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Cocoa, Postprandial Lipoproteins, And Inflammation in Type 2 Diabetes: A Secondary Data Analysis

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COCOA, POSTPRANDIAL LIPOPROTEINS, AND INFLAMMATION IN TYPE 2
DIABETES: A SECONDARY DATA ANALYSIS

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Abstract

Background: Type 2 Diabetes (T2D) is associated with dyslipidemia and chronic low-grade inflammation, which greatly increases the risk of cardiovascular disease (CVD) in this population. Hyperlipidemia and inflammation in the postprandial state substantially contribute to atherosclerosis development and CVD risk, and have shown to be exacerbated by high-fat meals. Dietary cocoa is a rich source of flavanols that is associated with favorable effects on cardiovascular health, such as improvements in the lipid profile and chronic inflammation, in non-diabetic adults.

Objective: To conduct a secondary data analysis using unpublished data from a previously conducted clinical study to determine whether acute cocoa supplementation reduces postprandial metabolic stress, through improvements in serum markers of lipid metabolism and inflammation, in obese T2D adults after a high-fat fast-food-style meal.

Methods: Adults with T2D [n = 18; age (mean ± SE): 56±3y; BMI (kg/m²): 35.3±2.0; 14 women; 4 men] were randomly assigned to receive cocoa beverage (960 mg total polyphenols; 480 mg flavanols) or flavanol-free placebo with a high-fat fast-food-style breakfast [766kcal, 50g fat (59% energy)] in a crossover trial. After an overnight fast, participants consumed the breakfast with cocoa or placebo, and blood sample collection [serum apoB, apoA1, NEFA, IL-1β, IL-6, and IL-18] was conducted at fasting, 1, 2, 4, and 6 h postprandial time points.

Results: Cocoa significantly decreased serum IL-18 levels at the 1, 4, and 6-h postprandial time points compared to placebo (p < 0.001), but had no effects on serum apoB, apoA1, NEFA, IL-1β, or IL-6 levels.

Conclusion: Cocoa may play a protective role against the development and progression of CVD through selective anti-inflammatory effects on postprandial IL-18.

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Chapter 1: Introduction

More than 30 million Americans currently suffer from Type 2 Diabetes (T2D), with greater than 1.5 million new cases arising each year (American Diabetes Association [ADA], 2017). Diabetes greatly increases an individual's risk of developing cardiovascular disease (CVD), the leading cause of death in the United States and worldwide (ADA, 2017). Increasing evidence suggests that hyperlipidemia and inflammation during the postprandial (post-feeding) state influences the development and progression of atherosclerosis, adversely affecting CVD risk. Serum lipids and inflammatory biomarkers considered to be predictive of CVD risk are often elevated in T2D, and may be exacerbated in the postprandial state following the ingestion of a high-fat meal (Lopez-Miranda, Williams, & Lairon, 2007; Perez-Martinez et al., 2016; Tomkin & Owens, 2017). Diets with high polyphenol content have shown to have favorable effects on human health and may limit the incidence of disease associated with inflammation and metabolic stress, such as T2D and CVD (Aprotosoaie et al., 2016). Cocoa is an especially rich dietary source of flavanols, a subclass of polyphenols, and several previous studies suggest cocoa flavanols have anti-diabetic and anti-atherogenic effects (Ramos, Martin, & Goya, 2017). However, randomized controlled trials observing the effects of cocoa flavanols on postprandial metabolism are extremely limited. Therefore, the purpose of this study is to examine the effects of cocoa powder consumption on selected markers of postprandial lipid metabolism and inflammation following a high-fat fast-food-style meal in T2D participants.

Alternate Hypotheses:

1. Acute cocoa beverage consumption will significantly decrease postprandial apoB and NEFA, and increase postprandial apoA1 serum concentrations following a high-fat fast-food-style meal compared to placebo.
2. Acute cocoa beverage consumption will significantly decrease postprandial serum levels of IL-1 β , IL-6, and IL-18 following a high-fat fast-food-style meal compared to placebo.

Chapter 2: Review of the Literature

Prevalence of Diabetes and Cardiovascular Disease

Type 2 Diabetes (T2D) is a metabolic disease characterized by chronic hyperglycemia due to both insulin resistance (IR) and insulin insufficiency. The pathogenesis of T2D is believed to be multifactorial, with poor nutrition, physical inactivity, and obesity (especially in the intra-abdominal region) being the most strongly associated risk factors. T2D accounts for 90-95% of all adult diabetes diagnoses and is currently the 7th leading cause of death in the United States, killing approximately 1.5 million people per year. The number of mortalities stemming from T2D is expected to double by year 2030 (ADA, 2017).

Diabetes greatly increases an individual's risk of developing CVD, the leading cause of death in the United States and worldwide. Diabetic adults are 2-4 times more likely to develop and die prematurely from CVD compared to individuals without diabetes (Khavandi et al., 2017; Wu & Parhofer, 2014). CVD currently accounts for approximately 1/3 of all US deaths, with coronary artery disease and stroke being responsible for more than 60% of these mortalities (American Heart Association [AHA], 2017). It is predicted that by year 2030, greater than 23.6 million people worldwide will die from CVD each year (World Health Organization [WHO], 2017).

Risk factors strongly associated with atherosclerotic cardiovascular disease (ASCVD) include obesity, excess central adiposity, poor dietary intakes, physical inactivity, chronic hyperglycemia, hypertension and dyslipidemia, all of which are often observed in individuals with T2D (AHA, 2017; WHO 2017).

Pathophysiology of Type 2 Diabetes

Glucose homeostasis is maintained and regulated through a negative feedback loop, primarily involving the hormones insulin and glucagon. When blood glucose levels are elevated, insulin is

secreted by the beta cells of the pancreas to stimulate the uptake of glucose by the insulin-sensitive tissues, such as the hepatic, skeletal muscle, and adipose tissues. Glucose uptake occurs due to insulin's effect on the intracellular insulin-dependent GLUT4 glucose transporters. Insulin signals the translocation of the GLUT4 transporters from the interior of the cell to the cellular membrane in order to transport glucose from the blood into the cell. Once cellular glucose uptake has occurred, the glucose can then be oxidized and used for energy, converted to and stored as glycogen in either the skeletal muscle or liver (glycogenesis), or converted into triglyceride and stored in the adipose tissue (lipogenesis). Blood glucose levels then return to normal and insulin levels are suppressed. Glucagon has the opposing effect of insulin and acts to increase blood glucose levels when they have declined, as they do in times of fasting. When blood glucose levels are low, glucagon is secreted by the alpha cells of the pancreas, stimulating the endogenous production of glucose (gluconeogenesis) through the breakdown of glycogen (glycogenolysis) and/or adipose tissue (lipolysis). If necessary, gluconeogenesis can also occur through the breakdown of body proteins into amino acids that can be converted to glucose. The process of gluconeogenesis restores blood glucose levels and glucagon is suppressed.

Insulin Resistance and Insulin Insufficiency

All forms of diabetes share the common characteristic of chronic hyperglycemia stemming from defects in insulin action, insulin production, or both (Nelms, 2016). The manifestation of T2D requires the presence of both insulin resistance—the hypo-responsiveness of the insulin-sensitive tissues to insulin, and insulin insufficiency—the progressively deficient production of insulin (Kahn, Cooper, & Del Prato, 2014). The development and clinical presentation of T2D is generally gradual and insidious, with insulin resistance often occurring for several years prior to

the clinical onset of the disease. Clinical presentation of T2D is largely associated with a decline in insulin production (Nelms, 2016).

When alterations in glucose metabolism initially arise, the beta cells are often functioning properly but the insulin-sensitive tissues have started to become insensitive to the insulin being secreted. It is thought that this desensitization to insulin is, in part, caused by insufficient insulin receptors on the normally insulin-sensitive target cells, preventing or decreasing the intracellular signaling and translocation of the GLUT4 receptors to the cell membrane (Nelms, 2016). This results in decreased glucose uptake by the insulin-sensitive tissues and leads to an increase in insulin output by the beta cells to try to compensate for the rising blood glucose levels. Over time, the beta cells become incapable of keeping up with the chronically elevated blood glucose levels and no longer produce enough insulin to maintain normal blood glucose concentrations, resulting in impaired glucose tolerance. The progressive deterioration in beta cell function, and reduction in the number of β -cells, further elevates plasma glucose levels and contributes to the evolution from impaired glucose tolerance to T2D (Kahn, 2014).

