K. Does flavanol intake influence mortality from nitric oxide-dependent processes? Ischemic heart disease, stroke, diabetes mellitus, and cancer in Panama. *Int J Med Sci.* 2007;4(1):53-58.

Bazzano, L. A., He, J., & Ogden, L. G. et al. Fruit and vegetable intake and the risk of cardiovascular disease in US adults: the first National Health and Nutrition Examination Survey Epidemiologic Follow-up Study. *Am J Clin Nutr.* 2002;76:93-99.

Benn, T., Kim, B., & Park, Y. et al. Polyphenol-rich blackcurrant extract exerts hypocholesterolaemic and hypoglycaemic effects in mice fed a diet containing high fat and cholesterol. *Br J Nutr.* 2015;113:1697-1703.

Bhagwat, S., Haytowitz, D. B., & Holden, J. M. USDA database for the flavonoid content of selected foods. USDA Nutrient Data Laboratory. http://www.ars.usda.gov/SP2UserFiles/Place/80400525/Data/Flav/Flav_R03-1.pdf. Published December 2013. Accessed July 15, 2015.

Bohn, T. Dietary factors affecting polyphenol bioavailability. *Nutr Rev.* 2014;72(7):429-452.

Bogani, P., Galli, C., Villa, M., & Visioli, F. Postprandial anti-inflammatory and antioxidant effects of extra virgin olive oil. *Atherosclerosis.* 2007;190*:*181-186.

Borch-Johnson, K. Neil, A., Balkau, B., et al. Glucose tolerance and mortality: comparison of WHO and American Diabetes Association diagnosis criteria. *The Lancet.* 1999;354(9179)*:*617-621.

Bordiga, M., Locatelli, M., Travaglia, F., Coisson, J. D., Mazza, G., & Arlorio, M. Evaluation of the effect of processing on cocoa polyphenols: antiradical activity, anthocyanins and procyanidins profiling from raw beans to chocolate. *Int J Food Sci Tech*. 2015;50:840-848.

Bouayed, J. & Bohn, T. Exogenous antioxidants- double-edged swords in cellular redox state. *Oxid Med Cell Longev.* 2010;3(4):228-237.

Brcanovic, J. M., Pavlovic, A. N., Mitic, S. S., et al. Cyclic voltammetric determination of antioxidant capacity of cocoa powder, dark chocolate and milk chocolate samples: correlation

with spectrophotometric assays and individual phenolic compounds. *Food Technol Biotechnol.* 2013;51(4):460-470.

Buijsse, B., Freskens, E. J. M., Kok, F. J., & Kromhout, D. Cocoa intake, blood pressure, and cardiovascular mortality: the Zutphen Elderly Study. *Arch Intern Med.* 2006;116:411-417.

Centers for Disease Control and Prevention (2013). Leading Causes of Death. Retrieved from http://www.cdc.gov/nchs/fastats/leading-causes-of-death.htm. Accessed August 4, 2015.

Chen, S.C., Judd, J.T., Kramer, M., Meijer, G.W., Clevidence, B.A., & Baer, D.J. Phytosterol intake and dietary fat reduction are independent and additive in their ability to reduce plasma LDL cholesterol. *J Am Oil Chem Soc.* 2009;44:273-281.

Choi, S. H., & Ginsberg, H. N. Increased very low density lipoprotein (VLDL) secreation, hepatic steatosis, and insulin resistance. *Trends Edocrinol Metab.* 2011;22(9):353-363.

Cohen, G. & Hochstein, P. Glutathione peroxidase: the primary agent for the elimination of hydrogen peroxide in erythrocytes. *Biochem.* 1963;2(6):1420-1428.

Cordero-Herrera, I., Martin, M. A., Goya, L., & Ramos, S. Cocoa in take ameliorates hepatic oxidative stress in young Zucker diabetic fatty rats. *Food Res Int.* 2015;69:194-201.

Daimon, M., Oizumi, T., Saitoh, T. et al. Decreased serum levels of adiponectin are a risk factor for the progression to type 2 diabetes in the Japanese population. *Diabetes Care.* 2003;27(7):2015-2020.

Derdemezis, C. S., Kiortsis, D. N., & Tsimihodimos, V. et al. Effect of plant polyphenols on adipokine secretion from human SGBS adipocytes. *Biochem Res Int.* 2011;285618:1-5.

Dillinger, T. L., Barriga, P., Escárcega, S., Jimenez, M., Diana, S. L. & Grivetti, L. E. Food of the gods: Cure for humanity? A cultural history of the medicinal and ritual use of chocolate. *J Nutr.* 2000;130:2057S-2072S.

Djoussé, L., Hopkins, P. N., & Arnett, D. K. et al. Chocolate consumption is inversely associated with calcified atherosclerotic plaque in the coronary arteries: The NHLBI Family Heart Study. *Clin Nutr.* 2011;30:38-43.

Dower, J., Gelaijnse, J., Gijsbers, L., Schalkwijk, C., & Kromhout, D. et al. Supplementation of pure flavonoids epicatechin and quercetin affect some biomarkers of endothelial dysfunction and inflammation in (pre)hypertensive adults: a randomized, double-blind, placebo-controlled, crossover trial 1,2. *J Nutr.* 2015;145.7:1459-1463.

Duncan, B. B., Schmidt, M. I., Pankow, J. S., et al. Low-grade inflammation and the development of type 2 diabetes. *Diabetes*. 2003;52(7):1799-1805.

Engeli, S., Feldpausch, M., & Gorzelniak, K. et al. Association between adiponectin and mediators of inflammation in obese women. *Diabetes.* 2003;52(4):942-947.

Espisito, K., Marfella, R., & Ciotola, M. et al. Effect of a Mediterraniean-style diet on endothelial dysfunction and markers of vascular inflammation in the metabolic syndrome. *JAMA.* 2004;292(12):1440-1446.

Esposito, K., Nappo, F., Guigliano, F. et al. Meal modulation of circulating interleukin-18 and adiponectin concentrations in healthy subjects and in patients with type 2 diabetes mellitus. *A J Clin Nutr.* 2003;78:1135-1140.

Ferrero-Miliani, L, Nielsen, O.H., Andersen, P.S., & Girardin, S.E. Chronic inflammation importance of NOD2 and NALP3 in interleukin-1β generation. *Clin Exp Immunol.* 2006;147:227-235.

