ACUTE EFFECT OF COCOA ON VASCULAR FUNCTION IN CHRONIC KIDNEY DISEASE PATIENTS: A PILOT STUDY

by

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A thesis submitted to the Faculty of the University of Delaware in partial fulfillment of the requirements for the degree of Master of Science in Human Nutrition

Summer 2014

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ACKNOWLEDGMENTS

My family and friends for their love and for always listening.

Shannon Lennon-Edwards, PhD. for supporting me and holding me to high standards.

My fellow Vascular Physiology lab-mates for encouraging me and always having time to help.

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ABSTRACT

Chronic kidney disease (CKD) is associated with an increased prevalence of cardiovascular disease (CVD) and these individuals often die of CVD before reaching end-stage kidney disease. Dysfunction of the endothelial lining of the blood vessels may cause a worsening of kidney function and increase the CVD risk in this population. Dietary polyphenol consumption, specifically flavonoids, has been associated with reduced CVD risk. Cocoa is a rich source of flavanols, a flavonoid subclass, and has been shown to improve vascular function and prevent CVD in several populations, but not yet in CKD. The aim of this study was to determine the effects of acute cocoa ingestion on endothelial-dependent dilation in subjects with CKD. We hypothesized that brachial artery flow-mediated dilation (FMD), a measure of endothelial function would increase and arterial stiffness and wave reflection would decrease from baseline to two hours following ingestion of 26 g of cocoa (CO) as compared to a placebo (PL). Seven patients with CKD (4M/3F; 53.7 ± 5.1 y) completed the randomized, double-blind, placebo-controlled trial. FMD at baseline (CO 3.96 ± 0.8%, PL 5.05 ± 1.6%) and 2 h post ingestion (CO 5.12 ± 0.9%, PL 7.6 ± 2.8%) was not significantly different between the conditions ($p>0.05$). Further, the delta change in FMD was not different (CO Δ1.16 ± 0.9 %, PL Δ 2.55 ± 1.7%; $p>0.05$). Arterial stiffness as assessed by carotid-femoral pulse wave velocity did not significantly differ over time (CO Δ-0.61 ± 0.7 m/s, PL Δ 0.66 ± 0.4 m/s; $p>0.05$) nor did wave reflection as assessed by augmentation index (CO Δ-1.43 ± 2.7, PL Δ 1.71 ± 1.5%; $p>0.05$). In conclusion, our pilot study did not find any significant change in

vascular function following ingestion of 26 g of cocoa suggesting that this dose may not be sufficient to rescue vascular dysfunction in this group of patients with CKD.

Chapter 1

INTRODUCTION AND BACKGROUND

1.1 Chronic Kidney Disease Definition and Risk Factors

Chronic Kidney Disease (CKD) affects approximately 26 million Americans (National Kidney Foundation, 2013). Individuals at high risk include those with hypertension, diabetes, a family history of CKD, and those of African American and Hispanic ethnicity (National Kidney Foundation, 2013). CKD is clinically defined as kidney damage or decreased kidney function, for three or more months (Levey, 2003). Kidney function is measured by glomerular filtration rate (GFR), the rate that substances are cleared from the plasma by the glomeruli (Nelm, 2010).

The GFR can be determined by measuring filtration markers. However, GFR can be more easily obtained using equations to determine an estimated GFR (eGFR) (Stevens, 2006). Decreases in eGFR identify a decline in kidney function. The level of kidney function determines the stage of CKD. The National Kidney Foundations Kidney Disease Outcomes Quality Initiative (K/DOQI) classifies the stages of CKD as follows (National Kidney Foundation, 2002):

Table 1.1: Stages of Chronic Kidney Disease

Stage	Description	eGFR (mL/min/1.73m^2)
1	Kidney damage w/ normal or ↑eGFR	≥90
2	Kidney damage with mild ↓eGFR	60-89
3	Moderate ↓ eGFR	30-59
4	Severe ↓ eGFR	15-29
5	Kidney failure	<15 (or dialysis)

eGFR: estimated glomerular filtration rate

1.1.1 Pathophysiology.

In chronic kidney failure there is a progressive loss of functioning nephrons. Initial loss of nephrons leads to adaptive changes where those intact are able to increase blood flow, eGFR and urine output (Guyton, 2006). Eventually these compensatory changes cannot keep up and the remaining nephrons are damaged (Kaufman, 1975). The increasing demands from the body may cause sclerosis, connective tissue replacing normal tissue, of the tiny blood vessels of the glomeruli, and further reduce kidney function (Guyton, 2006). Increases in glomerular pressure and filtration demands exacerbate the progressive nephron loss (Taal, 2006).

Decreases in eGFR often do not appear until the functional nephrons are reduced by 70-75% (Guyton, 2006). Abnormalities, such as proteinuria, may be present before eGFR falls (NKF, 2002). Low eGFR, and thus decreased kidney function, is associated with mortality, regardless of age (Hallan, 2012). Additionally, life expectancy was 4.1 years shorter in CKD NHANES participants 50 years and older with eGFR < 60 mL/min/1.73m^2 compared to CKD age matched counterparts with eGFR $\geq$60 mL/min/1.73m^2 (US Renal Data system, 2012). At extremely low eGFR levels an individual faces kidney failure, which necessitates dialysis or transplant (Levey, 2003).

1.1.2 Impact of Cardiovascular disease in Chronic Kidney Disease

Detection and treatment at the early stages of CKD is imperative in slowing not only the onset of kidney failure, but also the incidence of cardiovascular disease (CVD) (Levey, 2003). CKD and CVD are closely related. Individuals with CKD have an increased prevalence for CVD compared to the non-CKD population. In 2010 approximately 29.6% of individuals with CKD stages 3-5 had CVD, compared to 5.5% in the non-CKD population (Foster, 2013). More specifically, individuals with CKD, aged 66 years and older, have a 12.5% risk for myocardial infarction and 43.6% risk of coronary heart failure compared 5.8% and 19.1% in non-CKD aged counterparts (US Renal Data system, 2012).

Individuals with stage 3-5 CKD will often die of CVD before reaching end stage renal disease (ESRD) (Levey, 2003). Early stages of CKD are prevalent in about 11% of the US adult population, which is considerably greater than the 0.1% of the population at stage 5 kidney failure (Sarnak, 2003).

1.1.2.1 CVD Risk Factors in CKD

Understanding the risk factors for CVD is important for individuals with CKD because they are more likely to die from CVD than kidney failure (Sarnak, 2003). The American Heart Association identifies several health behaviors and health factors that may contribute to CVD development. Health behaviors include smoking, physical inactivity, poor diet and being overweight or obese (Go, 2013). Other risk factors include a family history of CVD, age, high blood cholesterol levels, diabetes and high blood pressure (Go, 2013). The risk of developing CVD doubles with each 20/10-mmHg increment increase in BP above 115/75 mmHg (Chobanian, 2003). More than 85% of CKD patients have hypertension (Balla, 2013). High blood pressure can

damage the glomeruli and initiate sclerosis of the blood vessels in the kidney, limiting their ability to filter and excrete fluid effectively (Guyton, 2006). In the presence of one or several of these risk factors blood vessels may become inflamed and promote the formation of atherosclerotic plaques, which can lead to stroke or heart attack (Vita, 2002 & Go, 2013). In addition to these traditional risk factors, those with CKD face additional CVD risk. Oxidative stress, inflammation, endothelial dysfunction, L-arginine deficiency, low eGFR and vascular calcification are some factors that may accelerate atherosclerosis and CVD in individuals with CKD (Balla, 2013, Martens, 2011, & Schiffrin, 2007). Biomarkers for oxidative stress and inflammatory markers such as C-reactive protein (CRP) and interleukin-6 (IL-6) were found to be significantly greater in CKD patients not yet receiving renal replacement therapy compared to healthy controls (Oberg, 2004). Renal insufficiency has been independently associated with elevated CRP and IL-6 (Shlipak, 2003). Oxidative stress and inflammation processes promote endothelial dysfunction, and contribute to CKD progression and CVD (Higashi, 2009, Himmelfarb, 2004, & Zyga, 2013).

1.2 The Endothelium

Alterations in the normal functioning of blood vessels play a major role in the development and onset of CVDs (Vita, 2002). Blood vessels are lined by the vascular endothelium, made up of a single cell layer of endothelial cells. The major function of the endothelium is to maintain vascular homeostasis with the release of contracting factors such as endothelin-1 and angiotensin-II, and relaxing factors such as nitric oxide (NO) and prostacyclin (Verma, 2002). Endothelial cells (ECs) release these factors in order to regulate cell adhesion, vessel wall inflammation, smooth muscle function and vascular tone (Vita, 2002).

1.2.1 Nitric Oxide

One crucial endothelial derived relaxing factor (EDRF) is NO, which induces vasodilation. The calcium/calmodulin-dependent enzyme known as endothelial nitric oxide synthase (eNOS) produces NO from L-arginine. Activation of eNOS is induced in response to shear stress or agonists, which stimulate calcium influx and intracellular calcium release, respectively (Klabunde, 2005). Cofactors required for eNOS activation includes NADPH, tetrahydropbiopterin (BH_4) and FMN/FAD (Shimokawa, 1999). Once produced, NO diffuses into the smooth muscle and assists in cyclic GMP (cGMP) formation and subsequent relaxation of the smooth muscle (Klabunde, 2005). Additionally, the action of NO attenuates inflammation through the inhibition of platelet aggregation and leukocyte adhesion (Verma 2002). Direct and indirect vasodilatory effects and thromboresistant characteristics of NO make it essential to maintaining blood vessel health (Klabunde, 2005).

1.2.2 Endothelial Dysfunction

Impaired NO activity, availability, or synthesis, can lead to endothelial dysfunction. Without the protective actions of NO, blood vessels may become thrombotic, inflamed and constricted and more likely to form atherosclerotic lesions (Vita, 2002). Reduced endothelial NO production may be caused by an alteration of signal transduction, limited L-arginine availability, lack of eNOS cofactors or limited enzyme expression (Shimokawa, 1999). During atherosclerosis, NO is oxidized by the superoxide radical, which has a high affinity for NO, resulting in peroxynitrite formation. Formation of peroxynitrite in turn oxidizes tetrahydrobiopterin (BH_4), an important eNOS cofactor. Limited BH_4 results in uncoupling of eNOS and production of superoxide, rather than NO. This cycle is exacerbated as the increase in superoxide

continues to bind the now limited amount of viable NO, and produce hydrogen peroxide and peroxynitrite (Tiefenbacher, 2001). The early and late processes of atherosclerosis are promoted by endothelial dysfunction (Verma, 2002). The endothelium may be damaged due to shear stress, such as high blood pressure. Initially macrophages come to the site of injury, activate the endothelium, and promote eNOS synthesis. A gradient of chemoattractants allows for an influx of molecules into the endothelium. Uptake of innate and immune response cells, oxidized LDL cholesterol and smooth muscle cells contribute to chronic inflammation, and eventually plaque formation (Galkina, 2009). Overtime, the plaque may block an artery or an unstable plaque may rupture and block a smaller blood vessel (Verma, 2002).

Many mechanisms are at work during atherosclerosis however the gradual impairment of NO signaling limits endothelium relaxation (Shimokawa, 1999). In addition to the cascade of events that contribute to the atherosclerotic process, it has been noted that endothelium dependent relaxation is impaired in individuals with hypertension (Panza, 1990). Endothelial dysfunction and a lack of NO contribute to high blood pressure as demonstrated in eNOS knockout mice with a significantly greater blood pressure of 128±3 mmHg compared to 108±5 mmHg in wild type mice (Yang 1999, & Rudic, 1997). The vascular smooth muscle NO-cGMP signaling pathway is limited due to the lack of NO and this has been shown to contribute to the development of hypertension (Thoonen, 2013). Impairments to the eNOS /NO pathway limit NO availability and can contribute to abnormal vessel remodeling associated with CVD (Rudic, 1999).

Endothelial dysfunction is a particular concern for CKD patients. Endothelial dysfunction may worsen renal function (Segal, 2006). Individuals with chronic renal

failure demonstrated strong associations between oxidative stress levels and endothelial dysfunction (Annuk, 2001). Free radicals and oxidative stress promote inflammation and endothelial dysfunction in the CKD population (Himmelfarb, 2004). Endothelial function is further impaired by the presence of asymmetric dimethylarginine (ADMA), an eNOS inhibitor that accumulates in those with chronic renal failure (Vallance, 1992). ADMA accumulation limits NO production, further contributing to endothelial dysfunction. CKD progression is accelerated in conjunction with increased ADMA levels (Fliser, 2005). The role of endothelial dysfunction in CVD development makes it an important target to improve cardiovascular health. The presence of both traditional and non-traditional risk factors for CVD in CKD patients suggests that endothelial dysfunction is an area to address in this population (Moody, 2012).