Obesity, especially intra-abdominal adiposity, is strongly associated with the development of T2D (Kahn, 2014). As of year 2014, greater than 90% of those with T2D were overweight or obese and greater than 60% were obese (ADA, 2017). Environmental factors shown to heavily contribute to the development of obesity and central adiposity include physical inactivity, excessive energy intake, and nutrient composition of the diet—specifically, diets high in saturated fats and fructose, and low in fiber and omega 3 polyunsaturated fatty acids (Kahn, 2014).

Diabetic Dyslipidemia

Dyslipidemia, characterized by elevated fasting and postprandial plasma concentrations of triglyceride (TG) and small, dense low-density lipoprotein (sdLDL) particles, along with low plasma levels of high-density lipoprotein cholesterol (HDL-C), is commonly observed in individuals with T2D (Khavandi et al., 2017). These lipid abnormalities are believed to largely contribute to the markedly increased risk of ASCVD exerted on those with T2D (Wu & Parhofer, 2014). Lipoproteins transport lipids throughout the body for uptake and use by the cells of the periphery, largely the muscle and adipose tissues. Chylomicrons (CM) and very low-density lipoproteins (VLDL) are both rich in TG, with the main differences between them being that CM transport exogenous sources of lipid and VLDL transport endogenous sources of lipid. Lipolysis of both CM- and VLDL-TG is dependent on the action of lipoprotein lipase (LPL). Once LPL has hydrolyzed the majority of the CM-TG, the remaining remnant particles are cleared from circulation by the liver. The lipolysis of VLDL-TG results in smaller, denser IDL particles, which are then further hydrolyzed and converted into cholesterol-rich LDL particles. The main function of LDL is to deliver cholesterol to the peripheral cells. In contrast, HDL are involved in reverse cholesterol transport, which serves to transport excess cholesterol from the cells back to the liver to be recycled or excreted (Feng et al., 2017; Khavandi et al., 2017).

Apolipoproteins are proteins found on the surface of all lipoproteins and are both structural and functional components of the lipoprotein. Two important functional roles of apolipoproteins involve the activation of enzymatic reactions and allowing for the binding of the lipoprotein to its designated receptor on the surface of the peripheral cells (Khavandi et al., 2017).

Apolipoprotein-B (apoB) is the main protein component of CM, VLDL, IDL, and LDL particles and promotes the uptake of TG and cholesterol into the peripheral tissues and liver (Hem, Kumar & Tamang, 2014). Apolipoprotein A-1 (apoA1) is the main protein component of HDL particles

and facilitates the transfer of cholesterol from the tissues into HDL particles. Plasma apoB levels are positively correlated and plasma apoA1 are inversely correlated with IR and T2D. Since all apoB-lipoproteins are considered atherogenic and apoA1-containing HDL particles are considered anti-atherogenic, elevated serum levels of apoB and low serum levels of apoA1 are associated with an increased risk of ASCVD (Hem, Kumar & Tamang, 2014).

ApoB

High serum LDL-C concentration is considered an independent risk factor of ASCVD and the lowering of LDL-C levels is a primary goal of medical treatment to prevent ASCVD and subsequent major cardiac events. However, focusing exclusively on LDL-C levels has shown to be less than optimal at reducing the risk of ASCVD and major cardiovascular events. It is estimated that 50% of all patients with ASCVD have LDL-C levels within or even below the recommended range of 70-100 mg/dL, yet still experience a major CV event (Ramjee, Sperling, & Jacobson, 2011; Varvel et al., 2015). This residual risk is due, at least in part, to the discordance between serum concentrations of LDL-C and atherogenic apoB-containing lipoproteins. This discordance is related to the considerable variability of cholesterol levels that can exist within the LDL particle (LDL-P). Having a predominant amount of cholesterol-depleted, sdLDL particles results in a lower concentration of LDL-C but a greater concentration of apoB particles in the blood. Since apoB is a constituent of all atherogenic lipoproteins, the total serum apoB concentration represents the total number of atherogenic particles in the blood. It is estimated that 34% of the population should be considered high-risk for ASCVD due to IR and elevated serum apoB concentrations, yet are overlooked as a result of having optimal LDL-C levels (Varvel et al., 2015). Therefore, the assessment of serum apoB levels may be more

effective in identifying ASCVD risk and preventing CV events in those with T2D (Harper & Jacobson, 2010; Varvel et al., 2015; Wilkins et al., 2016).

ApoA1

A low level of serum HDL-C (<40mg/dL for men and <50mg/dL for women) is considered an independent risk factor for ASCVD, whereas a high level of serum HDL-C (>60mg/dL) is considered to be cardio-protective. Reverse cholesterol transport (RCT), along with the anti-inflammatory, anti-oxidant, and anti-thrombotic properties of HDL, constitute its anti-atherogenic effects. However, the measurement of HDL-C quantity is not always indicative of HDL-P functionality (Eren, Yilmaz, & Aydin, 2012; Florvall, Basu, & Larsson, 2006; Sharif et al., 2016). Despite having optimal HDL-C levels, some individuals still develop ASCVD and experience a major CV event. It is estimated that almost 50% of those who experience a major CV event have both LDL-C and HDL-C levels within an optimal range. This supports the notion that not all HDL protects against the development of ASCVD and its related morbidity and mortality (Eren et al., 2012). In fact, Sharif et al (2016) found that in patients with T2D, elevated levels of HDL-C were associated with an increased risk of CV events and mortality when LDL-C levels were less than 80 mg/dL, indicating that higher levels of HDL-C are not always advantageous (Sharif et al, 2016). Also, a study in mice revealed that dysfunctional hepatic HDL-P receptors lead to high levels of serum HDL-C but also to the rapid development of extreme atherosclerosis (Eren et al, 2012). Results from these and other studies suggest that HDL-P functionality may be more indicative of ASCVD risk than HDL-C quantity (Eren et al., 2012; Florvall et al., 2006; Sharif et al., 2016). ApoA1 largely influences HDL-P functionality and recent evidence implies that apoA1 may be a stronger biomarker than HDL-C in predicting CV-related morbidity and mortality (Eren et al., 2012; Florvall et al., 2006; McQueen et al.,

2008; Sharif et al., 2016). ApoA1 normally accounts for approximately 70% of all HDL-P proteins and is thought to be widely responsible for the anti-atherogenic properties of HDL (Eren et al., 2012). Modification of the apoA1 composition of the HDL-P leads to a dysfunctional HDL-P with a decreased ability to remove excess cholesterol from both the peripheral cells and the cholesterol-rich macrophages within the arterial wall. Modified and dysfunctional HDL-P transforms from anti-inflammatory, cardio-protective to pro-inflammatory, atherogenic molecules. Chronic systemic inflammation and oxidative stress, dyslipidemia and glycation of apoA1, all often seen in T2D, are positively associated with the modification of apoA1 and the resulting dysfunction of HDL-P.