Fisher, ND., Hughes, M., Gerrhard-Herman, M., & Hollenberg, NK. Flavanol-rich cocoa induces nitric-oxide dependent vasodilation in humans. *J Hypertens.* 2003;21(12):2281-2286.

Flammer, A. J., Mermann, F., & Sudano, I. et al. Dark chocolate improves coronary vasomotion and reduces platelet reactivity. *Circulation.* 2007;116:2376-2382.

Forstermann, U. & Li, H. Therapeutic effect of enhancing endothelial nitric oxide synthase (eNOS) expression and preventing eNOS uncoupling. *Br J Pharmacol.* 2011;164:213-223.

Forstermann, U. & Sessa, W. C. Nitric oxide synthases: regulation and function. *Eur Heart J.* 2011;33(7):829-837.

Fraga, C. G., Galleano, M., Verstraeten, S. V., & Oteiza, P. I. Basic biochemical mechanisms behind the health benefits of polyphenols. *Mol Aspects Med.* 2010;31:435-445.

Gallagher, A. The effect of improved blood glucose control on post-prandial metabolism and markers of vascular risk in people with type 2 diabetes. *Diabetes Res Clin Pract.* 2005;67*:*196-203.

Garoufi, A., Vorre, S., Tsentidis, C., et al. Plant Sterols-enriched diet decreases small dense LDL-cholesterol levels in children with hypercholesterolemia: a prospective study. *Ital J Pediatr*. 2014;40(42):1-6.

Goodman, H. M. (2009). *Basic Medical Endocrinology, 4th ed.* Burlington, MA: Elsevier.

Grassi, D., Necozione, S., Lippi, C., et al. Cocoa reduced blood pressure and insulin resistance and improves endothelium-dependent vasodilation in hypertensives. *JAMA.* 2005;46:398-405.

Greenberg, A. S., Nordan, R. P., & McIntosh, J. et al. Interleukin 6 reduces lipoprotein lipase activity in adipose tissue of mice in vivo and in 3T3-L1 adipocytes: a possible role for interleukin 6 in cancer cachexia. *Cancer Res.* 1992;52:4113-4116.

Gropper, S. S. & Smith, J. L. (2013). *Advanced Nutrition and Human Metabolism, 6th ed.* Belmount, CA: Wadsworth, Cengage Learning.

Gu, Y., Yu, S., Lambert, J.D. Dietary cocoa ameliorates obesity-related inflammation in high fat-fed mice. *Eur J Nutr.* 2014;53:149-158.

Guerre-Millo, M. Adiponectin: an update. *Diabetes Metab*. 2008;24:12-18.

Hak, E., Stenhouwer, C.D.A., Bots, M.L., et al. Associations of C-reactive protein with measures of obesity, insulin resistance, and subclinical atherosclerosis in healthy, middle-aged women. *Arterioscler Thromb Vasc Biol.* 1999;19:1986-1991.

Halliwell, B. Biochemistry of oxidative stress. *Biochem Soc Trans.* 2007;35(5):1147-1150.

Hanefeld, M., Fischer, S., Julius, U., et al. Risk factors for myocardial infarction and death in newly detected NIDDM: the Diabetes Intervention Study, 11 year follow-up. *Diabetologia.* 1996;39:1777-1583.

Hanhineva, K., Torronen, R., Bondia-Pons, I. et al. Impact of dietary polyphenols on carbohydrate metabolism. *Int J Mol Sci.* 2010;11:I1365-1402.

Harwood, M. L., Ziegler, G. R., Hayes, J.E. Tolerance for High Flavanol Cocoa Powder in Semisweet Chocolate. *Nutrients*. 2013;5:2258-2267.

Haytowitz, D.B., Bhagwat, S., Hamly, J et al. Sources of flavonoids in the U.S. diet using USDA's updated database on the flavonoid content of selected foods. Agriculture Research Service. http://www.ars.usda.gov/SP2UserFiles/Place/80400525/Articles/AICR06_flav.pdf Published 2014. Accessed April 14, 2015.

Heath, R.B., Karpe, F., Milne, R.W., Burge, G.C., Wootton, S.A., & Frayn, K.N. Dietary fatty acids make a rapid and substantial contribution to VLDL-triacylglycerol in the fed state. *Am J Phys Endocrin Metab*. 2006;292:E732-E739.

Heber, D., Zhang, Y., & Yang, J. et al. Green tea, black tea, and oolong tea polyphenols reduce visceral fat and inflammation in mice fed high-fat, high-sucrose obesogenic diets. *J Nutr,* 2014;144(9):1385-1493.

Heilbronn, L.K., Noakes, M., & Clifton, P.M. Energy restriction and weight loss on very-low-fat diets reduce c-reactive protein concentrations in obese, healthy women. *Arterioscler Thromb Vasc Biol.* 2001;21:968-970.

Heiss, C., Dejam, A., Kleinbongard, P., Schewe, T., Sies, H., & Kelm. M. Vascular effects of cocoa rich in flavan-3-ols. *AMA.* 2003;290(8):1030-1031.

Heiss, C., Kleinbongard, P., Dejam, A. et al. Acute consumption of flavanol-rich cocoa and the reversal of endothelial dysfunction in smokers. *JACC.* 2005;46(7):1277-1283.

Helm, M. Nitric oxide metabolism and breakdown. *Biochem.* 1999;1411:273-289.

Herder, C., Baumert, J., & Zierer, A. et al. Immunological and cardiometabolic risk factors in the prediction of type 2 diabetes and coronary events: MONICA/KORA Augsburg case-cohort study. *PLOS.* 2011;6(6):1-9.

Hermann, F., Spieker, L.E., Ruschitzka, F. et al. Dark chocolate improves endothelial and platelet function. *Heart.* 2006;92:119-120.

Hlebowicz, J., Darwiche, G., Bjorgell, O., & Almer, L. Effect of cinnamon on postprandial blood glucose, gastric emptying, and satiety in healthy subjects. *Am J Clin Nutr.* 2007;85:1552-1556.

Hyson, D., Rutledge, J. C., & Berglund, L. Postprandial lipemia and cardiovascular disease. *Curr Atheroscler Rep.* 2003;5:437-444.

Ingelson, E., Hulthe, J., & Lind, L. Inflammation markers in relation to insulin resistance and the metabolic syndrome. *Eur J Clin Invest.* 2008;38(7):502-509.

Kaotoge, S. et al. C-reactive protein concentration and risk of coronary heart disease, stroke, and mortality: an individual participant meta-analysis. *Lancet.* 2010;375:132-140.