1.3 CVD Prevention in CKD

Prevention of or minimizing CVD in CKD is critical to improving survival. While pharmacologic therapies are often the primary method of treatment, it is useful to consider modifiable factors of CVD that do not require drug therapy. Modifiable factors such as tobacco use, physical inactivity, poor nutrition, being overweight or obese, dyslipidemia and having high blood pressure serve as important targets for treating CVD (Go, 2013). In particular, improving diet is one area that can impact several of these factors. Limiting intake of added sugars supports energy balance and weight management and lowering sodium intake can improve blood pressure (Nishida, 2004). High blood pressure is indicative of increased vascular stiffness and potential structural remodeling of arteries, which contributes to CVD (Giles, 2005). Fat intake, specifically avoidance of trans fatty acids and adequate polyunsaturated fatty acid

intake, can improve lipid profiles (Nishida, 2004). Improving one's lipid profile is important, as increased LDL cholesterol levels can result in greater oxidation of LDL and contribute to inflammation and plaque formation, leading to atherosclerosis (Galkina, 2009). CKD patients are considered in the "highest risk group" for CVD events (Sarnak, 2003). For this reason identification of effective interventions to delay CVD progression or development can be beneficial when applied to CKD patients.

1.4 Flavonoids

Polyphenols are a large family of phytochemicals found in many plant foods. The majority of antioxidants that are provided through the diet come from polyphenols (Scalbert, 2005). Polyphenols are classified as phenolic acids or flavonoids (Habauzit, 2012). There are more than 5000 flavonoid compounds, which are separated into six categories. Flavonoids are found in chocolate, berries, tea, celery, citrus fruits, broccoli, apples, and more. Flavonoid consumption has been associated with reduced CVD risk and inversely associated with coronary heart disease risk (Engler, 2006). Additionally, cardiovascular mortality is reduced in those who consume high amounts of flavonoids (Mink, 2007 & Liu, 2003). The cardioprotective impact of flavonoids includes an improved blood lipid profile, decreased blood pressure, inhibition of platelet activation and improved endothelial function (Haubauzit, 2012).

The influence of flavonoids on cardiovascular health is the result of both antioxidant effects and anti-inflammatory mechanisms (Enger, 2006). The antioxidant role of polyphenols is mediated by their structure and includes the reduction of free radicals and reactive species (Heim, 2002 & Stoclet, 2004). When they are unreduced, reactive oxygen species and free radicals can oxidize cellular proteins and lipids (Heim, 2002). Reactive species are responsible for the oxidation of LDL cholesterol

that can build up and contribute to atherosclerosis and CVD (Leeuwenburgh, 1997). Flavonoids have the ability to transfer electrons from free radicals, activate antioxidant enzymes, and inhibit oxidases (Heim, 2002). In addition to antioxidant properties, polyphenols enhance the production of the vasodilators NO and prostacyclin, while inhibiting pro-angiogenic factors and vasoconstrictors (Stoclet, 2004). They mediate NO production and improve endothelial function by increasing eNOS activity and expression. Also, they may prevent the migration of vascular cells into the endothelium, therefore delaying plaque progression (Stoclet, 2004). In a 16-year follow-up prospective study on post-menopausal women, flavonoids in the diet, specifically from cocoa, were associated with significantly reduced CVD associated mortality (Mink, 2007). In an observational study of elderly men in the Netherlands, high cocoa intake was inversely associated with blood pressure as well as cardiovascular and all-cause mortality after 15 years (Buijsse, 2006). In addition to these prospective studies, habitual dark chocolate intake over 18 weeks resulted in significantly decreased BP in older adults with elevated blood pressure (Taubert, 2007). All flavonoids in the diet offer some health benefits, however these studies identified cocoa intake as having a particularly notable role.

1.4.1 Cocoa

Cocoa contains flavanols, a flavonoid subclass, along with plant sterols and fiber (Fernandez, 2011). The flavanols in cocoa are in the forms (-)-epicatechin (see Figure 1.1) and (+)-catechin (Engler, 2004). Though flavanols are found in other foods such as apricots, cherries and tea, cocoa contains a much higher concentration (Fernandez, 2011). Processing of cocoa into different types of chocolate can influence the flavonoid concentration (Engler, 2004). Ten grams of dark chocolate contain

between 120-150 mg of polyphenols (Fernandez, 2011). This amount is increased about five times in cocoa powder, but reduced in milk chocolate counterparts (Fernandez, 2011). A 40-g serving of milk chocolate, the size of approximately one chocolate bar, contains about 394 mg of flavonoids while dark chocolate contains approximately 951 mg (Engler, 2004).

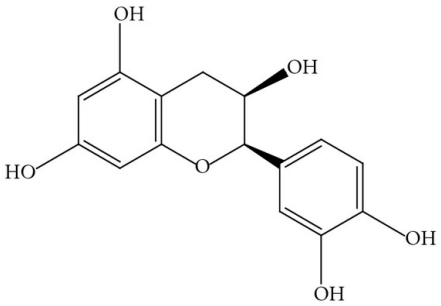

Figure 1.1: Epicatechin structure. (Rozza, 2012)

1.4.1.1 Mechanism of Cocoa's Action

Cocoa, a rich source of flavonoids, provides antioxidant and anti-inflammatory benefits. Flavanols may improve vasodilation, prevent LDL oxidation, attenuate platelet activation and reduce insulin resistance (Fernandez 2011 & Stoclet, 2004). Despite the demonstrated effects, it is still uncertain whether flavanols are the bioactive compound in flavonoids that mediate these responses (Heiss, 2006).

Epicatechin is the abundant flavanol in cocoa and is believed to contribute to the cardioprotective effects of cocoa. In healthy male adults, epicatechin was an independent predictor of improved endothelial function, as demonstrated through improved brachial artery FMD, following acute ingestion of cocoa (Schroeter, 2006). Additionally, chronic consumption demonstrated higher urinary excretion of

epicatechin and NO metabolites, demonstrating the long-term influence of epicatechin on NO production (Schroeter, 2006). Epicatechin may influence NO production in several ways. One, it may increase eNOS activity and lower arginase, resulting in increased availability of L-arginine for NO synthesis (Taubert, 2007 & Schnorr, 2008). Two, epicatechin may signal the release of NO while simultaneously inhibiting oxidation and nitration reactions that occur in the presence of excess NO (Engler, 2006). NO is preserved by limiting superoxide scavenging molecules through the inhibition of NADPH oxidase (Steffen, 2007). NO mediated mechanisms are supported by the demonstration that cocoa does not improve brachial artery FMD when eNOS is inhibited (Monahan, 2012 & Schroeter, 2006)

Epicatechin may demonstrate other cardioprotective benefits as well. It has been shown to inhibit ACE or angiotensin converting enzyme activity, which may improve blood pressure (Actis-Goretta, 2006). Further, evidence for an anti-inflammatory role in CVD has been explored. Cocoa may reduce cytokine activity at the site of endothelial injury and therefore, elicit a weaker inflammatory response (Fernandez, 2011). During inflammation, matrix metalloproteinases (MMPs) migrate to the smooth muscle layer and contribute to plaque formation and decreased stability of the plaque. Cocoa may inhibit the activation of the MMPs, thereby counteracting plaque formation (Fernandez, 2011). Epicatechin may also inhibit the activation of nuclear factor-kappa B (NF-κB), a transcription factor whose chronic activation is associated with inflammatory diseases (Fraga, 2011). In summary, the epicatechin component of cocoa appears to have the potential to provide cardiovascular protection and/or decrease CVD risk.

1.4.1.2 Cardioprotection and Cocoa Ingestion

Acute and chronic cocoa consumption has demonstrated improvements in vascular health in a variety of populations. Cocoa ingestion has been shown to improve endothelial function as measured by FMD, maintain vascular tone, improve blood pressure, decrease wave reflection and improve the lipid profile in healthy adults and in those with chronic disease (Fernandez 2011, Engler 2004 & 2006, & Vlachapolous, 2005). Acute studies have examined the vascular response over a few hours while studies of chronic ingestion have ranged from thirty days to six weeks of cocoa consumption.

Chronic studies of cocoa consumption utilizing a disease population have been promising. In diabetic patients, thirty days of cocoa intake containing 963 mg of flavanols, showed continuous sustained improvements in brachial artery FMD compared to baseline FMD (Balzer, 2008). Further, stage 1 hypertensive individuals with excessive body weight also demonstrated improved endothelial function following a four-week intervention of 50 g of 70% cocoa per day (Nogueira, 2012). Chronic cocoa ingestion has demonstrated improved vascular function in healthy populations as well. Healthy, overweight adults consuming 22 grams of cocoa per day had significant improvements in FMD at the end of a six-week trial (Njike, 2009). These studies support cocoa intake as a therapeutic strategy to improve endothelial function in a variety of populations.

Acute ingestion of cocoa also improved endothelial function in healthy, overweight adults, diabetics and smokers (Faridi, 2008, Balzer, 2008 & Heiss, 2005). Adults consuming 100 grams of dark chocolate (80% cocoa) for three days had significant improvements in FMD and wave reflection compared to consumption of flavanol- and cocoa-free white chocolate (Grassi, 2012). Compared to white chocolate,

dark chocolate attenuated the endothelial dysfunction that typically follows hyperglycemia, which was induced through an oral glucose tolerance test (Grassi, 2012). Overweight adults showed significant improvements in FMD following consumption of both dark chocolate (22g cocoa) and 22 grams of cocoa powder, compared with a placebo (Faridi, 2008). Healthy young adults showed cardiovascular protection following acute ingestion of 74g of cocoa from dark chocolate. This consumption was significantly related to improvements in FMD, decreases in wave reflection as assessed by augmentation index (Vlachopoulos, 2005). In healthy older adults, (n=23; body mass 72.7±2.3 kg) cocoa drink consumption dose dependently increased FMD (Monahan, 2011). This randomized, placebo controlled study compared measures of vascular function pre-ingestion and 1- and 2- hours post-ingestion of an experimental drink. Drinks containing 5 g, 13 g, and 26 g of cocoa significantly improved FMD compared to the placebo. The 26 g cocoa drink elicited the greatest increase, suggesting a dose dependent response. FMD increases from pre-ingestion to post-ingestion were greatest 2 hours after drink ingestion (Monahan, 2011).

Review of the literature suggests vascular improvements following both acute and chronic cocoa ingestion may be mediated by epicatechin, the most abundant flavanol in cocoa. Improvements in FMD correlated with increased epicatechin levels in the blood (Heiss, 2005). Epicatechin blood levels also dose dependently increased in response to cocoa ingestion (Monahan, 2011). Cocoa ingestion did not significantly increase plasma antioxidant levels, further supporting the role of mechanisms beyond an antioxidant effect in improving endothelial functioning (Vlachopoulos, 2005). Cocoa intake also increased circulating NO by almost fifty percent (Heiss, 2005).

Therefore, the presence and action of the flavanol epicatechin in cocoa likely plays a vital role in improving vascular health. In addition to benefits from chronic cocoa consumption, acute consumption demonstrates the biggest response with higher doses and peaks two hours post consumption of cocoa (Monahan, 2011).

1.5 Aim and Hypothesis

Individuals with CKD are more likely to die from CVD than reach end-stage kidney disease (Sarnak, 2003). Traditional risk factors alone do not account for the high incidence of CVD death in this population. Endothelial dysfunction is a precursor to the development of atherosclerosis and has been suggested to play a role in the increased CV risk (Shimokawa, 1999 & Vita, 2002). Dysfunction of the endothelium may impair the availability of the vasodilator NO, and promote plaque development and high blood pressure (Vita, 2002 & Verma, 2002). This makes vascular health an important target for interventions aimed at improving cardiovascular health and particularly in CKD.

Improving diet is a modifiable lifestyle factor that can target CV health and disease risk (Go, 2013). Finding support for dietary interventions is important because it provides individuals with non-invasive and non-pharmacologic ways to improve health. Therefore, we chose a dietary intervention as a potential therapeutic intervention to improve endothelial dysfunction and blood vessel health.

The benefits of cocoa ingestion have been demonstrated in a variety of populations, including those with CVD risk factors. Cocoa consumption has been shown to significantly improve endothelial function in smokers, stage I hypertensive individuals, overweight individuals and older adults (Heiss, 2005, Nogueira, 2012, Faridi, 2008 & Monahan, 2011). Review of the literature suggests that chronic and

acute cocoa consumption increases FMD, reduces systolic BP and may reduce stroke risk by 8%, coronary artery disease mortality by 5% and all-cause mortality by 4% (Hooper, 2008). The benefits of cocoa have yet to be evaluated in individuals with CKD, who are considered to be in the "highest risk" group for CV events (Levey, 2003). For this reason it is important to identify ways to improve cardiovascular health in this population.