ApoB/ApoA1 Ratio

The ratio of apoB to apoA1 (apoB/apoA1) represents the balance between total atherogenic apoB-containing lipoprotein particles and anti-atherogenic HDL-P in the blood. Many studies have shown the apoB/apoA1 ratio to be a stronger predictor of ASCVD compared to traditional lipid markers or its individual constituent apolipoprotein values (Kaneva et al., 2015; Krintus et al., n.d.; Lu et al., 2011; Tamang et al., 2014; Upadhyay, 2015). In fact, some studies have shown the apoB/apoA1 ratio to be the only serum lipid value with the ability to differentiate between patients with and without coronary artery disease (CAD), even when these patients present with normal serum lipid concentrations (Kaneva et al., 2015). A study by Tamang et al (2014) evaluated the predictive power of the apoB/apoA1 ratio in determining CVD risk by comparing several different lipid parameters between adults with and without CVD (Tamang et al., 2014). Significant results were observed between the CVD and non-CVD groups for apoB (107 vs. 91 mg/dL) and apoB/apoA1 ratio (1.00 vs. 0.84), but not for apoA1 or LDL-C. (Tamang et al, 2014). Krintus et al (n.d.) compared apolipoprotein concentrations and the apoB/apoA1 ratio to

conventional lipid markers in adult women with and without acute coronary syndromes (ACS). ApoB/apoA1 ratio values were significantly higher (0.62 vs. 0.5) and apoA1 values were significantly lower (132 vs. 155 mg/dL) in ACS patients compared to controls, though there were no significant differences between LDL-C or apoB concentrations. Additionally, 83% of the healthy control subjects in this study had an apoB/apoA1 ratio value indicative of low-risk for CVD, whereas 55% of the ACS patients had an apoB/apoA1 ratio value that indicated moderate to high risk of CVD (Krintas et al., n.d.).

Type 2 Diabetes, Alterations in Postprandial Lipid Metabolism, and NEFA

There has been a large amount of research conducted examining the relationship between lipid metabolism and the development of ASCVD. Serum lipid measurements are often performed in the fasting state, which mostly reflects endogenous lipid and lipoprotein metabolism. However, many with obesity and T2D spend a substantial amount of time in the fed state due to the daily consumption of three or more meals, sometimes along with snacks. Abnormalities in postprandial lipid metabolism are commonly seen in individuals with T2D, even when fasting lipid levels are within normal range. Given that most with T2D are in the postprandial state for a large portion of their day and that postprandial dyslipidemia is commonly observed, the assessment of postprandial lipid metabolism in this population is highly relevant to predicting ASCVD risk (Annuzi et al., 2008; Khavandi, 2017; Lopez-Miranda et al., 2007; Perez-Martinez et al., 2016). Several studies have shown that chronic over-nutrition, diets high in saturated fat and/or sucrose, and diets low in fiber, lead to an increase in the postprandial production and decreased circulatory clearance of apoB-particles in T2D (Annuzi 2008; Boren & Williams, 2016; Khavandi, 2017; Lopez-Miranda et al., 2007; Perez-Martinez et al, 2016). Elevated postprandial apoB-particle production and secretion is positively associated with IR, and is often

seen in obese, insulin-resistant individuals with and without T2D (Annuzzi, 2008; Khavandi, 2017). One possible mechanism to explain this response in those with IR is the suppression of insulin's anti-lipolytic effect in the adipose tissue. Insulin levels are typically elevated in the early postprandial state, suppressing both adipose tissue lipolysis and hepatic apoB-particle production. In IR and T2D this action of insulin is altered, resulting in heightened concentrations of non-esterified fatty acids (NEFA) from adipose tissue and hepatic-derived apoB-particles (Lopez-Miranda et al., 2007; Khavandi, 2017). Insulin resistance also inhibits apoB-particle lipolysis by lipoprotein lipase (LPL), prolonging the circulatory residence time of these particles. The prolonged circulatory time and delayed clearance of these particles from circulation results in the formation of both small, dense LDL particles and TG-rich HDL particles. TG-rich HDL particles are smaller than cholesterol-rich HDL particles and are more readily catabolized, resulting in the low plasma HDL-C concentrations commonly observed in T2D. Meals containing between 30-50 grams of fat are usually required to witness exaggerated elevations in postprandial TG-rich apoB-particle production, and it is not uncommon for meals in westernized societies to contain, on average, 20-70 grams of fat per meal. Consumption of consecutive high-fat meals further contributes to postprandial dyslipidemia, resulting in TG-rich apoB-particles levels remaining well above baseline for up to 8 hours following each meal (Lopez-Miranda et al., 2007).

Type 2 Diabetes is also associated with elevated fasting and postprandial NEFA levels. NEFA are derived from the lipolysis of both adipocyte and apoB-lipoprotein TG, and serum levels are typically elevated in the fasted state and suppressed in the postprandial state due to the anti-lipolytic effect of insulin. However, NEFA levels are often elevated in the fed state following a high-fat meal (Frayn, 2005; Karpe, Dickmann & Frayn, 2011). In the fasted state, adipose tissue

lipolysis is almost entirely responsible for serum NEFA concentrations but, after a high-fat meal, spillover fatty acids from the hydrolysis of CM-TG can account for up to 40-50% of postprandial serum NEFA concentrations (Karpe et al., 2011). NEFA that are not taken up by the tissues “spillover” into the blood and are shunted to the liver. Elevated levels of plasma NEFA and the subsequent increase in hepatic TG derived from NEFA heighten hepatic apoB-lipoprotein production and secretion, further exacerbating the postprandial dyslipidemia occurring in T2D (Frayn, 1998; Khavandi et al., 2017; Lambert & Parks, 2012). Elevated NEFA levels are also frequently observed non-diabetic, IR states, likely due to the reduced sensitivity of the adipose tissue to insulin and a failure of insulin to suppress lipolysis in the postprandial period (Karpe et al., 2011). While it is not yet clear whether elevated NEFA concentrations are independent risk factors for ASCVD, they have shown to be positively associated with many established risk factors, such as IR, VLDL and TG levels, blood pressure, and endothelial dysfunction (Frayn, 2005; Karpe et al., 2011; Xiong, Xu & Huang, 2015). Xiong et al (2015) examined the relationship between serum NEFA and CVD mortality in elderly men with chronic kidney disease (CKD). Though they did not find a relationship between serum NEFA and renal function, they did reveal that serum NEFA were independent predictors of CVD-related mortality in elderly men with CKD (Xiong et al., 2015).

Clinical Importance of Serum Lipids and Apolipoproteins

ApoB

The American Diabetes Association/American College of Cardiology (ADA/ACC) position statement suggests an apoB goal of <80 mg/dL for those considered highest-risk and <90 mg/dL for those considered high-risk for ASCVD (Harper & Jacobson, 2010). Slightly different recommendations exist, with The American Association of Clinical Chemistry (AACC)

recommending an apoB goal of <80 mg/dL for high-risk patients and the Canadian Cardiovascular Society suggesting an apoB goal of <80 mg/dL for high- and moderate-risk patients (Ramjee et al., 2011). Mayo Clinic Medical Laboratories considers plasma apoB levels less than 90mg/dL desirable, 110-119 mg/dL borderline high, 120-139mg/dL high, and ≥ 140 mg/dL very high risk for CAD. They also suggest a therapeutic goal of <90 mg/dL for individuals with moderate to high risk of developing CAD and <80 mg/dL for those at very high risk of CAD (Mayo Clinic Medical Laboratories, n.d.). Results from a study performed by Adeli et al (2015), which sought to create comprehensive reference values representative of healthy Canadians aged 3-79 years, found median serum concentrations of apoB to be 90 mg/dL (Adeli et al., 2015). Results from other recent clinical trials support the use of an apoB goal of <80 mg/dL in high-risk patients for reducing the risk of major CV events and ASCVD-related mortality (Harper & Jacobson, 2010; Ramjee et al., 2011).

ApoA1

According to Mayo Clinic – Mayo Medical Laboratories, optimal serum concentrations of apoA1 are those greater than 120mg/dL for males and 140mg/dL for females (Mayo Clinic Medical Laboratories, n.d.). Serum concentrations falling below these values are considered high-risk for CVD development. Results from a study performed by Adeli et al (2015), which sought to create comprehensive reference values representative of healthy Canadians aged 3-79 years, found median serum concentrations of apoA1 in both males and females aged 40-79 years to be above the previously mentioned reference values stated from Mayo Clinic Medical Laboratories (Adeli et al., 2015). 210 samples of serum apoA1 were collected for both males and females aged 40-79 years. Serum concentrations of apoA1 among this population ranged from

120-200mg/dL in males, with the median serum concentration being 140 mg/dL, and 110-230 mg/dL in females, with the median serum concentration being 160 mg/dL (Adeli et al., 2015).