Karim, M., McCormick, K., & Kappagoda, T. Effects of cocoa extracts on endothelium-dependent relaxation. *J Nutr. 2000;130:*2105S-2108S.

Kang, D. H. & Kang, S. W. Targeting cellular antioxidant enzymes for treating atherosclerotic vascular disease. *Biol Ther.* 2013;21(2):89-96.

Karpe, F. Postprandial lipoprotein metabolism and atherosclerosis. *J Intern Med.* 1999;246:341-355.

Karpe, F., Steiner, G., Olivecron, T., Carlson, L.A., & Hamsten, A. Metabolism of triglyceride-rich lipoproteins during alimentary lipemia. *J Clin Invest.* 1993;91:748-758.

Keidar, S., Heinrich, R., Kaplan, M., Hayek, T., & Aviram, M. Angiotensin II administration to atherosclerotic mice increases macrophage uptake of oxidized LDL: a possible role for interleukin-6. *Atheroscler Throm Vasc Biol.* 2001;21:1464-1469.

Kelm, M. Nitric oxide metabolism and breakdown. *Biochem Biophys Acta.* 1999;1411:273-289.

King, A. & Young, G. Characteristics and occurrence of phenolic phytochemicals. *J Am Diet Assoc.* 1999;99:213-219.

King, D. E., MAinous, A. G., Buchanan, T. A., & Pearson, W. S. C-reactive protein and glycemic control in adults with diabetes. *Diabetes Care.* 2003;25(5):1535-1539.

Kodydkova, J., Vavrova, L., Kocik, M., & Zak, A. Human catalase, its polymorphisms, regulation and changes of its activity in different disease. *Folio Biol.* 2014;60:153-167.

Kopp, H.P., Kopp, C.W., Festa, A., et al. Impact of weight loss on inflammatory proteins and their association with the insulin resistance syndrome in morbidly obese patients. *Arterioscler Thromb Vasc Biol.* 2003;23:1042-1047.

Krakoff, J., Funahashi, T & Stehouwer, C. et al. Inflammatory markers, adiponectin, risk of type 2 diabetes in the Pima Indian. *Diabetes Care.* 2003;26(6):1745-1751.

Kyle, J.A.M., Sharp, L., Little, J., Duthrie, G.G., & McNeill, G. Dietary flavonoid intake and colorectal cancer: a case-control study. *Br J Nutr.* 2010;103:429-436.

Lagathu, C., Bastard, J., Auclair, M., Maachi, M., Capeau, J., & Caron, M. Chronic interleukin-6 (IL-6) increased IL-6 secretion and induced insulin resistance in adipocyte: prevention by rosiglitazone. *Biochem Biophys Res Commun.* 2003;311:372-379.

Lambert, J. E., & Parks, E. J. Postprandial metabolism of meal triglycerides in humans. *Biochimica et Biophysica Acta.* 2012*;*1821:721-726.

Landberg, R., Sun, Q., Rimm, E. B., Cassidy, A., Scalbert, A. et al. Selected Dietary Flavonoids Are Associated with Markers of Inflammation and Endothelial Dysfunction in U.S. Women1,2. *J Nutr.* 2011;141.4:618-625.

Larson, S. C., Virtamo, J., & Wolk, A. Chocolate consumption and risk of stroke. *Nuerol.* 2012;79:1223-1229.

Lee, K.W., Kim, Y.J., Lee, H.J., & Lee, C.H. Cocoa has more phenolic phytochemicals and a higher antioxidant capacity than teas and red wine. *J Agric Food Chem.* 2003;51:7292-7295.

Leray, V., Freuchet, B., & Le Bloch, J. et al. Effect of citrus polyphenol- and curcumin-supplementation on inflammatory state in obese rats. *J Nutr.* 2011;106.S1:S198-201.

Levine, A. B., Punihaole, D., & Levine, T. B. Characterization of the role of nitric oxide and its clinical applications. *Cardiology.* 2012;122:55-68.

Li, H., Horke, S., & Forstermann, U. Vascular oxidative stress, nitric oxide and atherosclerosis. *Atherosclerosis.* 2014;237:208-219.

Liu, X., Millert, M.J.S., & Joshi, M.S. et al. Diffusion-limited reaction of free nitric oxide with erythrocytes. *J Biol Chem.* 1998;273(30):18709-18713.

Liu, Y., Zhan, J., Liu, X., Wang, Y., Ji, J., & He, Q. Dietary intake and risk of type 2 diabetes: a meta-analysis of prospective cohort studies. *Clin Nutr.* 2014;33:59-63.

Maki, K.C., Lawless, A.L., Reeves, M.S. et al. Lipid effects of a dietary supplement softgel capsule containing plant sterols/stanols in primary hypercholesterolemia. *Nutrition*. 2013;29:96-100.

Maleyki, M.J.A., & Ismail, A. Antioxidant properties of cocoa powder. *J Food Biochem. 2010;34:*111-128.

Mangge, H., Becker, K., Fuchs, D., & Gostner, J. Antioxidants, inflammation and cardiovascular disease. *World J Cardiol.* 2014;6(6):462-477.

Marchand, L.L., Murphy, S.P., Hankin, J.H., Wilkens, L.R., & Kolonel, L.N. Intake of flavonoids and lung cancer. *J Natl Cancer Inst.* 2000;92(2):154-160.

Marfella, R., Cacciapuoti, F., & Siniscalchi, M. et al. Effect of moderate red wine intake on cardiac prognosis after recent acute myocardial infarction of subjects with type 2 diabetes. *Diabet Med.* 2006;23:974-981.

Mates, J., Perez-Gomez, C., & Nunez de Castro, I. Antioxidant enzymes and human diseases. *Clin Biochem.* 1999;32(8):595-603.

Mathur, S., Devaraj, S., Grundy, S.M., & Jialal, I. Cocoa products decrease low density lipoprotein oxidative susceptibility but do not affect biomarkers of inflammation in humans. *J Nutr*. 2002:132(12):3663-3667.

Matsumoto, C., Petrone, A. B., Sesso, H. D., Gaziano, J. M., & Djousse, L. Chocolate consumption and risk of diabetes mellitus in the Physicians' Health Study. *Am J Clin Nutr.* 2015;101:362-267.

Medina-Remon, A, Tresserra-Rimbau, A., & Pons, A. et al. Effects of total dietary polyphenols on plasma nitric oxide and blood pressure in a high cardiovascular risk cohort. The PREDIMED randomized trial. *Nutr Metab Cardiovasc Dis.* 2015;25:60-67.