Therefore, the following aim and hypothesis is proposed:

Specific aim: to determine the effects of acute cocoa ingestion on endothelial dependent dilation and arterial stiffness in subjects with CKD.

Hypothesis: We hypothesized that there would be an increase in brachial artery flow-mediated dilation and a decrease in arterial stiffness and wave reflection from baseline to two hours following ingestion of 26 g cocoa (CO) as compared to placebo (PL).

Chapter 2

METHODS

2.1 Subjects

Subjects, both men and women, aged 18-80 years with CKD stage 3-5 were recruited for participation. Stages 3-5 of CKD are defined as a glomerular filtration rate (eGFR) $\leq$60 mL/min/1.73m^2, $\geq$ 15 mL/min/1.73m^2. Subjects were excluded for diabetes, smoking, dialysis treatment, history of angina or myocardial infarction, uncontrolled hypertension, abnormal liver enzymes, pulmonary or autoimmune diseases, and cancer. They were recruited in collaboration with Nephrology Associates, P.A. in Newark, Delaware. Subjects were initially screened for participation by phone prior to visit 1. The exclusion criteria for the study were reviewed and the study was explained to the potential subjects. If subjects qualified and were interested in participating, they were asked to arrive to visit 1 in a fasted state.

2.2 Visit 1

This visit to the Vascular Physiology Lab (room 128 of the Health Sciences Complex) was approximately 45 minutes in length. Subjects arrived having fasted for at least 12 hours and no exercise for the previous 24 hours. Subjects were first consented and then asked to fill out a medical history questionnaire. Subjects were instructed on the protocol for testing visits. Subjects were screened and medically cleared for participation by a registered nurse (NP) at the Nurse Managed Health

Center (NMHC). The screening included a physical exam including height, weight, an EKG, and blood pressure measurements. A blood sample and urine specimen was collected for analysis of a complete blood count, comprehensive metabolic profile, and lipid levels. Blood samples were placed in a centrifuge and plasma was stored frozen at -80C for measuring inflammatory markers. The following data from the medical history questionnaire was used for study purposes: height, weight, age, sex, blood pressure, history of CVD (includes hypertension, heart disease, stroke, blood clots), high cholesterol, diabetes, smoking, and any medications the subject was taking and diet related information. If the screening did not identify any exclusion criteria or medical conditions that prohibited participation in the study, subjects were invited to participate in the experimental protocol. This included subjects coming back to the laboratory on two different occasions.

2.3 Visit 2

This visit to the Vascular Physiology Lab was approximately three and a half hours in length. Subjects were asked to not eat food for 4 hours, drink alcohol or caffeine for 12 hours and not exercise for 24 hours prior to the visit. Subjects were asked to bring and/or wear shorts to this visit. When the subjects arrived they rested in a supine position for 15 minutes and three measurements of blood pressure were taken. Then blood vessel function was assessed (see section 2.5). Subjects had a blood pressure cuff placed on their forearm and an ultrasound image taken of the brachial artery. The cuff was inflated for five minutes and images were captured for up to two minutes following the cuff deflation. Next a cuff was placed around the brachial artery to capture central waveforms and determine wave reflection. Lastly, a cuff was placed

around the upper thigh and a tonometer was used to capture carotid waveform and measure arterial stiffness.

2.3.1 Drink Ingestion

After blood vessel function measurements were performed subjects ingested one eight-ounce drink containing either a placebo or cocoa powder. The day that the placebo or experimental drink was administered was randomized. The cocoa packets contained 26g of cocoa and were obtained from a cocoa distributor (Hershey, PA). The nutritional content of the experimental drinks is outlined (Monahan, 2011):

Table 2.1: Nutrition information of experimental drinks

Component	Placebo	26 g cocoa
Cocoa, g	0	26
Calories, kcal	122	96
Fat, g	4.4	4.0
Carbohydrates, g	16	16
Sugars, g	13	7
Protein, g	5.8	5.8
Total polyphenols, mg	330	1470
Epicatechin, mg	0.0	96.0

The experimental drink was prepared immediately following the vascular measurements using a handheld blender and 8 ounces of warm water.

2.3.2 Post-drink Ingestion

Two hours following drink ingestion the measures of blood vessel function mentioned above were repeated. Lastly, vascular endothelium-independent vasodilatory function was determined by measuring brachial artery dilation in response to oral nitroglycerin (sublingual nitroglycerin 0.4 mg). The nitroglycerin

tablet was placed under the tongue and ultrasound images were repeated for 10 minutes. Blood pressure and heart rate was also be monitored during this time. Nitroglycerin response only took place once each visit. Before the nitroglycerin tablet was given, 3 standing blood pressure measurements were taken. If the systolic pressure was not greater than 100, nitroglycerin was not administered. If they systolic pressure was greater than 100, the subject was instructed to lay supine. Three more blood pressure measurements were taken and the subject rested for 15 minutes. Then, a baseline image of the brachial artery was recorded. Next, one tablet of .4 mg nitroglycerin was placed underneath the tongue of the subject. An image was recorded for 10 minutes and blood pressure and heart rate was measured at 2, 5, and 10 minutes in the arm not being imaged. After the imaging, subjects remained in the laboratory for an additional 30 minutes to monitor heart rate and blood pressure in response to the nitroglycerin.

2.4 Visit 3

This visit to the Vascular Physiology Lab took place at the same time of day as Visit 2 and at least 72 hours after Visit 2. This visit was approximately three and a half hours in length. Subjects went through the identical protocol and measurements outlined for Visit 2. The drink ingestion at this visit was the opposite of the drink randomly assigned for the first visit. This randomization allowed for each subject to ingest each drink type only once. Study visits are outlined in Figure 2.1.

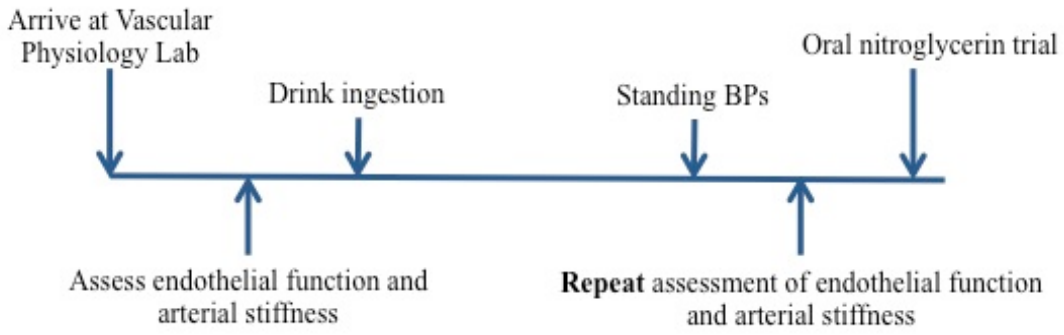

Figure 2.1: Timeline of experimental visits.

2.5 Methods to Determine Blood Vessel Function

Blood pressure was determined with triplicate measurements of brachial artery cuff BP by oscillometric sphygmomanometry. Brachial artery (left) vasodilatory responses to hyperemia were measured using a 10 MHz linear phased array ultrasound transducer (Logic e, GE). After recording baseline images, a cuff was inflated just below the elbow to 200 mmHg for 5 minutes. After release of the cuff, images were recorded for 2 minutes following cuff release. Flow mediated dilation (FMD) was used as a measure of endothelial-dependent function and expressed as a percent change from baseline normalized for shear rate calculated from the blood flow velocity and vessel diameter data.

Pulse wave analysis was used to determine wave reflection as assessed by augmentation index (AIx). A central aortic pressure waveform was synthesized from the measured brachial artery pressure waveform using a generalized transfer function (SphygmoCor XCEL, AtCor Medical, Sydney, Australia). The amplitude of central systolic arterial wave reflection was estimated by AIx, which was obtained from the configuration of the generated aortic pressure waveform. In adults above approximately 20 years of age and of average height, a distinct inflection point (Pi)

occurs in systole either before or after peak systolic pressure (Ps). AIx is calculated as (Ps-Pi)/(Ps-Pd) where Ps = systolic pressure, Pd = diastolic pressure, and Pi = pressure at the inflection point. AIx is a highly reproducible parameter, simple to measure and an indicator of systemic arterial stiffness.

Carotid to femoral pulse wave velocity (PWV) was measured by simultaneously recording a carotid and femoral pressure wave. A cuff placed around the upper thigh captured femoral wave and a tonometer captured carotid waveform (SphygmoCor Px, AtCor Medical, Sydney, Australia). The distance from the carotid measurement point to the sternal notch was subtracted from the distance from the sternal notch to the femoral measurement point and used as propagation distance. The time delay from the peak of the R wave on the ECG to the upstroke of the carotid pressure wave was subtracted from the time delay from the peak of the R wave on the ECG to the upstroke of the femoral pressure wave. PWV was calculated as propagation distance/time delay. PWV is a measure of regional stiffness of the aorta.

2.6 Statistical Analysis

The primary variable of interest was brachial artery FMD. A 2x2 repeated measures ANOVA (treatment x time) was performed to test for an effect of treatment (cocoa vs. placebo), time, and the interaction between treatment and time. Pearson's correlations were used to compare FMD to eGFR, age and epicatechin. Statistical significance was set at $p<0.05$.

Chapter 3

RESULTS

3.1 Subject Characteristics

Seven patients with CKD (4M/3F) completed the randomized, double-blind, placebo-controlled trial. Average eGFR of the subjects was 50.4 ± 3.9 mL/min/1.73m^2 classifying them as stage 3 CKD. Subjects ranged from 1 to 6 years since CKD diagnosis. Subject age ranged from 32 to 73 years, with an average of 53.7 ± 5.1 years. Average BMI was 29.6 ± 2.5 kg/m^2 placing them in the overweight but borderline obese range. Average blood pressures fell in the pre-hypertensive range although several were treated with blood pressure lowering medications. Total cholesterol was borderline high and LDL cholesterol was above optimal. Subject characteristics are presented in Table 3.1.

Subjects had a variety of co-morbidities including high blood pressure, high cholesterol, atrial fibrillation, depression and kidney stones, however none were diabetic. Subjects took anywhere from 1-5 medications. Four subjects were on lipid lowering medications such as lipitor, lovaza, and/or fenofibrate. Five were on blood pressure lowering medications such as lisinopril and spironolactone. Three subjects were on both blood pressure and lipid lowering medications.

Table 3.1: Subject characteristics

Variable	**(*n*=7)**
Sex	4M/3F
Age, yr	53.7 ± 5.1
Height, cm	167.5 ± 1.5
Weight, kg	83.7 ± 8.0
BMI, kg/m^2	29.6 ± 2.5
Systolic BP, mmHg	120.3 ± 6.6
Diastolic BP, mmHg	77.4 ± 5.3
Total cholesterol, mmol/l	210.4 ± 29.1
LDL cholesterol, mmol/l	128.3 ± 20.5
HDL cholesterol, mmol/l	59.7 ± 11.6
Triglycerides, mg/dL	53.7 ± 23.1
Glucose, mg/dL	97 ± 5.7
Blood urea nitrogen, mg/dL	24.9 ± 2.4
Creatinine, mg/dL	1.45 ± 0.08
eGFR, $mL/min/1.73m^2$	50.4 ± 3.9
Hemoglobin A1c, %	5.81 ± 0.13

Data given as mean ± SEM. BMI, body mass index; BP, blood pressure; eGFR, estimated glomerular filtration rate; F, female; M, male.

3.2 Epicatechin Intake

Subject's average epicatechin intake was 76.1 ± 27.8 mg per week. Epicatechin content of frequently consumed foods is presented in Table 3.2. The greatest sources of epicatechin were from dark chocolate, red wine, apple, and apple juice in this group of subjects. Subject's average weekly consumption was less than the amount found in our cocoa drink.

Table 3.2: Epicatechin content of typical foods consumed.