ApoB/ApoA1 Ratio

An apoB/apoA1 ratio value of greater than 0.9 for males and 0.8 for females is widely accepted to be an indication of high-risk for ASCVD development (Kaneva et al., 2015; Krintus et al., n.d.; Mayo Clinic Medical Laboratories, n.d.; Tamang et al., 2014). An apoB/apoA1 ratio value of 0.6-0.8 indicates a moderate risk and a value of less than 0.6 represents a low risk of CAD development and related CV events in women (Mayo Clinic Medical Laboratories, n.d.; Krintus et al., n.d.). In men, apoB/apoA1 values between 0.7-0.9 represent a moderate risk and a value below 0.7 represents a low risk of CAD and related CV events (Mayo Clinic Medical Laboratories, n.d.; Krintus et al., n.d.). A study by Kaneva et al (2015) examining the relationship of the apoB/apoA1 ratio with other lipid parameters in seemingly healthy, middle-aged men with normolipidemia found that 81% of the study population had an apoB/apoA1 ratio value of less than 0.9 (median value of 0.48), supporting the apoB/apoA1 reference values previously mentioned (Kaneva et al., 2015).

NEFA

While it is generally agreed upon that increased NEFA levels, especially in the postprandial period, are positively associated with IR, obesity, and CVD risk, suggested reference values vary within the literature and between laboratories. Frayn (2005) and Karpe et al (2015) propose NEFA concentrations in the range of 0.1-1.0 mmol/L and 0.3-0.6 mmol/L, respectively, to be normal and of low-risk in disease development (Frayn, 2005; Karpe et al., 2015). Mayo Clinic Medical Laboratories suggests a reference range of 0.0-0.72 mmol/L (Mayo Clinic Medical Laboratories, n.d.).

Type 2 diabetes, Inflammation, and Relation to ASCVD

Type 2 Diabetes is considered a chronic, low-grade inflammatory state, evidenced by the overproduction of several different pro-inflammatory cytokines, such as interleukin 1 beta (IL-1 β), interleukin 6 (IL-6), and interleukin 18 (IL-18). Chronically heightened production of pro-inflammatory cytokines brings about an inflammatory environment within the vasculature and is considered to be the foundation of atherosclerosis development and progression (Peiro et al., 2017). Meals high in saturated fat, sucrose, and/or total energy content appear to be the main triggers of the postprandial inflammatory response, with an exaggerated response seen in obese and T2D individuals (Telle-Hansen et al., 2017). Since these pro-inflammatory molecules are thought to directly contribute to the development of CVD, they present as useful biomarkers to help determine CVD risk. Elevated levels of circulating pro-inflammatory cytokines, such as IL-1 β , IL-6, and IL-18 are positively associated with T2D, obesity, and ASCVD, and their concentrations may be exacerbated by the ingestion of high-fat meals (Esposito et al., 2003; Fogarty et al., 2014).

Serum Inflammatory Markers of Interest

IL-6

IL-6 is a pro-inflammatory cytokine largely produced and secreted by the adipose tissue, endothelial cells, and activated leukocytes. IL-6 plays a critical role in the acute-phase inflammatory response and is believed to directly contribute to atherosclerosis development and progression (Ridker, 2016). Elevated levels of IL-6 are commonly observed in obesity, IR and T2D, and are associated with a greater risk of both ASCVD and all-cause mortality (Derosa & Maffioli, 2016). It has been shown that each SD increase in log IL-6 results in a 25% greater risk of a major adverse cardiac event (Ridker, 2016). Fernandez-Real et al (2001) examined the

association between serum IL-6 concentrations, IR, and blood pressure in 228 healthy, non-medicated obese subjects and found IL-6 levels to be positively associated with both fat mass and IR in non-smokers of both sexes, IR in male subjects, and blood pressure in female subjects (Fernandez-Real et al., 2001). Bastard et al (2000) compared serum IL-6 levels of obese diabetic women to both obese and lean non-diabetic women. This study discovered that the obese diabetic subjects had significantly higher IL-6 levels compared to both the obese non-diabetic subjects and lean controls, and that the obese non-diabetic subjects had significantly higher IL-6 levels compared to lean controls (3.58 ± 0.51 vs. 2.78 ± 0.30 vs. 0.39 ± 0.06 pg/mL) (Bastard et al., 2000). This study also found IL-6 serum levels to be positively correlated with degree of obesity and IR, and after weight loss resulting from three weeks on a very low calorie diet, the non-diabetic obese subjects saw an increase in insulin sensitivity and a significant decrease in serum IL-6 levels (2.78 ± 0.3 vs. 2.32 ± 0.19 pg/mL) (Bastard et al., 2000).

IL-1 β

IL-1 β is a pro-inflammatory cytokine produced by monocytes and macrophages in the adipose tissue, pancreatic beta cells, and aortic endothelium. IL-1 β is believed to heavily contribute to the initiation and progression of atherosclerosis through its potent ability to stimulate many secondary inflammatory mediators, such as IL-6, enhancing monocyte attraction and adhesion to the epithelium (Peiro et al., 2017; Ridker, 2016). The adhered monocytes release additional pro-inflammatory cytokines, including IL-1 β , resulting in further monocyte recruitment and adhesion to the area. Human islet cell studies have demonstrated an elevated production and secretion rate of IL-1 β in a hyperglycemic environment, indicating IL-1 β has a causal role in β -cell dysfunction and apoptosis, resulting in insufficient insulin production in T2D (Dinarello, Donath & Mandrup-Poulsen, 2010). Cholesterol crystals deposited within the arterial wall have also shown

to enhance IL-1 β production, linking dyslipidemia to vascular inflammation and ASCVD (Ridker & Luscher, 2014). Because IL-1 β is a potent driver of IL-6 and CRP production, both which have shown to be predictive of ASCVD and major CV event risk, its inhibition has become a promising therapeutic target to reduce inflammation, ASCVD, and CV event rates (Ridker, 2016). In fact, pharmacologic inhibition of IL-1 β activity has shown to reduce circulating IL-6 and hsCRP levels by more than 50% and the incidence of major CV events by up to 14% in high-risk CVD patients (Ridker et al., 2017; Ridker & Luscher, 2014). Even though circulating IL-1 β concentrations are typically low, and often times undetectable, they have shown to be up to five times greater in patients with various autoinflammatory diseases (Dinarello et al., 2010).

IL-18

IL-18 is a pro-inflammatory cytokine that is largely produced and secreted from visceral adipose tissue. Numerous animal models have established a causal role of IL-18 in the progression of atherosclerosis, primarily due to its initiation of other pro-inflammatory, atherogenic cytokines (Ballak et al, 2015; Landberg et al., 2011). In humans, plasma levels of IL-18 are positively correlated with degree of IR and are often elevated in obese, T2D, and CAD patients (Ballak et al., 2015; Dezayee, 2011; Nakamura et al., 2015; Suchanek et al., 2005). Elevated levels of IL-18 are associated with an increased risk of ASCVD in individuals with established T2D, and are considered to be a strong predictor of cardiovascular-related and all-cause mortality (Dezayee, 2011; Suchanek et al., 2005). Nakamura et al (2015) examined if serum IL-18 levels could be used to predict atherosclerosis in T2D patients. This study found that IL-18 levels were significantly higher in those with T2D compared to age-matched healthy controls (179 ± 62 vs. 121 ± 55 pg/mL) and were positively correlated with various parameters of atherosclerosis

(Nakamura et al., 2015). Suchanek et al (2005) compared serum IL-18 levels in CAD patients with and without T2D to healthy age and BMI-matched controls. Serum concentrations were significantly greater in CAD subjects with T2D compared to CAD subjects without T2D and compared to healthy controls (mean of 500 vs. 430 vs. 250 pg/mL) (Suchanek et al., 2005).