Mellor, D. D., Sathyapalan T., Kilpatrick, S., Beckett, S., and Atkin, S. L. High-cocoa polyphenol chocolate improves HDL cholesterol in Type 2 diabetes patients. *Diabet Med*. 2010:1318-1321.

Mendall, M. A. C-reactive protein and its relation to cardiovascular risk factors: a population based cross sectional study. *BMJ*. 1996;312:1061.

Mero, N., Syvanne, M., & Taskinen, M. Postprandial metabolism in diabetes. *Atherosclerosis*. 1998;141:S53-S55.

Marrelli, M., Loizzo, M. R., Nicoletti, M., Menichini, F., & Conforti, F. Inhibition of key enzymes linked to obesity by preparations from Mediterranean dietary plants: effects on alpha-amylase and pancreatic lipase activities. *Plant Foods Hum Nutr.* 2013;68:340-346.

Mink, P. J., Scrafford, C. G., Barraj, L. M., Harnack, L., Hong, C., Nettleton, J. A., & Jacobs, D. R. Flavonoid intake and cardiovascular disease mortality: a prospective study in postmenopausal women. *Am J Clin Nutr.* 2007;85:895-909.

Monagas, M., Khan, N., & Andres-Lacueva, C. et al. Effect of cocoa powder on the modulation of inflammatory biomarkers in patients at high risk of cardiovascular disease. *Am J Clin Nutr.* 2009;90:1144-1150.

Mostofsky, E., Levitan, E. B., Wolk, A., & Mittleman, M. A. Chocolate intake and incidence of heart failure: a population-based prospective study of middle-aged and elderly women. *Circ Heart Fail.* 2010;3:612-616.

Muniyappa, R., Hall, G., & Kolodziej, T. L. et al. Cocoa consumption for 2 wk enhances insulin-mediated vasodilation without improving blood pressure or insulin resistance in essential hypertension. *Am J Clin Nutr.* 2008;88:1685-1696.

Murase, T., Yokoi, Y., Misawa, K., et al. Coffee polyphenols modulate whole-body substrate oxidation and suppress postprandial hyperglycemia, hyperinsulinaemia and hyperlipidaemia. *Br J Nutr.* 2012;107:1757-1765.

Mursu, J., Voutilainen, S., Nurmi, T., et al. Dark chocolate consumption increases HDL cholesterol concentrations and chocolate fatty acids may inhibit lipid peroxidation in healthy humans. *Free Radic Biol Med.* 2004;37(9):1352-1359.

National Diabetes Statistics Report. (2014). *National Center for Chronic Disease Prevention and Health Promotion.* Retrieved from http://www.cdc.gov/diabetes/pubs/statsreport14/national-diabetes-report-web.pdf. Accessed July 7, 2015.

National Diabetes Surveillance System (2012). *Centers for Disease Control and Prevention.* Retrieved from http://gis.cdc.gov/grasp/diabetes/DiabetesAtlas.html. Accessed August 4, 2015.

Nishida, M., Funahashi, T., & Shimomura, I. Pathophysiological significance of adiponectin. *Med Mol Morphol.* 2007;40:55-67.

Njike, V. Y., Faradi, Z., & Shuval, K. et al. Effects of sugar-sweetened and sugar free cocoa on endothelial function in overweight adults. *Int. J. Cardiology.* 2011;149:83-88.

O'Keefe, J. H. & Bell, D. S. H. Postprandial hyperglycemia/hyperlipidemia (postprandial dysmetabolism) is a cardiovascular risk factor. *Am Cardiol.* 2007;100:899-904.

Parsaeyan, N., Mozaffari-Khosravi, H., Absalan, A., & Mozayan, R. Beneficial effects of cocoa on lipid peroxidation and inflammatory markers in type 2 diabetic patients and investigation of probable interactions of cocoa active ingredients with prostaglandin synthase-2 (PTGS-2/COX-2) using virtual analysis. *J Diabetes Metab Disord.* 2014;13(30):1-9.

Perez-Berezo, T., Franch, A., Castellote, C., Castell, M., & Perez-Cano, P. J. Mechanisms involved in down regulation of intestinal IgA in rats by high cocoa intake. *Nutr Biochem.* 2012;23:834-844.

Pitocco, D., Zaccardi, F., Di Stasio, E. et al. Oxidative stress, nitric oxide, and diabetes. *Diabet Stud.* 2010;7(1):15-25.

Pranhan, A. D., Manson, J. E., Rifai, N., Buring, J. E., & Ridker, P. M. C-reactive protein, interleukin 6, and the development of type 2 diabetes mellitus. *JAMA*. 2001;286(3):327-334.

Qin, B., Polansky, M., Sato, Y., Adeli, K., & Anderson, R. Cinnamon extracts inhibit the postprandial overproduction of apolipoprotein B48 –containing lipoproteins in fructose-fed animals. *J Nutr Biochem.* 2009;20:901-908.

Rahman, I., Biswas, S. K., & Kirkham, P. A. Regulation of inflammation and redox signaling by dietary polyphenols. *Biochem Pharmacol.* 2006;72:1439-1452.

Ramos- Romero, S., Perez-Cano, F. J., & Ramiro-Puig, E. et al. Cocoa intake attenuates oxidative stress associated with rat adjuvant arthritis. *Pharmacol Res.* 2012;66(3):207-212.

Ramirez-Sanchez, I., Taub, P. R., & Ciaraldi, T. P. et al. (-)-Epicatichin rich cocoa mediated modulation of oxidative stress regulators in skeletal muscle of heart failure and type 2 diabetes patients. *Int J Cardiol.* 2013;168:3982-3990.

Rassaf, T. & Kelm, M. Cocoa flavanols and the nitric oxide-pathway: targeting endothelial dysfunction by dietary intervention. *Cardiology.* 2008;5(3-4):273-278.

Rein, Dietrich et al. Epicatechin in human plasma: In vivo determination and effect of chocolate consumption on plasma oxidation status. *J Nutr*. 2000;130*:* 2109S-2114S.

Richard, C., Couture, P., Desroches, S., & Lamarche, B. Effect of the Mediterranean Diet with and without weight loss on markers of inflammation in men with metabolic syndrome. *Obesity.* 2013;21:51-57.