Food	Typical serving size	Epicatechin content (mg)
Dark chocolate	40 g	33.8
Milk chocolate	40 g	4.4
Red wine	6 fl oz	18.1
Raspberries	½ c	2.3
Blueberries	½ c	0.434
Green tea	10 fl oz	25
Apple	1 medium	14.7
Red grapes	1 c	1.4
Apple juice	10 fl oz	14.1

3.3 Acute Effects of Cocoa Ingestion on Measures of Vascular Function

FMD did not significantly differ when evaluating the interaction between experimental drink and time (p=0.60; Fig. 3.1). Individual FMD responses to each experimental drink are shown in Figure 3.2. As seen, there is greater variability in individual responses to the placebo drink compared to cocoa; however, the mean percentage change in FMD did not significantly differ between the placebo or cocoa trial (2.55 ± 1.7% *v* 1.16 ± 0.9%; p=0.60; Fig. 3.3). A closer look at the individual FMD responses shows two subjects had a very different response from the other five

subjects to the placebo drink. Their baseline FMD was higher and increased dramatically at 2 hours post-ingestion. When these two subjects were removed and the FMD data was re-analyzed with the remaining subjects, the delta change in FMD was greater in the cocoa trial as hypothesized although not significantly (PL, 0.25 ± 1.3% *v* CO, 2.23 ± 0.7%; p=0.345). It should be noted that the average age of these two subjects was 34 yrs while the average of the remaining 5 was 61.6 yrs making these two outliers the youngest subjects in this pilot study.

Brachial artery diameters and shear rate area under the curve (AUC) is presented for pre-ingestion and 2 hours post-ingestion in Table 3.2. There was no difference in pre-drink baseline brachial artery diameters between the trials. Further, there was no significant difference between brachial peak diameters between the trials. Average baseline diameters were larger than what is seen in the literature. Upon closer examination one subject had a significantly larger brachial diameter of about 0.75 cm, which was two standard deviations greater than the mean. When this subject was removed from the data set average baseline diameters before drink ingestion were in the normal limit for placebo (0.384 ± 0.04 cm) and cocoa (0.372 ± 0.03 cm). Additionally, baseline brachial diameter at two hours after ingestion changed similarly after removal of this subject (PL 0.349 ± 0.03, CO 0.365 ± 0.03 cm). Average peak brachial diameter at baseline (PL 0.410 ± 0.04, CO 0.389 ± 0.03 cm) and two hours (PL 0.385 ± 0.04, CO 0.382 ± 0.03 cm) also shifted upon removal of the subject. Shear rate AUC, an estimate for the shear stimulus for dilation, did not differ between the placebo and cocoa trials.

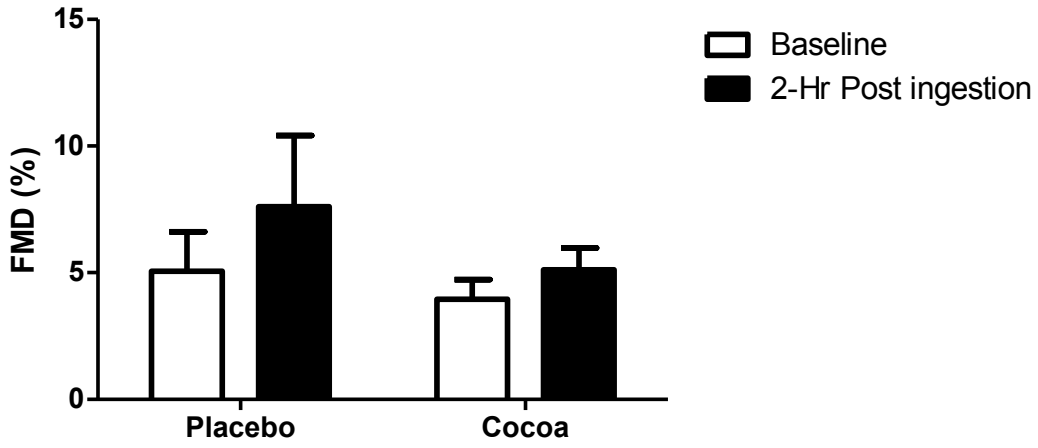

Figure 3.1: Brachial artery flow-mediated dilation at baseline and two hours after drink ingestion. Values are mean ± SEM.

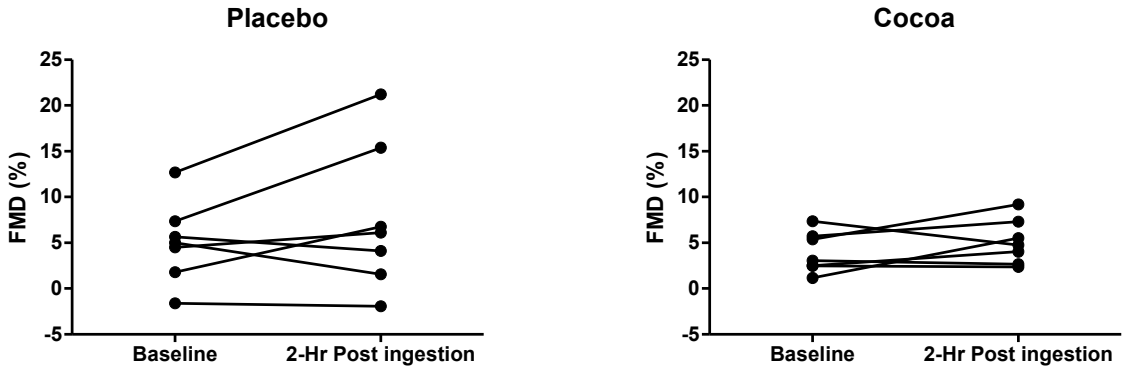

Figure 3.2: Individual responses to experimental drinks at baseline and after ingestion. FMD, flow mediated dilation.

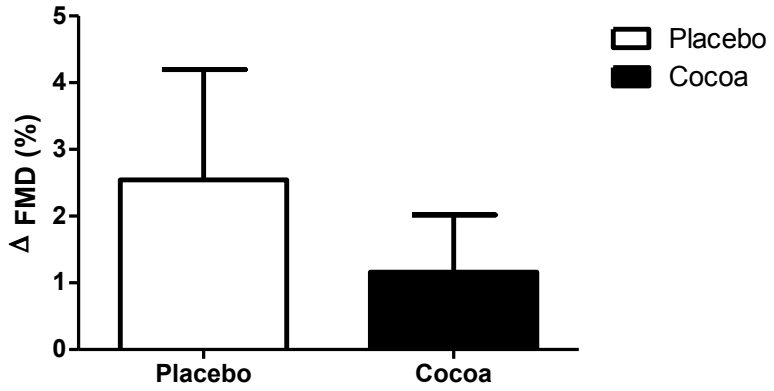

Figure 3.3: Mean percentage change in brachial artery flow-mediated dilation during the cocoa and placebo trials. Values are mean ± SEM. FMD, flow-mediated dilation.

Table 3.3: Vascular Measurements during the cocoa and placebo trials.

Drink	Time Point	Brachial baseline diameter, cm	Brachial peak diameter, cm	Shear, AUC
Placebo	Baseline	0.437 ± 0.06	0.457 ± 0.06	13073.9 ± 2354.4
	2-Hr post ingestion	0.405 ± 0.06	0.433 ± 0.06	12448.8 ± 3910.8
Cocoa	Baseline	0.431 ± 0.06	0.446 ± 0.06	15729.4 ± 3651.3
	2-Hr post ingestion	0.414 ± 0.05	0.435 ± 0.06	31501.7 ± 15100.7

Values are mean ± SEM. AUC, area under the curve.

Endothelial-independent dilation in response to sublingual nitroglycerin did not significantly differ between treatments but did trend toward decreased dilation following the cocoa drink compared to placebo (16.7 ± 3.6% *v* 25.3 ± 6.1%; p=0.067; Fig. 3.4).

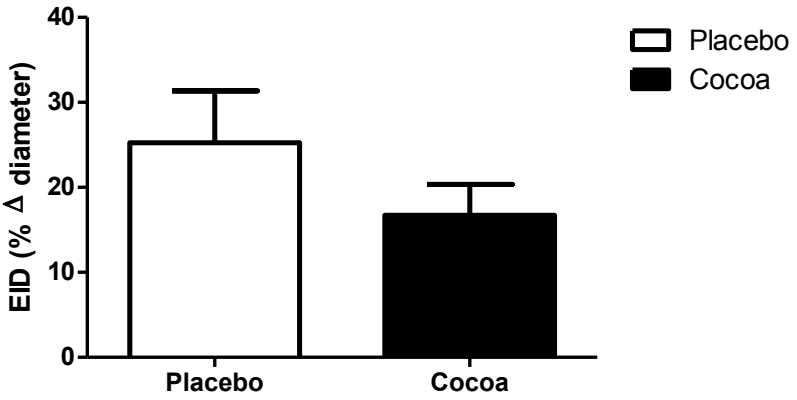

Figure 3.4: Endothelial independent dilation of the brachial artery during the cocoa and placebo trials. Values are mean ± SEM. EID, endothelial-independent dilation.

There was no significant treatment and time interaction for AIx (treatment x time effect; p=0.286; Fig. 3.5). In addition, PWV did not differ between drink and time (treatment x time effect; p=0.210; Fig. 3.6).

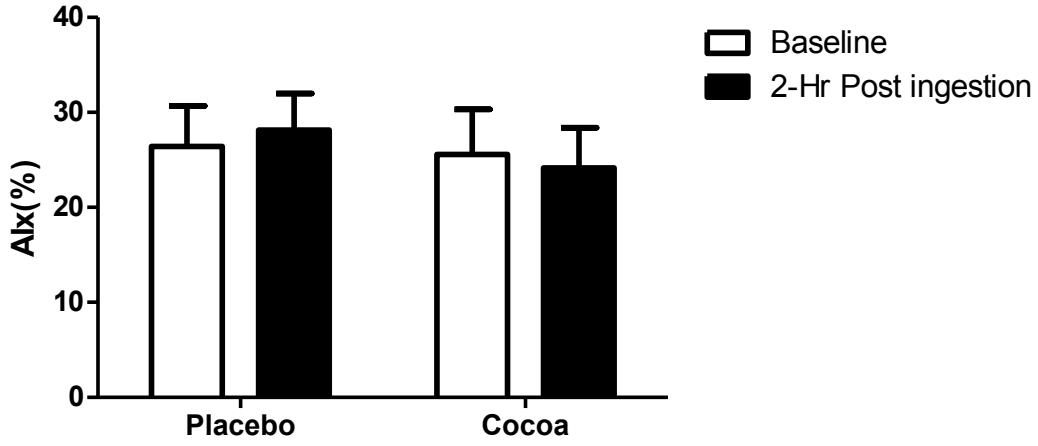

Figure 3.5: Wave reflection at baseline and two hours after drink ingestion. Wave reflection was assessed by augmentation index. Values are mean ± SEM. AIx, augmentation index.

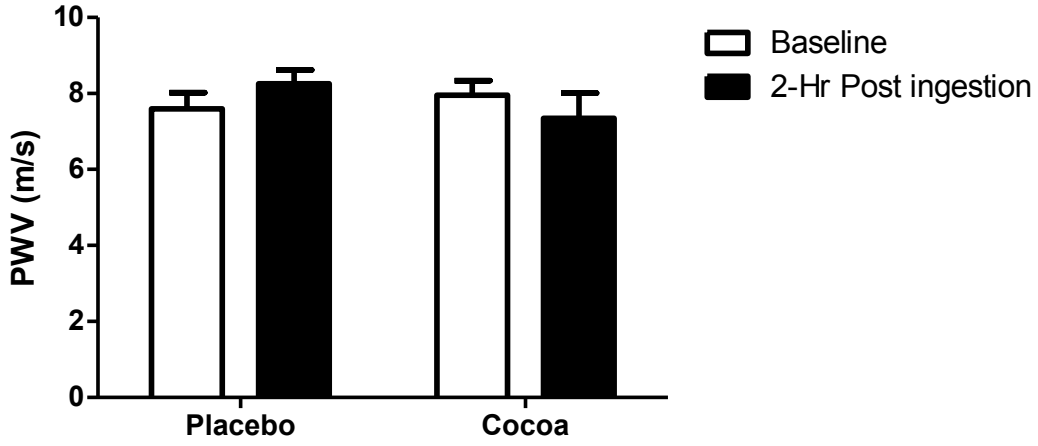

Figure 3.6: Pulse wave velocity at baseline and two hours after drink ingestion. Values are mean ± SEM. PWV, pulse wave velocity.

We were interested in determining if age affected our FMD results as our subject age range was wide (32 to 73 years). Therefore, correlations were run to determine if there was any relationship between age and FMD. There was no significant correlation between the delta change in FMD in response to cocoa and age nor average weekly intake of epicatechin. There was also no correlation between estimated GFR and baseline FMD. However, the correlation between age and baseline FMD nearly reached significance ($r= -0.75$, $p= 0.052$).