Clinical Importance of Serum Inflammatory Markers of Interest

IL-6

Mayo Clinic Medical Laboratories suggests IL-6 serum values be less than or equal to 1.8 pg/mL in healthy individuals (Mayo Clinic Medical Laboratories, n.d.). Some studies measuring IL-6 levels in healthy subjects have found mean serum concentrations within this range (Bastard et al., 2000; Davison et al., 2012). However, a study by Kleiner et al (2013), which sought to create reference guidelines for several serum cytokines in healthy individuals, found the mean IL-6 serum concentration of healthy adults (n=35) to be approximately 11 pg/mL (5-16 pg/mL) (Kleiner et al., 2013). Several other studies have also found mean serum concentrations of healthy controls to be well above the reference value stated by Mayo Clinic Medical Laboratories. Measurement of plasma IL-6 levels in 228 healthy, obese subjects revealed a mean concentration of 6.4 pg/mL (1.0-13.1 pg/mL) in men and 5.8 pg/mL (1.8-14 pg/mL) in women (Fernandez-Real et al., 2001). In healthy, non-obese controls, Kokkonen et al (2010) and Sarria et al (2014) found baseline IL-6 levels to be 4.6 pg/mL (1.1-10.8 pg/mL) and 4.3 pg/mL (.50), respectively (Kokkonen et al., 2010; Sarria et al., 2014).

IL-1 β

The expression of IL-1 β is limited in healthy individuals and is often low or below the level of detection even in patients with inflammatory disease (Dinarello et al., 2010; Peiro et al, 2017). Mayo Clinic Medical Laboratories suggests IL-1 β serum values be less than 1.0 pg/mL in

healthy persons (Mayo Medical Laboratories, n.d.). In the before mentioned study by Kleiner et al (2013), serum IL-1 β concentrations were below the limit of detection, which was 3.2 pg/mL, in all healthy sample subjects (total subjects, n = 72; subjects \geq 18 years of age, n =35) (Kleiner et al., 2013). Spranger et al (2003) also noted undetectable IL-1 β serum levels in 62% of study patients (n=565), with a mean of 0.57 ± 0.93 in case subjects (developed T2D within 2-3 years of follow-up) and 0.47 ± 0.79 in healthy, age and sex-matched control subjects (Spranger et al., 2003). However, other studies examining IL-1 β serum values in healthy control subjects found slightly elevated mean baseline concentrations of 2.6 (0.8-3.9) pg/mL and 3.43 (0.32) pg/mL (Kokkonen et al., 2010; Sarria et al., 2014).

IL-18

To our knowledge, there are no reference values established for IL-18. However, Kleiner et al (2013) found that all healthy adult subjects (n=35) had serum IL-18 concentrations of less than 200 pg/mL, with the mean being below 100 pg/mL (Kleiner et al., 2013). Other studies comparing serum IL-18 levels of T2D or CAD patients to healthy control subjects found mean concentrations of 121-284 pg/mL in the healthy study participants (Landberg et al., 2011; Nakamura et al., 2015; Suchanek et al., 2005).

Dietary Polyphenols

Polyphenols are a structural category of organic chemicals characterized by the existence of multiple phenolic units. Several thousand polyphenols have been identified and many of them are found in plant foods, such as fruits, vegetables, tea, coffee, and cocoa beans (Manach et al., 2004). Polyphenols are classified into subgroups according to their chemical structure. The five main classes of polyphenols are: Flavonoids, Phenolic Acids, Stilbenes, Lignans, and Others. There is strong evidence to support the notion that diets with a high polyphenol content have

favorable effects on human health and may limit the incidence of disease associated with inflammatory and oxidative stress, such as T2D and ASCVD (Aprotosoaie et al., 2016). Possible biological effects of polyphenols associated with a reduction in chronic disease include: Correction of hyperglycemia due to the augmentation of insulin secretion and sensitivity; Improvement of dyslipidemia by the lowering of LDL-C, total cholesterol and TG, and elevation of HDL-C concentrations; Amelioration of endothelial dysfunction through the enhancement of flow-mediated dilation (FMD), nitric oxide (NO) levels; Reduction of blood pressure; and the lessening of inflammatory and oxidative stress due to the inhibition of LDL oxidation and reduced levels of pro-inflammatory cytokines (Merone & McDermott, 2017; Tome-Carneiro & Visioli, 2016). Suggested mechanisms of action related to the positive effects of polyphenol-rich diets are the ability of polyphenols to modulate cell-signaling pathways and act as antioxidants to defend against oxidative stress (Manach et al., 2004).

Cocoa

Flavonoids are the largest subclass of dietary polyphenols and are predominantly found in cocoa, coffee, tea, red wine, and many fruits. Cocoa is an especially rich source of flavonoids, having the largest flavanol (a subclass of flavonoids) content by weight of all foods, with flavanols comprising ~12-18% of raw, dried cocoa beans (Aprotosoaie et al., 2016; Grassi, Desideri & Ferri, 2013). Dietary sources of cocoa generally containing the largest amount of flavanols are ground cocoa and dark chocolate (Aprotosoaie et al., 2016). A large body of research suggests that cocoa flavanols may have anti-diabetic and anti-atherogenic effects that alleviate the hallmarks of T2D, such as hyperglycemia, dyslipidemia, chronic inflammation, hypertension, and endothelium dysfunction, therefore reducing the risk of ASCVD (Grassi et al., 2013; Ramos

et al., 2017). The most abundant flavanol found in cocoa and cocoa-derived products is (-)-epicatechin (EC) and EC can likely be credited for the beneficial metabolic effects of cocoa. A number of systematic reviews and meta-analyses examining the effects of cocoa on human health have been conducted over the past 10 years (Aprotosoai et al., 2016; Ellinger & Stehle, 2016; Ludovici et al., 2017; Ramos et al., 2017; Tome-Carneiro & Visioli, 2016). The large majority of recent meta-analyses generated demonstrate that moderate cocoa consumption, defined as up to 6oz. of flavanol-rich cocoa products per week, has a beneficial effect on IR, serum lipids, and systemic inflammation (Ramos et al., 2017). These positive effects may in-turn reduce the risk of ASCVD development. Also, several epidemiological studies have shown an inverse relationship between consumption of flavanol-rich chocolate and risk of premature cardiovascular and all-cause mortality (Ludovici et al., 2017).

Anti-diabetic effects of cocoa

In-vitro, experimental animal, human intervention, and epidemiological studies conducted over the past decade provide strong support for the anti-diabetic effects of cocoa flavanols. The most common anti-diabetic actions of cocoa supported by in-vitro studies include enhanced levels of insulin secretion, a decreased degree of IR, and mitigation of abnormal lipid values (Ramos et al., 2017). Attenuation of β -cell damage and death, and heighten levels of β -cell replication, have been credited for the enhanced insulin secretion observed. Improvements in IR were attributed to heightened levels of glucose transporters (GLUT2 & GLUT4) and key proteins involved in the insulin-signaling pathway, resulting in improved glucose uptake by both liver and skeletal muscle cells, and decreased glucose production in hepatic cells. Cocoa also exhibited an insulinomimetic effect in adipocytes, decreasing the rate of FA synthesis and increasing the rate of FA oxidation, alleviating lipid accumulation (Ramos et al., 2017). Most studies using

experimental animal models to investigate the effects of cocoa flavanols on hyperglycemia and T2D reveal promising results. In fact, dietary cocoa supplementation has shown to be one of the most compelling nutritional methods used over the last decade in order to prevent and treat T2D (and decrease the risk of CVD) in experimental rats and mice (Ramos et al., 2017). Minimized deterioration and enhanced function of β -cells, decreased production of pro-inflammatory cytokines, heightened insulin sensitivity, and improved lipid metabolism are among the favorable effects of cocoa on T2D observed in experimental rat models (Ramos et al., 2017). Also, cocoa supplementation has not demonstrated any toxicity in experimental animals, even in studies lasting longer than three months (Ramos et al., 2017). Short-term consumption of flavanol-rich cocoa products in humans has also shown to ameliorate pathologies contributing to T2D, such as IR, pancreatic β -cell dysfunction, and insulin insufficiency (Grassi et al., 2013; Ramos et al., 2017).