Ridker, P.M., Hennekens, C.H., Buring, J.E., & Rifal, N. C-reactive protein and other markers of inflammation in the prediction of cardiovascular disease in women. *NEJM.* 2000;342(12):836-843.

Ridker, P.M., Buring, J.E., Shih, J., Matias, M., & Hennekens, C.H. Prospective study of c-reactive protein and the risk of future cardiovascular events among apparent healthy women. *Circulation*. 1998;98:731-733.

Rodriguez-Hernandez, H., Simental-Mendia, L. E., Rodriguez-Ramirez, G., & Reyes-Romero, M. A. Obesity and Inflammation: Epidemiology, Risk Factors, and Markers of Inflammation. *Int J Endocrinol.* 2013*:*1-11.

Rostami, A., Khalili, M., & Haghighat, N. et al. High-cocoa polyphenol-rich chocolate improves blood pressure in patients with diabetes and hypertension. *ARYA Atheroscler.* 2015;11(1):21-29.

Salado, C., Olaso, E., & Gallot, N. et al. Resveratrol prevents inflammation-dependent hepatic melanoma metastasis by inhibiting the secretion and effects of interleukin-18. *J Transl Med.* 2011;9(59):1-11.

Salazar, J., Martinez, M. S., & Chavez, M. et al. C-reactive protein: clinical and epidemiological perspectives. *Cardiol Res Pract.* 2014;2014:1-10.

Sasso, F.C., Carbonara, O., Nasti, R., et al. Glucose metabolism and coronary heart disease in patients with normal glucose tolerance. *AMA.* 2004;291(15):1857-1863.

Sanders, T. A. B. Dietary Fat and Postprandial Lipids. *Curr Atheroscler Rep.* 2003;5:445-451.

Sansone, R., Rodriguez-Mateos, A., & Heuel, J. et al. Cocoa flavanol intake improves endothelial function and Framingham Risk Score in healthy men and women: a randomized, controlled, double-masked trial: the Flaviola Health Study. *Br J Nutr.* 2015:114:1246-1255.

Scalbert, A., Johnson, I. T., & Saltmarsh, M. Polyphenols: antioxidants and beyond. *Am J Clin Nutr.* 2005;81:215S-217S.

Scalbert, A. & Williamson, G. Dietary intake and bioavailability of polyphenols. *J Nutr.* 2000;130:2073S-2085S.

Schramm, D.D., Karim, M., Schrader, H.R., et al. Food effects on the absorption and pharmacokinetics of cocoa flavanols. *Life Sciences.* 2003;73:857-869.

Schroeter, H., Heiss, C., Balzer, J., et al. (-)-Epicatechin mediates beneficial effects of flavanol-rich cocoa on vascular function in humans. *Proc Natl Acad Sci*. 2005;103(4):1024-1029.

Shen, C., Cao, J. J., & Dagda, R. Y. et al. Green tea polyphenols benefit body composition and improves bone quality in long-term high-fat diet-induced obese rats. *Nutr Res.* 2012;32:448-457.

Shiva, S. Nitrite: a physiological store of nitric oxide and modulator of mitochondrial function. *Redos Biol.* 2013;1:40-41.

Steffen, Y., Schewe, T., & Sies, H. (-)-Epicatechin elevates nitric oxide in endothelial cells via inhibition of NADPH oxidase. *Biochem Biophys Res Common.* 2007;359:828-833.

Stote, K. S., Clevidence, B. A., Novotny, J. A., Henderson, T., Radecki, S. V., & Baer, D. J. Effect of cocoa and green tea on biomarkers of glucose regulation, oxidative stress, inflammation and hemostasis in obese adults at risk for insulin resistance. *Eur J Clin Nutr.* 2012;66:1153-1159.

Taubert, D., Berkels, R., Roesen, R., & Klaus, W. Chocolate and blood pressure in elderly individuals with systolic hypertension. *AMA*. 2003;290(8):1029-1030.

Torronen, R., Sarkkinen, E., & Tapola, N. et al. Berries modify the postprandial plasma glucose response to sucrose in healthy subjects. *Br J Nutr.* 2010;103:1094-1097.

Trujillo, M. E., Sullivan, S., & Harten, I. et al. Interleukin-6 regulates human adipose tissue lipid metabolism and leptin production in vitro. *J Clin Endocrinol Metab.* 2004;89(11):5577-5582.

United States Department of Agriculture (2015). Food Groups. Retrieved from http://www.choosemyplate.gov/food-groups/. Accessed August 13, 2015.

Valko, M., Leibfritz, D., Moncol, J., Cronin, M., Mazur, M., & Telser, J. Free radical and antioxidants in normal physiological functions and human disease. *Int J Biochem Cell Biol.* 2007;39:44-84.

Vallance, P. & Collier, J. Biology and clinical relevance of nitric oxide. *BMJ.* 1994;309:453-457.

Venkatesan, B., Valente, A. J., & Venkatapuram, S. R. et al. Resveratrol blocks interleukin-18EMMPRIN cross-regulation and smooth muscle cell migration. *Am J Physiol.* 2009;297(2):H874-H886.

Visser, M., Bouter, L.M., McQuillan, G.M., Wener, M.H., & Harris, T.B. Elevated C-reactive protein levels in overweight and obese adults. *JAMA.* 1999;282(22):2131-2135.

Vlachopoulos C, Aznaouridis K, Alexopoulos N, et al. Effect of dark chocolate on arterial function in healthy individuals. *Am J Hypertens.* 2005;18:785-791.

Wan, Y., Vinson, J.A., Etherton, T.D., Proch, J., Lazarus, S.A., & Kris-Etherton, P.M. Effects of cocoa powder and dark chocolate on LDL oxidative susceptibility and prostaglandin concentrations in humans. *Am Soc Clin Nutr.* 2001;74:596-602.

Waterhouse, Andrew L.; Shirley, Joseph R.; Donovan, Jennifer L. Antioxidants in chocolate. *The Lancet.* 1996;348:834.

Weisberg, S.P., McCann, D., Desai, M., Rosenbaum, M., Leibel, R.L., & Ferrante, A.W. Obesity is associated with macrophage accumulation in adipose tissue. *J Clin Invest.* 2003;112:1796-1808.

Westphal, S. & Luley, C. Flavanol-rich cocoa ameliorates lipemia-induced endothelial dysfunction. *Heart Vessels.* 2011;26:511-515.

Woerle, H.J., Szoke, E., Meye, C., et al. Mechanisms for abnormal postprandial glucose metabolism in type 2 diabetes. *Am J Endocrinol Metab.* 2005;290:E67-E77.