Chapter 4

DISCUSSION

The purpose of this study was to determine the effects of acute cocoa ingestion on vascular function in individuals with stage 3-4 chronic kidney disease. The hypothesis tested was that there would be an increase in brachial artery FMD and a decrease in wave reflection and arterial stiffness in response to ingestion of 26 g of cocoa as compared to placebo. The primary finding of this study indicates that acute cocoa ingestion does not rescue vascular function in this sample of individuals with chronic kidney disease. There was no significant difference in EDD as assessed by brachial artery FMD following consumption of 26 g of cocoa as compared to placebo. Endothelial independent dilation (EID) did not significantly differ but trended towards being lower following cocoa. Further, when examining PWV, a marker of arterial stiffness, we saw no differences nor did we see any differences in wave reflection as assessed by AIx. In this pilot study, we studied a group of individuals with stage 3 CKD as indicated by an average eGFR of 50.4 ± 3.9 mL/min/1.73m^2. All subjects were free of diabetes however they had a variety of co-morbidities including hypertension, dyslipidemia, and atrial fibrillation. Further, our population had an average body mass index (BMI) of 29.6 ± 2.5 kg/m^2 classifying them as overweight/borderline obese.

4.1 Endothelial Function

We assessed FMD in our subjects both at baseline and 2 hours following consumption of the experimental drink. FMD has been shown to be an independent predictor of future cardiovascular events in a CKD population (Yilmaz, 2011). Our baseline FMDs averaged 4.5 ± 0.9% that is similar to other studies (Lilitkarntakul, 2011, Recio-Mayoral, 2011) in CKD patients. Indeed, Lilitkarntakul (2011) reported FMD values of 4.0 ± 2.6 and 4.2 ± 3.7 %, in non-diabetic, stage 3 and 4 CKD subjects, respectively while Recio-Mayoral et al., (2011) reported FMDs of 3.2% (1.6-4.8) in stage 3-4 CKD subjects.

While our FMD values are similar to previously published studies, an observational cohort study of early and late stage CKD patients found that FMD decreases as kidney function worsens (Yilmaz et al., 2006; 2011). Further, eGFR is correlated with FMD and thus endothelial function (Lilitkarntakul, 2012 & Recio-Mayoral, 2011). In the above-mentioned cohort, individuals with stage 3-5 CKD had FMD values of 6.8%, 6.2%, and 5.1%, respectively (Yilmaz, 2011). This is similar to earlier work by this group that showed declines in FMD (6.71 ± 0.38, 5.97 ± 0.52, 4.75 ± 0.63%) as CKD progressed from stage 3 to 5. Additionally, patients at stage 5 with renal failure have a significantly lower FMD (0.7 ± 0.5 %) compared to earlier stages and healthy controls (2.6% *v* 6.5%) (Lilitkarntakul, 2011 & Thambyrajah, 2000). One study comparing stage 3-4 CKD to age matched healthy controls found no significant difference in FMD (Ghanavatian, 2014) but found the greatest decrements in endothelial function likely to occur closer to stage 5. Previously, work in our lab has shown that FMD is significantly decreased in CKD (approximately 4.0%) compared to healthy controls (approximately 9.0%) (Kuczmarski, 2011). Our subjects, categorized

at stage 3 (average eGFR 50.4 ± 3.9 mL/min/1.73m^2) are likely on a similar trajectory of accelerated endothelial dysfunction as their disease progresses.

Although CKD patients are at great risk for endothelial dysfunction, few studies have assessed vascular function in response to supplementation in this population. Stage 3-4 CKD patients with low vitamin D status demonstrated improved FMD following 16 weeks of vitamin D supplementation. This intervention was able to improve FMD significantly from 3.1 ± 3.3 % to 6.1 ± 3.7 % (Chitalia, 2014). In an unblinded study of renal transplant patients, FMD significantly increased from baseline (3.2 ± 1.8 %) to five weeks (4.4 ± 2.0 %) after a soy protein diet where 25 g of animal protein was replaced with soy protein (Cupisti, 2007). Soy contains isoflavones, which are another flavonoid subclass.

To our knowledge, our pilot study is the first to examine the role of the flavonoid subclass of flavanols in CKD. Epicatechin, a flavanol in cocoa, has demonstrated improvements in FMD in many populations including overweight adults (Njike, 2009) and those with stage 1 hypertension (Nogueira, 2012). We did not find significant improvements in FMD following cocoa ingestion as compared to placebo (CO Δ1.2±0.86 %, PL Δ2.5±1.7 %). Our dose of 26 g cocoa powder, containing 96 mg of epicatechin previously demonstrated an increase in FMD 2 hours post-ingestion in healthy older adults (Monahan, 2011). It is possible that in an unhealthy population with poor vascular health, such as CKD, a higher acute dose of cocoa and epicatechin may have been necessary to yield similar results.

While FMD did not improve following ingestion of the cocoa drink, other factors such as elevated oxidative stress may have played a role in the lack of significant response to cocoa ingestion (see section 4.1.2) Most likely our subjects

entered the study visits with poorer vascular health compared to the healthy young and older adults, and overweight older adults studied in other acute interventions who saw a significant response to cocoa powder (Monahan, 2011 & Faridi, 2008) and solid dark chocolate (Vlachopoulos, 2005). A study by Balzer et al. (2008) showed that older adults aged 50-80 years with type-2 diabetes for at least 5 years had increased FMD following acute cocoa ingestion. However, only the high cocoa drink, with epicatechin almost twice our beverage (203mg *v* 96 mg), demonstrated significant increases in FMD at 2 hours compared to placebo (Balzer, 2008) suggesting our subjects may require a higher dose of epicatechin.

These findings, though conflicting with our results, suggest that diseased populations with high cardiovascular risk may warrant a higher dose of cocoa to see an effect on endothelial-dependent dilation. Only one study on cocoa flavanols yielded FMD results similar to ours. Farouque et al. (2006) studied the effect of both acute and chronic cocoa ingestion in individuals with coronary artery disease (CAD) (n=40). Subjects consumed either a flavonoid rich chocolate bar or cocoa beverage containing a total of 107 mg epicatechin or a placebo for 6 weeks daily. Vascular measurements were conducted at baseline, 90 minutes following ingestion of the first beverage, and at weeks 3 and 6. There were no significant increases in FMD at any of the time points. Other measures of endothelial function included forearm blood flow, systemic arterial compliance, cellular adhesion biomarkers in the blood, as well as lipid profile and blood pressure. None of these assessments showed significant improvements acutely or after 6 weeks (Farouque, 2006). This study is notable because not only was vascular function assessed in a variety of ways, but also the results mirror our findings. The authors noted that their subjects had numerous cardiovascular risk

factors compared to subjects in other studies, such as those found in individuals with hypertension (Noguiera, 2012). The vascular burden placed on CAD subjects, similar to CKD subjects, may be too great to elicit a beneficial effect from flavanol supplementation.

4.1.1 Endothelial Independent Dilation

Endothelial-independent dilation (EID) was assessed in response to sublingual nitroglycerin in four of the subjects. Nitroglycerin targets the smooth muscle and increases dilation independent of the endothelium. Dilation was decreased following the cocoa drink compared to placebo (16.7 ± 3.6% *v* 25.3 ± 6.1%). This trended towards significance (p=0.067).

Previously, healthy older adults showed similar EID responses to varying levels of cocoa (2, 5, 13, and 26 g) (Monahan, 2011). Similarly, in a group of individuals with type 2 diabetes, EID was similar between cocoa and placebo both acutely and over a 30-day intervention (Balzer, 2008). Similar effects of nitroglycerin on vessels at varying cocoa levels indicates that the cocoa induced vasodilation measured by FMD is in fact due to increased NO production in the endothelium, rather than increased response to NO (Monahan, 2011).

One study did find EID to be significantly decreased with chocolate consumption compared to control in healthy adults (n=17, age 28.9 years) (Vlachopoulus, 2005). It is important to point out that in that group resting brachial artery diameter at the time of nitroglycerin administration was significantly higher following the chocolate compared to the control (Vlachopoulus, 2005). In our subjects we also found resting brachial artery diameter to be greater following cocoa compared

to placebo (0.472 ± 0.12 vs 0.295 ± 0.02 cm; p=0.308) however this was not significant.

4.1.2 Epicatechin Mechanisms of Action

Cocoa contains several compounds such as epicatechin, theobromine, caffeine and magnesium, which may elicit numerous benefits (Ellam, 2013). Epicatechin has been identified as the constituent responsible for improving vascular function through endothelial dependent dilation; with the isolated compound eliciting the same improvement in vasodilation as cocoa powder (Schroeter, 2006). Epicatechin has been shown to directly increase NO levels *in vitro* in human endothelial cells (Brossette, 2011). Although more research is warranted in determining all of the underlying mechanisms behind the benefits of cocoa, a few important functions demonstrate epicatechin's ability to improve bioavailability and bioactivity of nitric oxide.

First, epicatechin activates eNOS through the calcium-calmodulin pathway. It helps eNOS dissociate from the cell membrane, thereby mediating coupling with calcium for activation (Ramirez-Sanchez, 2010). Epicatchin helps decrease superoxide concentration in the endothelial cell in two ways; it scavenges superoxide molecules (Ruijters, 2013) and its metabolites limit superoxide generation by inhibiting endothelial NADPH oxidase (Steffen, 2007). Finally, epicatchin lowers arginase activity, resulting in greater availability of L-arginine, a NO precursor (Schnorr, 2008). Increased NO improves EDD, and consistent epicatechin intake may eventually help down regulate the arginase gene and sustain vasodilatory effects (Schnorr, 2008). Plasma total epicatechin is correlated with increases in EID following cocoa ingestion (Heiss, 2005 & Monahan, 2011).

Epicatechin plays an important role in maintaining NO concentrations and activating eNOS, however L-arginine is the limiting substrate of NO production. L-arginine synthesis occurs in the kidney and accounts for most of the endogenous supply throughout the body (Wu, 1998). In those with impaired renal function uptake of L-arginine's precursor, cirtulline, and subsequent release of L-arginine from the kidney is reduced (Tizianello, 1980).

In individuals with CKD normal plasma levels of L-arginine, impaired transport of L-arginine into cells may mask a deficiency (Baylis, 2007). There is a high prevalence of hyperhomocysteinemia in CKD (Nerbass, 2006) that decreases the expression of the cationic amino acid transporter (CAT-1), which brings L-arginine into the cell (Jin, 2007). In CKD there is an increase in uremic toxins because the kidneys cannot filter them as readily (Liabeuf, 2011). L-arginine transport into endothelial cells may be inhibited in the presence of uremic toxins (Wagner, 2002). The eNOS inhibitor ADMA is also increased in CKD (Vallance, 1992 & Yilmaz, 2006) and competes with L-arginine for CAT-1 transport into endothelial cells (Closs, 1997). Additionally, ADMA inhibits eNOS (Valance, 2005) and limits NO production in that way as well. These unique characteristics that are present in CKD support the idea that limited NO availability is in part due to limited presence of the precursor L-arginine in the endothelial cells. This may explain why cocoa was not be able to elicit vasodilatory benefits in our CKD population because there is not enough precursor to stimulate NO production.

4.1.2.1 Epicatechin Intake

During a 4-week study on the effects of daily cocoa ingestion in hypertensive adults, participants completed a 3-day food record and were instructed to avoid other

flavonoid containing foods throughout the intervention (Noguiera, 2012). A randomized double-blind 6-week crossover controlled trial in overweight adults did not collect diet records but instructed participants to avoid flavonoid containing foods for 24 hours before each testing day (Njike, 2009). We did not instruct our subjects to avoid any foods because they arrived to the visits fasted for 4 hours. This is similar to other acute studies on adults (Monahan, 2011 & Balzer, 2008).

Although we did not control for flavonoid intake the day before the visits we collected information about typical epicatechin intake. Average weekly epicatechin intake of subjects was 76.1 ± 27.8 mg. Intake ranged from 2.72 mg to 224 mg per week. Common sources of epicatechin consumed by subjects included milk and dark chocolate, apples, wine, and raspberries (see Table 3.2 in the results section). Subjects ranged from consuming foods daily to just a few times a month. It is important to note that diet intake is variable, especially between seasons.