Effects of cocoa on serum lipids

Both animal and human studies have indicated that flavanol-rich cocoa and cocoa products impose a beneficial effect on serum lipid profiles (Aprotosoie et al., 2016; Basu et al., 2015; Cordero-Herrera et al., 2015; Gutierrez-Salmean et al., 2014; Neufingerl et al., 2013; Parsaeyan et al., 2014; Rostami et al., 2015; Tokede, Gaziano & Djousse, 2011). The majority of the RCTs conducted to examine the effects of cocoa or cocoa flavanols on serum lipids have used non-diabetic participants, with only two reporting this relationship in subjects with T2D (Basu et al., 2015; Rostami et al., 2015) and studies observing the effects of cocoa flavanols on apolipoprotein metabolism are scarce (Lee et al., 2017; Neufingerl et al., 2013; Rostami et al., 2015). Basu et al (2015) examined the effect of 20 grams cocoa powder (480 grams of total flavanols) served along with a high-fat fast-food-style breakfast on postprandial serum lipids and

other markers of metabolic stress. This study concluded that HDL-C levels were significantly higher during the 6-hour postprandial period after the cocoa intervention compared to placebo ($p=0.011$), however there was no treatment effect found for LDL-C or TG levels (Basu et al., 2015). Rostami (2015) conducted a RCT to examine the effects of cocoa polyphenols on serum apoA1 and apoB concentrations in hypertensive patients with T2D. This study found that daily consumption of 25 grams dark chocolate (450mg total flavonoids) for eight weeks resulted in a significant decrease from baseline in apoB (-4.46 ± 9.44 pg/mL, $p = .012$) and increase from baseline in apoA1 ($+4.56 \pm 12.36$ pg/mL, $p = .045$), whereas no significant differences were observed in the placebo group (Rostami et al., 2015). However, even though the change from baseline was statistically significant for these variables following the dark chocolate intervention, the effect size was small. Lee et al (2017) examined the effect of regular consumption of sixty-one grams of dark chocolate and cocoa (CHOC), and sixty-one grams of dark chocolate and cocoa combined with forty-three grams almonds (CHOC + ALD), on apoB and apoA1 concentrations in healthy overweight and obese participants. Each treatment period lasted for four weeks and diets were controlled for the entire duration of the study (isocaloric, weight maintenance diets). In this study, neither the CHOC nor the CHOC + ALD treatment significantly influenced apoA1 levels compared to the control diet, but apoB concentrations and the apoB/apoA1 ratio were significantly lower in the CHOC + ALD compared to the control diet (apoB: 101.9 ± 2.4 vs. 107.5 ± 2.4 mg/dL, $p=0.01$; apoB/apoA1: 0.7 vs. 0.8, $p=0.02$) (Lee et al., 2017). Neufingerl et al. (2013) also performed a RCT to examine the effects of cocoa and theobromine on serum lipoproteins and apolipoproteins. Healthy participants were given one beverage per day for four weeks containing 6 grams cocoa powder (150 mg theobromine, 325 mg flavonoids), pure theobromine (850 mg), cocoa plus theobromine (1000 mg theobromine,

325 mg flavonoids), or placebo. Results showed that pure theobromine significantly increased serum HDL-C (6.2 ± 1.6 mg/dL, $p < 0.0001$) and apoA1 (11 ± 2 mg/dL, $p < 0.0001$), and decreased LDL-C (-7.0 ± 3.1 mg/dL, $p = 0.016$) and apoB (-5.0 ± 2.0 mg/dL, $p = 0.002$) from baseline following the four week intervention, whereas cocoa powder alone did not have a significant effect on these variables (Neufingerl, 2013).

Effects of cocoa on inflammation

Cocoa flavanols have shown powerful anti-inflammatory and antioxidant properties in-vitro. Antioxidant and anti-inflammatory functions that have been exhibited in-vitro are inhibited platelet activation, diminished LDL oxidation, repressed synthesis and expression of pro-inflammatory cytokines, and increased production of anti-inflammatory factors (Aprotosoae et al., 2016; Ellinger & Stehle, 2016; Parsaeyan et al., 2014). In high-fat-fed obese C57BL/6J mice, supplementation with 8% cocoa powder for 10 weeks reduced IL-6 production by 30.4% compared to high-fat-fed mice not receiving cocoa supplementation (Gu, Yu & Lambert, 2014). Human studies examining the effects of cocoa consumption on serum pro-inflammatory cytokines, especially IL-6, IL-1 β , and IL-18, in a diabetic population are extremely limited (Ellinger & Stehle, 2016; Goya et al., 2016). A RCT in subjects with T2D and hyperlipidemia indicated that consumption of 10 g of cocoa powder, twice per day for six weeks, reduced serum IL-6 levels by 32% (2.5 ± 0.9 vs. 1.7 ± 0.7 pg/ml) (Parsaeyan et al., 2014). This same study also revealed a significant improvement in total cholesterol (16.6% decrease; 246mg/dl to 205mg/dl), LDL-C (17.54% decrease; 135mg/dl to 111mg/dl) and TG (13.3% decrease; 226mg/dl to 195 mg/dl) levels after the six weeks of dietary cocoa supplementation, which was significantly less than the reductions seen in the control group (total cholesterol 5%, LDL-C 4%, and TG 3%). Another RCT conducted by Stote et al. (2012) investigated the effect of cocoa on various serum

markers of inflammation following an OGTT in obese individuals with impaired glucose tolerance. This study found that daily consumption of two moderate-flavanol content cocoa drinks (400 mg flavanols) for five days significantly decreased IL-6 concentrations following the OGTT compared to the low-flavanol (180 mg flavanols) group (2.1 vs. 2.8 pg/mL) (Stote et al., 2012). To our knowledge, there have not been any human intervention studies conducted looking at the effect of cocoa ingestion on IL-1 β in T2D: however, Sarria et al (2014) examined this effect in healthy and moderately hypercholesterolemic subjects and found that consumption of two, fifteen-gram servings of cocoa powder per day (416 mg polyphenols) for four weeks significantly reduced IL-1 β concentrations in the moderately hypercholesterolemic subjects compared to placebo (-0.95 vs. -0.33 pg/mL, $p=0.011$) (Sarria et al., 2014). This study also found that 4 weeks of cocoa supplementation significantly increased HDL-C and decreased blood glucose levels (Sarria et al., 2014).

To our awareness, there have not been any published studies examining the effect of cocoa consumption on serum IL-18 levels, however, there have been studies conducted exploring this effect using other high-polyphenol foods or diets. An observational study by Landberg et al (2011) reported that higher intake of total flavonoids and flavonoids from citrus fruits were inversely correlated with IL-18 concentrations in healthy US women (Landberg et al., 2011). In this study, mean IL-18 concentrations in the greatest total flavonoid intake quintile were 8% lower than those in the lowest total flavonoid intake quintile (55 vs. 276 pg/mL). Among the foods assessed in this study, tea, apples and citrus fruits were the greatest contributors to total flavonoid intake (Landberg et al., 2011). Another study in healthy adults examined the effect of sweet bing cherries on IL-18 levels and found that consumption of 280 grams of sweet bing cherries per day for twenty-eight days reduced IL-18 levels by 8.1% (Kelley et al., 2013). In

adult patients with Metabolic Syndrome, two years of a Mediterranean-style diet (high in dietary polyphenols, fiber, and unsaturated fatty acids) significantly reduced IL-18 levels compared to the control group (11% vs. 2% reduction, $p=0.3$) (Esposito et al., 2014). Another study examining the effect of a Mediterranean-style diet on IL-18 concentrations found that three years of this style diet significantly lowered IL-18 levels (-10.5% from baseline) compared to the control diet in elderly, white males considered to be at high-risk of developing CVD ($p=0.012$) (Troseid et al., 2009). In contrast to these results, Marfella et al (2006) found no significant change in IL-18 levels after twelve months on a Mediterranean-style diet in T2D adults who had recently experienced their first myocardial infarction; however, this same style diet in conjunction with moderate red wine consumption at meals significantly lowered IL-18 levels compared to the non-wine group (mean change of -61.7 ± 5.1 vs. -28.2 ± 3.8 pg/mL) (Marfella et al., 2006). Lastly, in obese, T2D adults, an eight-week high-fiber diet that also contained a large amount of coffee (≥ 5 cups/d) resulted in a modestly significant decrease in IL-18 levels compared to the low-fiber, no coffee control group ($p<0.05$) (Nowotny et al., 2014).