Yang, Y., Ju, D., & Zhang, M. Interleukin-6 stimulates lipolysis in porcine adipocytes. *Endocr.* 2008;33:261-269.

Yi, D., Tan, X., & Zhao, Z. et al. Reduced risk of dyslipidaemia with oolong tea consumption: a population-based study in southern China. *Br J Nutr.* 2014;111:1421-1429.

Yki-Jarvinen, H. & Utriainen, T. Insulin-induced vasodilation: physiology or pharmacology? *Diabetologia.* 1998;41:369-379.

Yudkin, J. S., Kumari, M., Humphries, S. E., & Mohamed-Ali, V. Inflammation, obesity, stress and coronary heart disease: is interlekin-6 the link? *Atherosclerosis*. 2000;148:209-214.

Yudkin, J. S., Stehouwer, C.D.A., Emeis, J. J., & Coppack, S. W. C-reactive protein in healthy subjects: associations with obesity, insulin resistance, and endothelial dysfunction: a potential role for cytokines originating from adipose tissue. *Artioscler Thromb Vasc Biol.* 1999;19:972-978.

Zamora-Ros, R., Forouhi, N. G., & Sharp, S. J. et al. The association between dietary flavonoid and lignin intakes and incident type 2 diabetes in European populations. *Diabetes Care.* 2013;36:3961-3970.

APPENDICES

Oklahoma State University Institutional Review Board

Date:	Monday, June 27, 2011
IRB Application No	HE1130
Proposal Title:	Postprandial Effects of Polyphenol-rich Cocoa Beverage on Glucose, Insulin, Lipids, Oxidative Stress and Inflammation in Type 2 Diabetic Patients
Reviewed and Processed as:	Expedited

Status Recommended by Reviewer(s): Approved Protocol Expires: 5/31/2012

Principal Investigator(s):

Arpita Basu
301 HES
Stillwater, OK 74078

Timothy J. Lyons
OUHSC WP1345
Okla. City, OK 73104

The IRB application referenced above has been approved. It is the judgment of the reviewers that the rights and welfare of individuals who may be asked to participate in this study will be respected, and that the research will be conducted in a manner consistent with the IRB requirements as outlined in section 45 CFR 46.

☒ 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.

As Principal Investigator, it is your responsibility to do the following:

1. Conduct this study exactly as it has been approved. Any modifications to the research protocol must be submitted with the appropriate signatures for IRB approval.
2. Submit a request for continuation if the study extends beyond the approval period of one calendar year. This continuation must receive IRB review and approval before the research can continue.
3. Report any adverse events to the IRB Chair promptly. Adverse events are those which are unanticipated and impact the subjects during the course of this research; and
4. Notify the IRB office in writing when your research project is complete.

Please note that approved protocols are subject to monitoring by the IRB and that the IRB office has the authority to inspect research records associated with this protocol at any time. If you have questions about the IRB procedures or need any assistance from the Board, please contact Beth McTernan in 219 Cordell North (phone: 405-744-5700, beth.mcternan@okstate.edu).

Sincerely,

Shelia H. Kennison

Shelia Kennison, Chair
Institutional Review Board

Volunteers Needed

For a Study related to:

The Health Effects of Cocoa Drink

We are testing the effects of a cocoa drink on the increase of blood sugar, lipids and blood pressure after eating a high-fat breakfast.

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

- A waist circumference of greater than 40 inches in men or 35 inches in women
- Have been diagnosed with diabetes (HbA1c >6.5%)
- Triglycerides greater than 150 mg/dL or
- Total cholesterol greater than 200 mg/dL
- Blood pressure >120/80 mm Hg

Following an initial telephone questionnaire, eligible participants will be scheduled for a screening visit and 2 follow-up visits:

There is no charge to participate in the study. Study participants will receive compensation for each follow-up visit. Visits will take place at the OUHSC General Clinical Research Center, 1122 NE 13TH, or the Harold Hamm Oklahoma Diabetes Center, 1000 N. Lincoln Boulevard, Timothy Lyons, MD Principal Investigator.

For more information, please contact Dr. Arpita Basu at arpita.basu@okstate.edu
Or (405) 744-4437 (long distance from OUHSC)

Oklahoma State University IRB # HE 11-30

Consent Version, 03, 6/15/2011 *OUHSC IRB# 15913, OSU IRB # HE-11-30*

Consent Form
University of Oklahoma Health Sciences Center (OUHSC)
Oklahoma State University (OSU)

Title: Postprandial effects of Polyphenol-rich cocoa beverage on glucose, insulin, lipids, oxidative stress and inflammation in Type 2 diabetic patients.

Sponsor: The Hershey Company

Principal Investigator: Timothy J Lyons MD, FRCP (OUHSC)
Co-Principal investigator: Misti J Leyva, MS, RD (OUHSC)
Steve Blevins, MD, (OUHSC)
Sub-Investigators: Arpita Basu, PhD, RD (OSU)
Nancy Betts, PhD, RD (OSU)

This is a clinical trial (a type of research study). Clinical trials include only patients who choose to take part in them. Please take your time to make your decision. Discuss this with your family and friends.

Why Have I Been Asked To Participate In This Study?
You are being asked to take part in this trial because you are overweight and have been diagnosed with diabetes, having a large waist, high levels of bad lipids and higher than normal blood pressure. These conditions put you at an increased risk of developing heart disease and other health problems.

Why Is This Study Being Done?
The purpose of this study is to find out about the effects of cocoa on blood pressure, blood sugar and lipids, and certain blood tests affected by consuming a fast food-style breakfast meal. Cocoa has been previously shown to improve blood pressure and blood lipids.

What is the Status of the Drugs (Devices or Procedures) involved in this study?

Cocoa powder or control powder will be made into a drink with water and will be used in the study. The cocoa powder is a commercially available food and has not been approved by FDA as a drug.

How Many People Will Take Part In The Study?
About 25 people will take part in this study at this site.

What Is Involved In The Study?

You will receive cocoa on one study day and placebo (non-cocoa containing liquid) on the other day in addition to consuming breakfast meal on each of the two days of the study. The order in which you receive cocoa or placebo is randomized. Randomization means that you are put in a group by chance, like the flip of a coin. A computer program at the study sponsor will make this random assignment. Neither you nor the investigator will choose whether you get the cocoa or the placebo on each day.