4.2 Wave Reflection and Arterial Stiffness

We also evaluated arterial stiffness and wave reflection in our CKD subjects. We did not find any significant changes in wave reflection, as assessed by AIx nor in PWV, an indicator of arterial stiffness following cocoa ingestion. A study in a non-dialyzed CKD population free of established CVD and diabetes, assessing arterial stiffness demonstrated that AIx does not significantly differ across stages of CKD or compared to age matched healthy controls (Lilitkarntakul, 2011). Average baseline AIx of our subjects (26 ± 3.2%) was similar to those of the stage 3 CKD subjects of the previously mentioned cohort (24 ± 14%) (Lilitkarntakul, 2011). Futhermore, few studies have examined AIx in response to acute cocoa ingestion. Healthy young adults demonstrated significant decreases in AIx following cocoa ingestion (Vlachopoulos,

2005). However, these differences were greatest at 180 minutes, while our study assessed subjects 120 minutes after ingestion. Based on the limited assessment of wave reflection in response to CKD supplement interventions and in response to cocoa, it is not surprising that we too, did not see significant changes after acute cocoa ingestion.

Similar to the AIx data, our average baseline PWV (7.8 ± 0.29 m/s) is similar to stage 3 CKD patients similar to ours (7.0 ± 1.4 m/s) (Lilitkarntakul, 2011). It is important to note PWV increases significantly as renal function worsens (Lilitkarntakul, 2011). Cardiovascular risk increases by 10% with each 1 m/s increase in PWV (Vlachopoulos, 2010) so interventions that can attenuate the increase in PWV in a CKD population should be determined. Arterial stiffness is another measurement that has been understudied in response to an intervention in the CKD population as well as in response to an acute cocoa dose. Although vitamin D supplementation in CKD improved FMD over 16 weeks, PWV did not significantly change (Chitalia, 2014). Post-menopausal women with type 2 diabetes improved PWV over 1 year after supplementation with cocoa (Curtis, 2012). The cocoa supplement was enriched with flavanols and isoflavones, another flavonoid subclass, so the results may be due to a synergistic effect of the flavonoids (Curtis, 2012). Following acute cocoa ingestion in a group of healthy young adults, there were no changes in PWV, which is consistent with our findings (Vlachopoulos, 2005) Additionally, a meta-analysis of arterial stiffness data from flavonoid interventions found that although flavonoids improve arterial stiffness, flavanols may have a lesser effect compared to isoflavones and anthocyanins (Lilamand, 2014).

4.3 Perspectives

There are several potential mechanisms that could have influenced the results from our study. These include our small sample size, the dose of cocoa used, the stage of CKD in these subjects and finally, the recognition that CKD may create a unique physiologic environment that prevents cocoa from eliciting a response.

The small sample size is an important factor to consider when examining the results of our data. With a limited number of subjects (n=7) age ranged across 40 years; spanning from 32 to 73 years. Additionally, the eGFR of our subjects ranged across stage 3. Stage 3 CKD is defined as a moderate decrease in eGFR ranging from 30-59 mL/min/1.73m^2. Our subjects ranged from 32 to 59 mL/min/1.73m^2 thereby including individuals categorized as early stage 3 as well as those who were nearing stage 4. It may be that an individual in stage 4 may respond more to a nutritional supplement. With a small sample size, one subject has the ability to pull the data in one direction. This was seen in our own brachial diameter results. Our baseline brachial diameter at the baseline time point for placebo and cocoa was 0.437 ± 0.06 and 0.431 ± 0.06 cm, respectively. These values are higher than what is seen in the literature for healthy younger and older adults with baseline brachial diameter falling between 0.37 and 0.40 cm, respectively (Vlachapoulos, 2005 & Monahan, 2011). Upon examination of our diameters we found that one subject's diameters for both baseline and peak were greater than two standard deviations away from the mean. When this subject was removed diameters entered a range that one would expect, falling between 0.349 ± 0.03 and 0.384 ± 0.04 cm for baseline diameter.

The lack of a significant difference in the response to cocoa ingestion may have been due to the dose given. Our dose of 26 g cocoa with 96 mg epicatechin has previously been shown to improve FMD in a group of healthy older adults (n=23; 63 ±

2 yrs) (Monahan, 2011). At 2-hours post ingestion these individuals saw significant increases in FMD compared to the placebo and lower cocoa doses (2 and 5 g). A study in type 2 diabetics (n=10; 64.7 ± 9.9 yrs) examined the effects of varying levels of cocoa ingestion on FMD (Balzer, 2008). This study looked at a control beverage (16.8 mg epicatechin), a medium flavanol drink (78.9 mg epicatechin), and a high flavanol drink (203 mg epicatechin). In this population the high flavanol drink was the only drink to significantly improve FMD compared to baseline and the control drink. It is important to note that this dose of 203 mg epicatechin was almost double our dose of 96 mg epicatechin. One study conducted in older adults with coronary artery disease (n=40; 61 ± 8) did not find any improvements in endothelial function in response to a dose of 107 mg epicatechin (Farouque, 2006) that is similar to the dose used in our study. In this population FMD did not differ between groups or change from baseline to 90 minutes post-ingestion, nor at 3 and 6 weeks of daily ingestion. The results of these studies suggest that in a high-risk population, such as CKD, a higher dose of cocoa may be warranted to see an acute response in FMD.

Finally, those with CKD may have a unique physiologic state that is not seen in other diseases. With impaired renal function there is a reduced uptake of citrulline, L-arginine's precursor, by the kidneys (Tizianello, 1980). This decreases the kidney's subsequent release of L-arginine. Skeletal muscle breakdown may also contribute to the presence of L-arginine (Martens, 2011). While plasma levels of L-arginine remain normal, this may mask a cellular deficiency (Baylis, 2007). Impaired transport of L-arginine into endothelial cells may play an important role in endothelial dysfunction in this population (Baylis, 2007 & Martens, 2011). L-arginine is transported into the endothelial cell via cationic amino acid transporter (CAT-1). ADMA accumulates in

those with CKD (Yilmaz, 2006) and competes with L-arginine for CAT-1 transport into the cell (Closs, 1997). In addition, hyperhomocysteinemia is prevalent in CKD (Nerbass, 2006), and decreases the expression of CAT-1 (Jin, 2007). Uremic toxin accumulation in CKD (Liabeuf, 2011) may limit the transport of L-arginine into the cell (Wagner, 2002). Within the endothelial cell, the presence of ADMA acts as an eNOS inhibitor thereby further limiting NO production. These unique characteristics of CKD support the idea that L-arginine may be less available in the endothelial cell and therefore individuals with CKD are not able to sufficiently produce NO. These factors contribute to endothelial dysfunction in the CKD population, and may limit cocoa's vasodilatory mechanisms of action (see section 4.1.2).

In summary, our sample size, stage of CKD, and dose of cocoa may not have been large enough to see an acute response from cocoa in this population. Those with CKD have a unique physiological state that is not seen in other diseases and this may have played a role in our findings. It is unclear if targeting CKD patients at a later stage would elicit a response. At this time, we cannot draw conclusions about cocoa's ability to impact vascular function in the CKD population as more research is warranted.

4.4 Limitations

Several limitations may have contributed to the lack of effect in this study. First, because this was a pilot study there was a small sample size (n=7). Our subjects also had a wide age range (32 to 73 years). Time since CKD diagnosis was collected from six subjects and ranged from 1 to 6 years. We did not control for this because subjects pointed out they were not sure how long they had the disease prior to their

official diagnosis from a doctor. Finally, we did not measure oxidative stress, which may have been a valuable tool in studying the results in these subjects.

4.5 Future Directions

Future research in this population warrants the use of a higher dose of cocoa and perhaps a long-term intervention. Our subjects ranged from adults to older adults, ages 32 to 73 years. A more focused recruitment of just middle-aged adults (36-55 years) or just older adults (>55 years) may provide more consistent results across subjects. Also, the average eGFR of our subjects places them in stage 3 CKD (50.4 ± 3.9 mL/min/1.73m^2) but eGFR ranged across stage 3 from 32 to 59 mL/min/1.73m^2. Enrolling subjects as soon as they reach stage 3 (59 mL/min/1.73m^2) may be another criteria to create more homogeneity in a study sample.

CKD patients have a high risk of developing CVD and identifying potential therapies that may replace or augment pharmacological treatment deserve investigation (Levey, 2003). Recent reviews outline other potential dietary interventions that may benefit the CKD patient. Resveratrol, another polyphenol compound, has been shown to be cardioprotective in several populations but has not been investigated in the CKD population (Saldanha, 2013). Further, omega-3 polyunsaturated fatty acids may improve vascular aspects of CKD and should be studied (Lee, 2013). There is the need for additional research trials to identify methods of alleviating vascular burden in CKD.

4.6 Conclusion

In conclusion, we found that an acute dose of 26 g of cocoa powder with 96 mg of epicatechin was not sufficient to restore vascular function in a small sample of

CKD patients. Previous research in a CAD population with high vascular burden did not demonstrate benefit from cocoa supplementation (Farouque, 2006). These findings suggest that in a CKD population with significant vascular dysfunction, cocoa intervention earlier in the progression may be beneficial and additional research in this area is warranted.

REFERENCES

Actis-Goretta, L., Ottaviani, J. I., & Fraga, C. G. (2006). Inhibition of angiotensin converting enzyme activity by flavanol-rich foods. *Journal of Agricultural and Food Chemistry, 54*(1), 229-234. doi:10.1021/jf052263o

Annuk, M., Zilmer, M., Lind, L., Linde, T., & Fellstrom, B. (2001). Oxidative stress and endothelial function in chronic renal failure. *Journal of the American Society of Nephrology, 12*, 2747-2752.

Balla, S., Nusair, M. B., & Alpert, M. A. (2013). Risk factors for atherosclerosis in patients with chronic kidney disease: Recognition and management. *Current Opinion in Pharmacology, 13*(2), 192-199. doi:10.1016/j.coph.2012.12.001; 10.1016/j.coph.2012.12.001

Balzer, J., Rassaf, T., Heiss, C., Kleinbongard, P., Lauer, T., Merx, M., Kelm, M. (2008). Sustained benefits in vascular function through flavanol-containing cocoa in medicated diabetic patients a double-masked, randomized, controlled trial. *Journal of the American College of Cardiology, 51*(22), 2141-2149. doi:10.1016/j.jacc.2008.01.059; 10.1016/j.jacc.2008.01.059

Baylis, C. (2008). Nitric oxide deficiency in chronic kidney disease. *American Journal of Physiology.Renal Physiology, 294*(1), F1-9. doi: 00424.2007

Brossette, T., Hundsdorfer, C., Kroncke, K. D., Sies, H., & Stahl, W. (2011). Direct evidence that (-)-epicatechin increases nitric oxide levels in human endothelial cells. *European Journal of Nutrition, 50*(7), 595-599. doi: 10.1007/s00394-011-0172-9

Buijsse, B., Feskens, E. J., Kok, F. J., & Kromhout, D. (2006). Cocoa intake, blood pressure, and cardiovascular mortality: The zutphen elderly study. *Archives of Internal Medicine, 166*(4), 411-417. doi:10.1001/.411

Chitalia, N., Ismail, T., Tooth, L., Boa, F., Hampson, G., Goldsmith, D., et al. (2014). Impact of vitamin D supplementation on arterial vasomotion, stiffness and endothelial biomarkers in chronic kidney disease patients. *PloS One, 9*(3), e91363. doi: 10.1371/journal.pone.0091363

Chobanian, A. V., Bakris, G. L., Black, H. R., Cushman, W. C., Green, L. A., Izzo, J. L.,Jr. National High Blood Pressure Education Program Coordinating Committee. (2003). The seventh report of the joint national committee on prevention, detection, evaluation, and treatment of high blood pressure: The JNC 7 report. *JAMA : The Journal of the American Medical Association, 289*(19), 2560-2572. doi:10.1001/jama.289.19.2560

Closs, E. I., Basha, F. Z., Habermeier, A., & Forstermann, U. (1997). Interference of L-arginine analogues with L-arginine transport mediated by the y+ carrier hCAT-2B. *Nitric Oxide : Biology and Chemistry / Official Journal of the Nitric Oxide Society, 1*(1), 65-73. doi:S1089-8603(96)90106-8

Cupisti, A., Ghiadoni, L., D'Alessandro, C., Kardasz, I., Morelli, E., Panichi, V., et al. (2007). Soy protein diet improves endothelial dysfunction in renal transplant patients. *Nephrology, Dialysis, Transplantation : Official Publication of the European Dialysis and Transplant Association - European Renal Association, 22*(1), 229-234.

Curtis, P. J., Sampson, M., Potter, J., Dhatariya, K., Kroon, P. A., & Cassidy, A. (2012). Chronic ingestion of flavan-3-ols and isoflavones improves insulin sensitivity and lipoprotein status and attenuates estimated 10-year CVD risk in medicated postmenopausal women with type 2 diabetes: A 1-year, double-blind, randomized, controlled trial. *Diabetes Care, 35*(2), 226-232. doi:10.2337/dc11-1443

Ellam, S., & Williamson, G. (2013). Cocoa and human health. *Annual Review of Nutrition, 33*, 105-128. doi:10.1146/annurev-nutr-071811-150642

Engler, M. B., & Engler, M. M. (2004). The vasculoprotective effects of flavonoid-rich cocoa and chocolate. *Nutrition Research, 24*, 695-706.