Chapter 3: Methodology

This thesis study is a secondary data analysis of unpublished data from a previously conducted clinical study by Basu et al. (2015), which examined the effects of cocoa on serum lipoproteins, endothelial function, and inflammation following a high-fat fast-food-style meal in a T2D population. The study design has been previously published and is stated below (Basu et al., 2015). Approval for this secondary data analysis was obtained from the Institutional Review Board of the University of Nevada, Las Vegas.

Participants. The Institutional Review Boards of the Oklahoma University Health Sciences Center and the Oklahoma State University approved the original study protocol. Written informed consent was acquired from all enrolled participants. All study procedures were administered at the Harold Hamm Diabetes Center at the Oklahoma University Health Sciences Center. Four men and fourteen women with established, stable T2D for a minimum of five years, not on insulin therapy, and with a waist circumference of >89 cm for women or >102 cm for men were screened for inclusion in the study. T2D eligibility was confirmed according to the guidelines of the American Diabetes Association (ADA, 2017). Potential participants were excluded if they were <21 y of age; presented with preexisting conditions, such as cancer or coronary heart disease; were anemic or had abnormal liver, renal, or thyroid function on the basis of screening examinations; were consuming antioxidants or fish oil supplements on a regular basis; were current smokers or regular consumers of alcohol (no more than 1–2 drinks/wk.); were currently enrolled in a weight loss program; or were pregnant or lactating. Presence of CVD risk factors, such as elevated blood pressure or serum lipids, did not inhibit participation in the study. Each participant's regular oral medications were continued throughout the duration of the study.

Study design. Volunteers completed a randomized, double blind, crossover study in which they made two separate visits to the study site. They arrived to the site in a fasted state (10–12 h) and each visit lasted 6–7 h. The two study days were separated by a 1-wk washout phase. On each study day, blood draws were performed at baseline (fasting state) and then again at 30 (0.5h), 60 (1h), 120 (2h), 240 (4h), and 360min (6h) postprandial time points, starting from the time of completion of the provided meal and beverage. After the fasting blood draw, participants consumed a high fat fast-food style breakfast with either the cocoa or placebo beverage. The meal was prepared at the clinic and consumption of the test meal by participants was supervised. Participants were instructed to refrain from alcohol and caffeine for 24 h prior to each study visit, and foods or dietary supplements with high polyphenol content for 48 h prior to each study visit. With the exception of the previously stated instructions, participants were asked to maintain their usual diet, medications, and lifestyle for the entire duration of the study. Three-day food records (two weekdays and one weekend day) were collected at baseline, prior to the start of the study, and nutrient and food group intakes were analyzed with Nutritionist Pro version 3.2 (Axxya Systems).

Interventions. The nutritional composition of the cocoa and placebo powder has been previously published (Basu et al., 2015). The total polyphenol and flavanol content per serving was substantially higher in the cocoa powder compared to the flavanol-free placebo powder. The total polyphenol content was determined by the Folin-Ciocalteu assay as described by Singleton et al. (Singleton & Rossi, 1965), and the total flavanol content was determined by the 4-dimethylaminocinnamaldehyde assay as described by Payne et al. (Payne et al., 2010). Besides the cocoa, the test powder contained the following ingredients: sunflower oil, corn syrup solids, and $\leq 2\%$ food additives (carrageenan, vanillin, salt, sodium caseinate, aspartame, dipotassium

phosphate, monoglycerides, diglycerides, acesulfame potassium, soy lecithin, and silicon dioxide). The composition of the placebo powder mix was similar to that of the cocoa mix, except the cocoa was substituted with milk protein isolate. The fast-food–style breakfast meal consisted of two scrambled eggs (no added fat), hash brown potatoes (70 g), two buttermilk biscuits, butter (15 g), and a sausage patty (57 g), and provided a total of 766 kcal, 50 g total fat (25 g saturated fats, 12 g monounsaturated fats, 13 g polyunsaturated fats; 59% energy), 50 g carbohydrates (26% energy), 30 g proteins (16% energy), 465 mg cholesterol, and 2.4 g dietary fiber. All ingredients for the test meals were purchased from a local grocery store and prepared in the metabolic kitchen of the clinic each morning on both testing days. The cocoa and placebo mixes were reconstituted in warm water and were provided to the participants in closed lid cups along with the breakfast meal for supervised consumption.

Anthropometrics. On each day of the trial, after an overnight fast and before blood sampling, body weight was measured on a calibrated scale, height was measured with the use of a stadiometer, and waist circumference (60.05 cm) was measured at the superior iliac crest.

Biochemical variables. Freshly drawn blood samples were sent to the Oklahoma University Medical Center Laboratory for analysis of serum lipids (apoB, apoA1, and NEFA) and inflammatory markers (IL-1 β , IL-6, and IL-18). Analyses for the serum lipids were conducted with the use of automated diagnostic equipment (Abbott Architect Instruments) by enzymatic colorimetric methods that used commercially available kits according to manufacturer's protocols. Analyses for the inflammatory markers were conducted with the use of R&D Human Elisa kits according to the manufacturer's protocol.

Statistical analysis. For each outcome, change from baseline (i.e., the fasting, pre-intervention

time point) was calculated for each postprandial time point. Differences between intervention and placebo were analyzed with the use of paired *t*-tests. Data analyses were conducted with the use of IBM SPSS Statistics version 23.0 (IBM Corp.). Results corresponding to $P < 0.05$ are described as significant for the purposes of discussion. For this crossover design, between-intervention comparisons were performed at each time point with the use of generalized estimating equation analyses to account for the repeated measures in the same individual. Between-intervention comparisons across all time points, that is, for the entire 6-h time period, were performed with the use of generalized estimating equation analyses (with results indicated as overall *P*).

Chapter 4: Results

Baseline Characteristics. Out of twenty-six participants assessed for eligibility, eight were excluded due to not meeting inclusion criteria and 18 were enrolled and completed the study. All participants were obese ($\text{BMI} > 30\text{kg/m}^2$) with clinically diagnosed T2D. The majority of participants were taking oral hypoglycemic agents (83%) and antihypertensive agents (84%), but none were on insulin therapy. At baseline, participants had hyperglycemia ($\text{FBG} > 126\text{mg/dL}$), elevated blood pressure and LDL-C, and hsCRP levels considered at high risk for CVD ($>3.0\text{g/L}$). Baseline dietary analyses indicated that participant's usual diets were high in fat (37% total kcals) and low in fruits and vegetables. Baseline characteristics are presented in Table 2.

Postprandial Serum Lipids. Serum apoA1, apoB and NEFA concentrations, and the apoB:apoA1 ratio were not significantly different over the 6-h postprandial period following cocoa intervention compared with placebo. There was a significant effect of time on NEFA levels, with NEFA concentrations being significantly lower at the 1, 2, and 4-h postprandial time points compared to baseline ($p < 0.001$) (Table 3).

Postprandial Inflammatory Markers. Serum IL-6 and IL-1 β concentrations were not significantly different over the 6-h postprandial period following cocoa intervention compared to placebo. However, serum IL-18 levels were significantly lower at the 1, 4, and 6-h postprandial time points following cocoa intervention compared to placebo ($p < 0.001$) (Table 4, Figure 1).