If you take part in this study, you will have the following tests and procedures:

This is a 2- week study that will be conducted at the Harold Hamm Oklahoma Diabetes Center (HHODC) and the Clinical Research Center (CRC) at OUHSC. Participants recruited at OSU will need to drive to Oklahoma City for all visits.

Screening visit:
During your first visit we will do some tests and measurements to determine if you qualify for the study. This will involve:

- reading and signing the consent form;
- measuring your height, weight, blood pressure, and waist size
- drawing about 2 tablespoons of fasting blood for measuring your blood sugar, lipids, blood cell counts to do some tests to find out how well your cells, liver, kidney, and thyroid are working; and
- guidelines and forms for a 3-day food record.

If you qualify, we will let you know over the telephone and ask you to come back for 2 additional days, at least 7-10 days apart. Several tests will be done on each of these 2 days when you will be asked to remain at the study site from about 8AM to 2PM each day. We will provide you breakfast, lunch, beverages and water at the study site. During these hours in the 2 days of the study, you can only consume foods and beverages provided by the research staff and cannot eat or drink any outside food or beverage (that is, you cannot bring anything on your own to be consumed during those hours).

If you are in the cocoa group, you will consume 2 cups of cocoa beverage that will made by mixing 2 scoops of cocoa powder with water, along with fast-food style breakfast.

If you are in the control group, you will consume 2 cups of control beverage that will made by mixing 2 scoops of control (milk, vanilla) powder with water, along with fast-food style breakfast.

The breakfast meal will be made of 2 scrambled eggs, 1 hash brown patty, 2 junior size biscuits, and 2 tsp butter. The research staff will prepare the breakfast for you.

You will also keep a diary of everything you eat for 3 days of the week, during the first week of the study, before you come in for the 2 days of the study. You will also be asked to not consume alcohol and caffeine for 12 hours, and chocolates, berries, tea and wine

for 48 hours before each day of the study. If you qualify, we will also provide you a list of food items to avoid before each of the 2 study days.

The following procedures will be done on each of the 2 days of the study:

- Fasting: when you come in the morning after overnight fast, we will place a catheter in your vein called a heplock and draw about 1 tablespoon blood to determine your blood glucose and lipids. We will also do some additional tests to determine how well your blood vessels are doing. In order to do so, we will lay you down, place a cuff on your left and right arms which will be connected to a monitor and then measure your blood pressure and how your blood vessels are working
- Breakfast with cocoa or placebo beverage: you will be asked to eat the breakfast first and then drink the cocoa or placebo beverage in front of the research staff
- 30 min, 1 hour, 2 hours, 4 hours, and 6 hours after consuming the breakfast with cocoa or placebo beverage, we will draw about 1 tablespoon blood from the heplock to determine your blood glucose and lipids. We will also do some additional tests to determine how well your blood vessels are doing. In order to do so, we will lay you down, place a cuff on your left and right arms which will be connected to a monitor and then measure your blood pressure and how your blood vessels are working

We will provide you drinking water throughout these hours and lunch at the end of the study.

If you drink cocoa the first study day, you will get placebo the second study day. If you drink placebo the first study day, you will get cocoa the second study day. The total amount of blood drawn over the two days is 12 tablespoons.

There will be at least 7-10 days interval between the 2 days of the study.

How Long Will I Be In The Study?

We think that you will be in the study for 2 weeks. The duration of the screening will be between 0.5-1 hours and the 2 additional days will involve about 6-7 hours at the clinic on each day.
There may be anticipated circumstances under which your participation may be terminated by the investigator without regard to your consent. This may occur if you fail to follow the study requirements, such as, making follow-up visits to the study site, or your fasting glucose or blood pressure is too high. You can stop participating in this study at any time. However, if you decide to stop participating in the study, we encourage you to talk to the researcher and your regular doctor first.

What Are The Risks of The Study?

While on the study, you are at risk for these side effects. You should discuss these with the researcher and/or your regular doctor. There also may be other side effects that we cannot predict.

Likely: the risks involved with taking 2 cups cocoa or control drink may include stomach aches, gas, or headaches which may happen daily or less if you are not used to taking them.

Less likely: you may develop some allergy, if not used to taking cocoa powder.

There is also the risk involved with pain, bruising and infection during blood draw. There may be some discomfort in repeated blood draws by placing a catheter in your vein called a heplock, and tests for blood pressure on each day.

Are There Benefits to Taking Part in The Study?

If you agree to take part in this study, there is no direct medical benefit to you. We hope that the information learned from this study will benefit other participants with diabetes.

What Other Options Are There?

You may choose not to participate in the study.

What About Confidentiality?

Efforts will be made to keep your personal information confidential. You will not be identifiable by name or description in any reports or publications about this study. We cannot guarantee absolute confidentiality. Codes will be used instead of names and only authorized research persons will have access to those codes. The links between codes and personal information will be destroyed as soon as data collection is complete and we no longer require any further contact with the participants. Your personal information may be disclosed if required by law. You will be asked to sign a separate authorization form for use or sharing of your protected health information.
There are organizations that may inspect and/or copy your research records for quality assurance and data analysis. These organizations include the US Food & Drug Administration, the funding agency (The Hershey Company), and the OUHSC and OSU Institutional Review Boards.

What Are the Costs?

The study sponsor will pay for all costs related to your participation in this study.

Will I Be Paid For Participating in This Study?

You will not be paid for participating in this study but you will be reimbursed $30 per blood draw on each day; a total of $ 180 per day; $360 for the entire study.

What if I am Injured or Become Ill While Participating in this Study?

In the case of injury or illness resulting from this study, emergency medical treatment is available. No funds have been set aside by Oklahoma State University or University of Oklahoma Health Sciences Center, or the funding agency to compensate you in the event of injury.

What Are My Rights As a Participant?

Taking part in this study is voluntary. You may choose not to participate. Refusal to participate will involve no penalty or loss of benefits to which you are otherwise entitled. If you agree to participate and then decide against it, you can withdraw for any reason and leave the study at any time. You may discontinue your participation at any time without penalty or loss of benefits, to which you are otherwise entitled.

We will provide you with any significant new findings developed during the course of the research that may affect your health, welfare or willingness to continue your participation in this study.
You have the right to access the medical information that has been collected about you as a part of this research study. However, you may not have access to this medical information until the entire research study has completely finished and you consent to this temporary restriction. In case of any medical concern during the study, you will be provided with the medical information immediately so that you can consult with your primary physician.