Engler, M. B., & Engler, M. M. (2006). The emerging role of flavonoid-rich cocoa and chocolate in cardiovascular health and disease. *Nutrition Reviews, 64*(3), 109-118.

Faridi, Z., Njike, V. Y., Dutta, S., Ali, A., & Katz, D. L. (2008). Acute dark chocolate and cocoa ingestion and endothelial function: A randomized controlled crossover trial. *The American Journal of Clinical Nutrition, 88*(1), 58-63.

Farouque, H. M., Leung, M., Hope, S. A., Baldi, M., Schechter, C., Cameron, J. D., & Meredith, I. T. (2006). Acute and chronic effects of flavanol-rich cocoa on vascular function in subjects with coronary artery disease: A randomized double-blind placebo-controlled study. *Clinical Science (London, England : 1979), 111*(1), 71-80. doi:CS20060048

Fernandez-Murga, L., Tarin, J. J., Garcia-Perez, M. A., & Cano, A. (2011). The impact of chocolate on cardiovascular health. *Maturitas, 69*(4), 312-321. doi:10.1016/j.maturitas.2011.05.011; 10.1016/j.maturitas.2011.05.011

Fliser, D., Kronenberg, F., Kielstein, J. T., Morath, C., Bode-Boger, S. M., Haller, H., & Ritz, E. (2005). Asymmetric dimethylarginine and progression of chronic kidney disease: The mild to moderate kidney disease study. *Journal of the American Society of Nephrology : JASN, 16*(8), 2456-2461. doi:10.1681/ASN.2005020179

Foster, M. C., Rawlings, A. M., Marrett, E., Neff, D., Willis, K., Inker, L. A., et al. (2013). Cardiovascular risk factor burden, treatment, and control among adults with chronic kidney disease in the united states. *American Heart Journal, 166*(1), 150-156. doi:10.1016/j.ahj.2013.03.016; 10.1016/j.ahj.2013.03.016

Fraga, C. G., & Oteiza, P. I. (2011). Dietary flavonoids: Role of (-)-epicatechin and related procyanidins in cell signaling. *Free Radical Biology & Medicine, 51*(4), 813-823. doi:10.1016/j.freeradbiomed.2011.06.002; 10.1016/j.freeradbiomed.2011.06.002

Galkina, E., & Ley, K. (2009). Immune and inflammatory mechanisms of atherosclerosis. *Annual Review of Immunology, 27*, 165-197. doi:10.1146/annurev.immunol.021908.132620; 10.1146/annurev.immunol.021908.132620

Ghanavatian, S., Diep, L. M., Barany, P., Heimburger, O., Seeberger, A., Stenvinkel, P., et al. (2014). Subclinical atherosclerosis, endothelial function, and serum inflammatory markers in chronic kidney disease stages 3 to 4. *Angiology, 65*(5), 443-449. doi:10.1177/0003319713483000

Giles, T. D., Berk, B. C., Black, H. R., Cohn, J. N., Kostis, J. B., Izzo, J. L.,Jr, & Weber, M. A. (2005). Expanding the definition and classification of hypertension. *Journal of Clinical Hypertension (Greenwich, Conn.), 7*(9), 505-512.

Go, A. S., Mozaffarian, D., Roger, V. L., Benjamin, E. J., Berry, J. D., & Borden, W. B. (2013). Heart disease and stroke statistics--2013 update: A report from the american heart association. *Circulation, 127*(1), e6-e245. doi:10.1161/CIR.0b013e31828124ad; 10.1161/CIR.0b013e31828124ad

Grassi, D., Desideri, G., Necozione, S., Ruggieri, F., Blumberg, J. B., Stornello, M., & Ferri, C. (2012). Protective effects of flavanol-rich dark chocolate on endothelial function and wave reflection during acute hyperglycemia. *Hypertension, 60*(3), 827-832.

Guyton, A. C., & Hall, J. E. (2006). *Textbook of medical physiology*. (11th ed.). Philadelphia, PA: Elsevier Saunders.

Habauzit, V., & Morand, C. (2012). Evidence for a protective effect of polyphenols-containing foods on cardiovascular health: An update for clinicians. *Therapeutic Advances in Chronic Disease, 3*(2), 87-106. doi:10.1177/2040622311430006; 10.1177/2040622311430006

Hallan, S. I., Matsushita, K., Sang, Y., Mahmoodi, B. K., Black, C., & Ishani, A. (2012). Age and association of kidney measures with mortality and end-stage renal disease. *JAMA : The Journal of the American Medical Association, 308*(22), 2349-2360. doi:10.1001/jama.2012.16817

Heim, K. E., Tagliaferro, A. R., & Bobilya, D. J. (2002). Flavonoid antioxidants: Chemistry, metabolism and structure-activity relationships. *The Journal of Nutritional Biochemistry, 13*(10), 572-584.

Heiss, C., Kleinbongard, P., Dejam, A., Perre, S., Schroeter, H., Sies, H., & Kelm, M. (2005). Acute consumption of flavanol-rich cocoa and the reversal of endothelial dysfunction in smokers. *Journal of the American College of Cardiology, 46*(7), 1276-1283. doi:10.1016/j.jacc.2005.06.055

Heiss, C., Schroeter, H., Balzer, J., Kleinbongard, P., Matern, S., Sies, H., & Kelm, M. (2006). Endothelial function, nitric oxide, and cocoa flavanols. *Journal of Cardiovascular Pharmacology, 47 Suppl 2*, S128-35; discussion S172-6.

Higashi, Y., Noma, K., Yoshizumi, M., & Kihara, Y. (2009). Endothelial function and oxidative stress in cardiovascular diseases. *Circulation Journal: Official Journal of the Japanese Circulation Society, 73*(3), 411-418.

Himmelfarb, J. (2004). Linking oxidative stress and inflammation in kidney disease: which is the chicken and which is the egg? *Seminars in Dialysis, 17*(6), 449-454.

Hooper, L., Kroon, P. A., Rimm, E. B., Cohn, J. S., Harvey, I., Le Cornu, K. A., . . . Cassidy, A. (2008). Flavonoids, flavonoid-rich foods, and cardiovascular risk: A meta-analysis of randomized controlled trials. *The American Journal of Clinical Nutrition, 88*(1), 38-50.

Jin, L., Caldwell, R. B., Li-Masters, T., & Caldwell, R. W. (2007). Homocysteine induces endothelial dysfunction via inhibition of arginine transport. *Journal of Physiology and Pharmacology : An Official Journal of the Polish Physiological Society, 58*(2), 191-206.

Kaufman, J. M., Siegel, N. J., & Hayslett, J. P. (1975). Functional and hemodynamic adaptation to progressive renal ablation. *Circulation Research, 36*(2), 286-293.

Klabunde, R. E. (2005). *Cardiovascular physiology concepts*. Philadelphia, PA: Lippincott Williams & Wilkins.

Kuczmarski, J. M., Darocki, M. D., DuPont, J. J., Sikes, R. A., Cooper, C. R., Farquhar, W. B., et al. (2011). Effect of moderate-to-severe chronic kidney disease on flow-mediated dilation and progenitor cells. *Experimental Biology and Medicine, 236*, 1085-1092.

Lee, S. M., & An, W. S. (2013). Cardioprotective effects of omega -3 PUFAs in chronic kidney disease. *BioMed Research International, 2013*, 712949. doi:10.1155/2013/712949

Leeuwenburgh, C., Hardy, M. M., Hazen, S. L., Wagner, P., Oh-ishi, S., Steinbrecher, U. P., & Heinecke, J. W. (1997). Reactive nitrogen intermediates promote low density lipoprotein oxidation in human atherosclerotic intima. *The Journal of Biological Chemistry, 272*(3), 1433-1436.

Levey, A. S., Coresh, J., Balk, E., Kausz, A. T., Levin, A., Steffes, M. W., et al. (2003). National kidney foundation practice guidelines for chronic kidney disease: Evaluation, classification, and stratification. *Annals of Internal Medicine, 139*(2), 137-147.

Liabeuf, S., Drueke, T. B., & Massy, Z. A. (2011). Protein-bound uremic toxins: New insight from clinical studies. *Toxins, 3*(7), 911-919. doi:10.3390/toxins3070911

Lilamand, M., Kelaiditi, E., Guyonnet, S., Antonelli Incalzi, R., Raynaud-Simon, A., Vellas, B., & Cesari, M. (2014). Flavonoids and arterial stiffness: Promising perspectives. *Nutrition, Metabolism, and Cardiovascular Diseases : NMCD,* doi:S0939-4753(14)00046-5

Lilitkarntakul, P., Dhaun, N., Melville, V., Blackwell, S., Talwar, D. K., Liebman, B., et al. (2011). Blood pressure and not uraemia is the major determinant of arterial stiffness and endothelial dysfunction in patients with chronic kidney disease and minimal co-morbidity. *Atherosclerosis, 216*(1), 217-225. doi:10.1016/j.atherosclerosis.2011.01.045

Lilitkarntakul, P., Dhaun, N., Melville, V., Kerr, D., Webb, D. J., & Goddard, J. (2012). Risk factors for metabolic syndrome independently predict arterial stiffness and endothelial dysfunction in patients with chronic kidney disease and minimal comorbidity. *Diabetes Care, 35*(8), 1774-1780. doi:10.2337/dc11-2345

Liu, R. H. (2003). Health benefits of fruit and vegetables are from additive and synergistic combinations of phytochemicals. *The American Journal of Clinical Nutrition, 78*(3 Suppl), 517S-520S.

Martens, C. R., & Edwards, D. G. (2011). Peripheral vascular dysfunction in chronic kidney disease. *Cardiology Research and Practice, 2011*, 267257. doi:10.4061/2011/267257; 10.4061/2011/267257

Mink, P. J., Scrafford, C. G., Barraj, L. M., Harnack, L., Hong, C. P., Nettleton, J. A., & Jacobs, D. R.,Jr. (2007). Flavonoid intake and cardiovascular disease mortality: A prospective study in postmenopausal women. *The American Journal of Clinical Nutrition, 85*(3), 895-909.

Monahan, K. D., Feehan, R. P., Kunselman, A. R., Preston, A. G., Miller, D. L., & Lott, M. E. (2011). Dose-dependent increases in flow-mediated dilation following acute cocoa ingestion in healthy older adults. *Journal of Applied Physiology (Bethesda, Md.: 1985), 111*(6), 1568-1574. doi:10.1152/japplphysiol.00865.2011; 10.1152/japplphysiol.00865.2011

Monahan, K. D. (2012). Effect of cocoa/chocolate ingestion on brachial artery flow-mediated dilation and its relevance to cardiovascular health and disease in humans. *Archives of Biochemistry and Biophysics, 527*(2), 90-94. doi:10.1016/j.abb.2012.02.021; 10.1016/j.abb.2012.02.021

Moody, W. E., Edwards, N. C., Madhani, M., Chue, C. D., Steeds, R. P., Ferro, C. J., & Townend, J. N. (2012). Endothelial dysfunction and cardiovascular disease in early-stage chronic kidney disease: cause or association? *Atherosclerosis, 223*, 86-94.

National Kidney Foundation. (2013). Kidney Disease. Retrieved from: http://www.kidney.org/index.cfm

National Kidney Foundation. (2002). KDOQI Clinical Practice Guidelines for Chronic Kidney Disease: Evaluation, Classification and Stratification. *American Journal of Kidney Diseases, 39*, S1-S000,

Nelms, M., Sucher, K. P., Lacey, K., & Roth, S. L. (2010). *Nutrition Therapy and Pathophysiology* (2nd ed.). Belmont, CA: Wadsworth, Cengage Learning.

Nerbass, F. B., Draibe, S. A., Feiten, S. F., Chiarello, P. G., Vannucchi, H., & Cuppari, L. (2006). Homocysteine and its determinants in nondialyzed chronic kidney disease patients. *Journal of the American Dietetic Association, 106*(2), 267-270. doi:S0002-8223(05)01908-5

Nishida, C., Uauy, R., Kumanyika, S., & Shetty, P. (2004). The joint WHO/FAO expert consultation on diet, nutrition and the prevention of chronic diseases: Process, product and policy implications. *Public Health Nutrition, 7*(1A), 245-250.

Njike, V. Y., Faridi, Z., Shuval, K., Dutta, S., Kay, C. D., West, S. G., et al. (2011). Effects of sugar-sweetened and sugar-free cocoa on endothelial function in overweight adults. *International Journal of Cardiology, 149*(1), 83-88. doi:10.1016/j.ijcard.2009.12.010; 10.1016/j.ijcard.2009.12.010

Nogueira Lde, P., Knibel, M. P., Torres, M. R., Nogueira Neto, J. F., & Sanjuliani, A. F. (2012). Consumption of high-polyphenol dark chocolate improves endothelial function in individuals with stage 1 hypertension and excess body weight. *International Journal of Hypertension, 2012*, 147321. doi:10.1155/2012/147321; 10.1155/2012/147321

Oberg, B. P., McMenamin, E., Lucas, F. L., McMonagle, E., Morrow, J., Ikizler, T. A., & Himmelfarb, J. (2004). Increased prevalence of oxidant stress and inflammation in patients with moderate to severe chronic kidney disease. *Kidney International, 65*(3), 1009-1016. doi:10.1111/j.1523-1755.2004.00465.x

Panza, J. A., Quyyumi, A. A., Brush, J. E.,Jr, & Epstein, S. E. (1990). Abnormal endothelium-dependent vascular relaxation in patients with essential hypertension. *The New England Journal of Medicine, 323*(1), 22-27. doi:10.1056/NEJM199007053230105

Ramirez-Sanchez, I., Maya, L., Ceballos, G., & Villarreal, F. (2010). (-)-Epicatechin activation of endothelial cell endothelial nitric oxide synthase, nitric oxide, and related signaling pathways. *Hypertension, 55*(6), 1398-1405.

Recio-Mayoral, A., Banerjee, D., Streather, C., & Kaski, J. C. (2011). Endothelial dysfunction, inflammation and atherosclerosis in chronic kidney disease--a cross-sectional study of predialysis, dialysis and kidney-transplantation patients. *Atherosclerosis, 216*(2), 446-451. doi:10.1016/j.atherosclerosis.2011.02.017

Rozza, A. L., Hiruma-Lima, C. A., Tanimoto, A., & Pellizzon, C. H. (2012). Morphologic and pharmacological investigations in epicatechin gastroprotective effect. *Evidence Based Complementary and Alternative Medicine, 2012*, 708156. doi: 10.1155/2012/708156

Rudic, R. D., Shesely, E. G., Maeda, N., Smithies, O., Segal, S. S., & Sessa, W. C. (1998). Direct evidence for the importance of endothelium-derived nitric oxide in vascular remodeling. *Journal of Clinical Investigation*, *101*, 731-736.

Ruijters, E. J., Weseler, A. R., Kicken, C., Haenen, G. R., & Bast, A. (2013). The flavanol (-)-epicatechin and its metabolites protect against oxidative stress in primary endothelial cells via a direct antioxidant effect. *European Journal of Pharmacology, 715*(1-3), 147-153. doi:10.1016/j.ejphar.2013.05.029

Saldanha, J. F., Leal Vde, O., Stenvinkel, P., Carraro-Eduardo, J. C., & Mafra, D. (2013). Resveratrol: Why is it a promising therapy for chronic kidney disease patients? *Oxidative Medicine and Cellular Longevity, 2013*, 963217. doi:10.1155/2013/963217

Sarnak, M. J., Levey, A. S., Schoolwerth, A. C., Coresh, J., Culleton, B., Hamm, L. L., et al. (2003). Kidney disease as a risk factor for development of cardiovascular disease: A statement from the american heart association councils on kidney in cardiovascular disease, high blood pressure research, clinical cardiology, and epidemiology and prevention. *Circulation, 108*(17), 2154-2169. doi:10.1161/01.CIR.0000095676.90936.80

Scalbert, A., Johnson, I. T., & Saltmarsh, M. (2005). Polyphenols: Antioxidants and beyond. *The American Journal of Clinical Nutrition, 81*(1 Suppl), 215S-217S.

Schiffrin, E. L., Lipman, M. L., & Mann, J. F. (2007). Chronic kidney disease: Effects on the cardiovascular system. *Circulation, 116*(1), 85-97. doi:10.1161/CIRCULATIONAHA.106.678342

Schnorr, O., Brossette, T., Momma, T. Y., Kleinbongard, P., Keen, C. L., Schroeter, H., & Sies, H. (2008). Cocoa flavanols lower vascular arginase activity in human endothelial cells in vitro and in erythrocytes in vivo. *Archives of Biochemistry and Biophysics, 476*(2), 211-215. doi:10.1016/j.abb.2008.02.040; 10.1016/j.abb.2008.02.040

Schroeter, H., Heiss, C., Balzer, J., Kleinbongard, P., Keen, C. L., Hollenberg, N. K., et al. (2006). (-)-Epicatechin mediates beneficial effects of flavanol-rich cocoa on vascular function in humans. *Proceedings of the National Academy of Sciences of the United States of America, 103*(4), 1024-1029. doi:10.1073/pnas.0510168103

Segal, M. S., Baylis, C., Johnson, R. J. (2006). Endothelial health and diversity in the kidney. *Journal of the American Society of Nephrology, 17*, 323-324.

Shimokawa, H. (1999). Primary endothelial dysfunction: Atherosclerosis. *Journal of Molecular and Cellular Cardiology, 31*(1), 23-37. doi:10.1006/jmcc.1998.0841

Steffen, Y., Schewe, T., & Sies, H. (2007). (-)-Epicatechin elevates nitric oxide in endothelial cells via inhibition of NADPH oxidase. *Biochemical and Biophysical Research Communications, 359*(3), 828-833. doi:10.1016/j.bbrc.2007.05.200

Stevens, L. A., Coresh, J., Greene, T., & Levey, A. S. (2006). Assessing kidney function — measured and estimated glomerular filtration rate. *N Engl J Med, 354*(23), 2473-2483. doi:10.1056/NEJMra054415

Stoclet, J. C., Chataigneau, T., Ndiaye, M., Oak, M. H., El Bedoui, J., Chataigneau, M., & Schini-Kerth, V. B. (2004). Vascular protection by dietary polyphenols. *European Journal of Pharmacology, 500*(1-3), 299-313. doi:10.1016/j.ejphar.2004.07.034

Taal, M. W., & Brenner, B. M. (2006). Predicting initiation and progression of chronic kidney disease: Developing renal risk scores. *Kidney International, 70*(10), 1694-1705. doi:10.1038/sj.ki.5001794

Taubert, D., Roesen, R., Lehmann, C., Jung, N., & Schomig, E. (2007). Effects of low habitual cocoa intake on blood pressure and bioactive nitric oxide: A randomized controlled trial. *JAMA : The Journal of the American Medical Association, 298*(1), 49-60. doi:10.1001/jama.298.1.49

Thambyrajah, J., Landray, M. J., McGlynn, F. J., Jones, H. J., Wheeler, D. C., & Townend, J. N. (2000). Abnormalities of endothelial function in patients with predialysis renal failure. *Heart (British Cardiac Society), 83*(2), 205-209.

Thoonen, R., Sips, P. Y., Bloch, K. D., & Buys, E. S. (2013). Pathophysiology of hypertension in the absence of nitric oxide/cyclic GMP signaling. *Current Hypertension Reports, 15*(1), 47-58. doi:10.1007/s11906-012-0320-5; 10.1007/s11906-012-0320-5

Tiefenbacher, C. P. (2001). Tetrahydrobiopterin: A critical cofactor for eNOS and a strategy in the treatment of endothelial dysfunction? *American Journal of Physiology.Heart and Circulatory Physiology*, 280(6), H2484-8.

Tizianello, A., De Ferrari, G., Garibotto, G., Gurreri, G., & Robaudo, C. (1980). Renal metabolism of amino acids and ammonia in subjects with normal renal function and in patients with chronic renal insufficiency. *The Journal of Clinical Investigation, 65*(5), 1162-1173. doi:10.1172/JCI109771

U S Renal Data System, USRDS 2012 Annual Data Report: Atlas of Chronic Kidney Disease and End-Stage Renal Disease in the United States, National Institutes of Health, National Institute of Diabetes and Digestive and Kidney Diseases, Bethesda, MD, 2012.

Vallance, P., Leone, A., Calver, A., Collier, J., & Moncada, S. (1992). Endogenous dimethylarginine as an inhibitor of nitric oxide synthesis. *Journal of Cardiovascular Pharmacology, 20*(Suppl. 12). S60-S62.

Verma, S., & Anderson, T. J. (2002). Fundamentals of endothelial function for the clinical cardiologist. *Circulation*, *105*(5), 546-549.

Vita, J. A., & Keaney, J. F.,Jr. (2002). Endothelial function: A barometer for cardiovascular risk? *Circulation, 106*(6), 640-642.

Vlachopoulos, C., Aznaouridis, K., Alexopoulos, N., Economou, E., Andreadou, I., & Stefanadis, C. (2005). Effect of dark chocolate on arterial function in healthy individuals. *American Journal of Hypertension*, *18*(6), 785-791. doi:10.1016/j.amjhyper.2004.12.008

Wagner, L., Klein, J. D., Sands, J. M., & Baylis, C. (2002). Urea transporters are distributed in endothelial cells and mediate inhibition of L-arginine transport. *American Journal of Physiology.Renal Physiology, 283*(3), F578-82. doi:10.1152/ajprenal.00355.2001

Wu, G., & Morris, S. M.,Jr. (1998). Arginine metabolism: Nitric oxide and beyond. *The Biochemical Journal, 336*, 1-17.

Yang, X. P., Liu, Y. H., Shesely, E. G., Bulagannawar, M., Liu, F., & Carretero, O. A. (1999). Endothelial nitric oxide gene knockout mice: Cardiac phenotypes and the effect of angiotensin-converting enzyme inhibitor on myocardial ischemia/reperfusion injury. *Hypertension*, *34*(1), 24-30.

Yilmaz, M. I., Saglam, M., Caglar, K., Cakir, E., Sonmez, A., Ozgurtas, T., et al. (2006). The determinants of endothelial dysfunction in CKD: Oxidative stress and asymmetric dimethylarginine. *American Journal of Kidney Diseases : The Official Journal of the National Kidney Foundation, 47*(1), 42-50. doi:S0272-6386(05)01512-X

Yilmaz, M. I., Stenvinkel, P., Sonmez, A., Saglam, M., Yaman, H., Kilic, S., et al (2011). Vascular health, systemic inflammation and progressive reduction in kidney function; clinical determinants and impact on cardiovascular outcomes. *Nephrology, Dialysis, Transplantation : Official Publication of the European Dialysis and Transplant Association - European Renal Association, 26*(11), 3537-3543. doi:10.1093/ndt/gfr081

Zyga, S., & Kolovos, P. (2013). Cardiovascular disease and chronic inflammation in end stage kidney disease. *International Journal of Caring Sciences, 6*(1), 29-36.

Appendix A

APPROVAL LETTER

RESEARCH OFFICE

210 Hullihen Hall
University of Delaware
Newark, Delaware 19716-1551
Ph: 302/831-2136
Fax: 302/831-2828

DATE: July 8, 2013

TO: Shannon Lennon-Edwards, PhD
FROM: University of Delaware IRB

STUDY TITLE: [463317-1] Effect of Cocoa on Vascular Function in CKD Patients

SUBMISSION TYPE: New Project

ACTION: APPROVED
APPROVAL DATE: July 8, 2013
EXPIRATION DATE: June 18, 2014
REVIEW TYPE: Full Committee Review

Thank you for your submission of New Project materials for this research study. The University of Delaware IRB has APPROVED your submission. This approval is based on an appropriate risk/benefit ratio and a study design wherein the risks have been minimized. All research must be conducted in accordance with this approved submission.

This submission has received Full Committee Review based on the applicable federal regulation.

Please remember that informed consent is a process beginning with a description of the study and insurance of participant understanding followed by a signed consent form. Informed consent must continue throughout the study via a dialogue between the researcher and research participant. Federal regulations require each participant receive a copy of the signed consent document.

Please note that any revision to previously approved materials must be approved by this office prior to initiation. Please use the appropriate revision forms for this procedure.

All SERIOUS and UNEXPECTED adverse events must be reported to this office. Please use the appropriate adverse event forms for this procedure. All sponsor reporting requirements should also be followed.

Please report all NON-COMPLIANCE issues or COMPLAINTS regarding this study to this office.

Please note that all research records must be retained for a minimum of three years.

Based on the risks, this project requires Continuing Review by this office on an annual basis. Please use the appropriate renewal forms for this procedure.

If you have any questions, please contact Jody-Lynn Berg at (302) 831-1119 or jlberg@udel.edu. Please include your study title and reference number in all correspondence with this office.