Whom Do I Call If I have Questions or Problems?

If you have questions, concerns, or complaints about the study or have a research-related injury, contact Timothy Lyons, MD at 405-271-5896 (8AM-5PM), or 405-255-3340 (anytime), or Arpita Basu, PhD at 405-744-4437 (8AM-5PM), or 405-612-2414 (anytime).
If you cannot reach the Investigator or wish to speak to someone other than the investigator, contact Dr. Shelia Kennison, IRB Chair, 219 Cordell North, Stillwater, OK 74078, 405-744-3377 or irb@okstate.edu, or the OUHSC Director, Office of Human Research Participant Protection at 405-271-2045.

Okla. State Univ. IRB
Approved 6/27/11
Expires 5/31/12
IRB # HE-11-20

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JUN 1 8 2011

APPROVAL EXPIRES
MAY 3 1 2012

Signature:

By signing this form, you are agreeing to participate in this research study under the conditions described. You have not given up any of your legal rights or released any individual or entity from liability for negligence. You have been given an opportunity to ask questions. You will be given a copy of this consent document.

I agree to participate in this study:

PARTICIPANT SIGNATURE (age ≥18) *(Or Legally Authorized Representative)*	Printed Name	Date
SIGNATURE OF PERSON OBTAINING CONSENT	Printed Name	Date

IRB No.:15913

AUTHORIZATION TO USE or DISCLOSE
PROTECTED HEALTH INFORMATION FOR RESEARCH
An additional Informed Consent Document for Research Participation may also be required.
Form 2 must be used for research involving psychotherapy notes.

Title of Research Project: **Postprandial effects of Polyphenol-rich cocoa beverage on glucose, insulin, lipids, oxidative stress and inflammation in Type 2 diabetic patients.**

Leader of Research Team: **Timothy J Lyons, MD**

Address: **1000 N. Lincoln Blvd, Suite 2900, Oklahoma City, OK 73104**

Phone Number: **405-271-5896**

If you decide to join this research project, University of Oklahoma Health Sciences Center (OUHSC) researchers may use or share (disclose) information about you that is considered to be protected health information for their research. Protected health information will be called private information in this Authorization.

Private Information To Be Used or Shared. Federal law requires that researchers get your permission (authorization) to use or share your private information. If you give permission, the researchers may use or share with the people identified in this Authorization any private information related to this research from your medical records and from any test results. Information, used or shared, may include all information relating to any tests, procedures, surveys, or interviews as outlined in the consent form, medical records and charts, name, address, telephone number, date of birth, race, and government-issued identification number.

Purposes for Using or Sharing Private Information. If you give permission, the researchers may use your private information to assess the effects of cocoa on blood pressure, glucose and lipids after consuming a high-fat breakfast meal.

Other Use and Sharing of Private Information. If you give permission, the researchers may also use your private information to develop new procedures or commercial products. They may share your private information with the research sponsor, the OUHSC Institutional Review Board, auditors and inspectors who check the research, and government agencies such as the Food and Drug Administration (FDA) and the Department of Health and Human Services (HHS). The researchers may also share your private information with other researchers for future research projects.

Confidentiality. Although the researchers may report their findings in scientific journals or meetings, they will not identify you in their reports. The researchers will try to keep your information confidential, but confidentiality is not guaranteed. Any person or organization receiving the information

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based on this authorization could re-release the information to others and federal law would no longer protect it.

YOU MUST UNDERSTAND THAT YOUR PROTECTED HEALTH INFORMATION MAY INCLUDE INFORMATION REGARDING ANY CONDITIONS CONSIDERED AS A COMMUNICABLE OR VENEREAL DISEASE WHICH MAY INCLUDE, BUT ARE NOT LIMITED TO, DISEASES SUCH AS HEPATITIS, SYPHILIS, GONORRHEA, AND HUMAN IMMUNODEFICIENCY VIRUS ALSO KNOWN AS ACQUIRED IMMUNE DEFICIENCY SYNDROME (AIDS).

Voluntary Choice. The choice to give OUHSC researchers permission to use or share your private information for their research is voluntary. It is completely up to you. No one can force you to give permission. However, you must give permission for OUHSC researchers to use or share your private health information if you want to participate in the research and if you revoke your authorization, you can no longer participate in this study.

Refusing to give permission will not affect your ability to get routine treatment or health care from OUHSC.

Revoking Permission. If you give the OUHSC researchers permission to use or share your private information, you have a right to revoke your permission whenever you want. However, revoking your permission will not apply to information that the researchers have already used, relied on, or shared.

End of Permission. Unless you revoke it, permission for OUHSC researchers to use or share your private information for their research will never end. You may revoke your permission at any time by writing to:

Privacy Official
University of Oklahoma Health Sciences Center
PO Box 26901, Oklahoma City, OK 73190
If you have questions call: (405) 271-2511

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Giving Permission. By signing this form, you give OUHSC and OUHSC's researchers led by Timothy J Lyons, MD, permission to share your private information for the research project called Postprandial effects of Polyphenol-rich cocoa beverage on glucose, insulin, lipids, oxidative stress and inflammation in Type 2 diabetic patients.

Patient/Subject Name: _____

_____ _____
Signature of Patient-Subject or Parent if subject is a child — Date

Or

_____ _____
Signature of Legal Representative** — Date

**If signed by a Legal Representative of the Patient-Subject, provide a description of the relationship to the Patient-Subject and the Authority to Act as Legal Representative:

_____
OUHSC may ask you to produce evidence of your relationship.

A signed copy of this form must be given to the Patient-Subject or the Legal Representative at the time this signed form is provided to the researcher or his representative.

IRB No.: 15913

VITA

Megan Raeann Foster

Candidate for the Degree of

Master of Science

Thesis: THE EFFECTS OF DIETARY COCOA POWDER ON INFLAMMATORY MARKERS AND CATALASE ACTIVITY IN PARTICIPANTS WITH TYPE-2 DIABETES FOLLOWING A FAST FOOD STYLE MEAL CHALLENGE

Major Field: Nutritional Sciences

Biographical:

Education:

Completed the requirements for the Bachelor of Science in Nutritional Sciences at Oklahoma State University, Stillwater, OK in 2014.

Experience:

Graduate Teaching Assistant – January 2015 – December 2015

Graduate Research Assistant – Dr. Arpita Basu – January 2015 – December 2015

Professional Memberships:

Student Member – Academy of Nutrition and Dietetics

Student Member – American Society for Nutrition