TABLE 1 Composition of cocoa and placebo powder administered

	Cocoa	Placebo
Serving Size, <i>g</i>	20	12
Energy, <i>kcal</i>	67	66
Fat, <i>g</i>	4.7	5.0
Protein, <i>g</i>	2.7	1.1
Carbohydrates, <i>g</i>	9.6	4.0
Carbohydrate: Protein	3.6:1	3.6:1
Fiber, <i>g</i>	4.4	0.1
Total Polyphenols, <i>mg</i>	960	110
Total Flavanols, <i>mg</i>	480	<0.1
Proanthocyanidins (PAC) 1-10, <i>mg</i>	201	<0.001
Epicatechins, <i>mg</i>	40	0
Catechins, <i>mg</i>	18	0
Theobromine, <i>mg</i>	220	0
Caffeine, <i>mg</i>	21	0

Source: The Hershey Company (Hershey, PA, USA)

TABLE 2. Baseline characteristics of the study participants

Variable	Value
<i>N</i>	18
Age, <i>y</i>	56±3.2
Gender, <i>M/F</i>	4/14
Weight, <i>kg</i>	100±12
BMI, <i>kg/m²</i>	35.3±2.0
Waist Circumference, <i>inches</i>	45±1.8
Serum glucose, <i>mg/dL</i>	136±16
Serum insulin, <i>mU/L</i>	14.8±2.6
Insulin resistance (HOMA-IR)	3.5±0.9
Serum HbA _{1c} , %	8.2±0.6
Serum total cholesterol, <i>mg/dL</i>	188±11
Serum LDL cholesterol, <i>mg/dL</i>	112±11
Serum HDL cholesterol, <i>mg/dL</i>	46±2.5
Serum LDL:HDL	2.54±0.24
Serum triglycerides, <i>mg/dL</i>	140±13
Serum hsCRP, <i>mg/L</i>	5.3±1.2
Systolic blood pressure, <i>mm Hg</i>	144±5.5
Diastolic blood pressure, <i>mm Hg</i>	85±3.0
Small artery elasticity index, <i>mL/mmHg x 100</i>	5.6±3.6
Large artery elasticity index, <i>mL/mmHg x 10</i>	17±7.4
Medication/supplement use <i>n</i> (%)	
Insulin	0 (0)
Oral hypoglycemic agents	15 (83)
Statins/fibrates	5 (28)
CCBs	3 (17)
ACEIs/ARBs	10 (56)
Diuretics	2 (11)
Aspirin	6 (33)
Multivitamins/minerals	6 (33)
Botanical supplements	2 (11)
Macronutrient and food intake	
Energy, <i>kcal/d</i>	2216±198
Carbohydrates, <i>g/d</i>	263±38
Total fats, <i>g/d</i>	92±8
Protein, <i>g/d</i>	94±9
Fiber, <i>g/d</i>	30±9
Fruit servings, <i>g/d</i>	144±52
Vegetable servings, <i>g/d</i>	160±35

Mean ± SEMs for continuous variables; ACEIs/ARBs: angiotensin converting enzyme inhibitors/ angiotensin receptor blockers; CCBs: **calcium channel blockers**; HOMA-IR: homeostasis model assessment of insulin resistance; hsCRP: high sensitivity C-Reactive Protein.

TABLE 3: Serum apolipoprotein and NEFA values during the cocoa and placebo interventions.

Variable	Fasting	1hr	2hr	4hr	6hr	P-value
Apo-B (mg/dL)						Interaction: 0.58
Placebo	96 ± 4.0	93 ± 5.0	95 ± 4.0	97 ± 5.0	98 ± 4.0	Time: 0.70
Cocoa	96 ± 5.0	94 ± 5.0	97 ± 5.0	94 ± 5.0	100 ± 5.0	Treatment: 0.57
Apo-A1 (mg/dL)						Interaction: 0.26
Placebo	155 ± 6.0	167 ± 8.0	153 ± 8.0	162 ± 9.0	164 ± 8.0	Time: 0.06
Cocoa	158 ± 8.0	153 ± 7.0	155 ± 6.0	167 ± 8.0	166 ± 8.0	Treatment: 0.97
NEFA (mg/dL)						Interaction: 0.07
Placebo	0.64 ± .05	0.42 ± .05	0.36 ± .03	0.45 ± .03	0.71 ± .06	Time: <0.001*
Cocoa	0.62 ± .04	0.49 ± .04	0.44 ± .04	0.51 ± .05	0.65 ± .05	Treatment: 0.70
ApoB/ApoA1 Ratio						Interaction: 0.51
Placebo	0.63 ± .03	0.57 ± .04	0.66 ± .04	0.63 ± .05	0.62 ± .04	Time: 0.48
Cocoa	0.63 ± .04	0.63 ± .04	0.64 ± .04	0.59 ± .04	0.63 ± .05	Treatment: 0.92

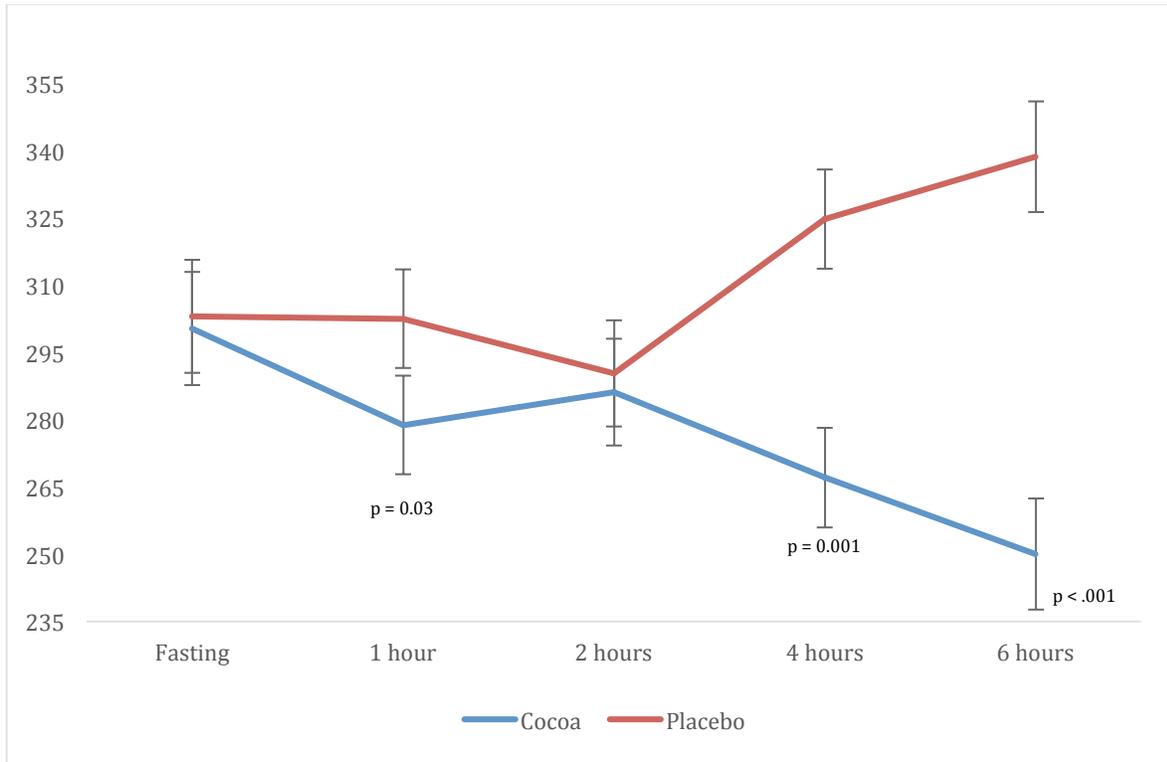
Data represented as mean ± SEM; Apo-B: apolipoprotein-B; Apo-A1: apolipoprotein-A1; NEFA: non-esterified fatty acids; ApoB/ApoA1 Ratio: ratio of apolipoprotein-B to apolipoprotein-A1; *Significant effect of time on NEFA levels over 6-h postprandial period in both treatment groups, P < 0.001

TABLE 4: Serum IL-6, IL-1 β , and IL-18 values during the cocoa and placebo interventions.

Variable	Fasting	1hr	2hr	4hr	6hr	P-value
IL-6, pg/mL						
Placebo	6 \pm 2.0	4 \pm 1.0	7 \pm 2.0	5 \pm 1.0	5 \pm 2.0	Interaction: 0.51 Time: 0.06
Cocoa	3 \pm 0.5	5 \pm 0.7	6 \pm 2.0	3 \pm 0.6	4 \pm 1.0	Treatment: 0.39
IL-1 β , pg/mL						
Placebo	3 \pm 1.0	2 \pm 0.4	3 \pm 1.0	3 \pm 1.0	4 \pm 0.6	Interaction: 0.77 Time: 0.55
Cocoa	3 \pm 0.4	5 \pm 2.0	3 \pm 0.4	6 \pm 1.0	4 \pm 0.4	Treatment: 0.15
IL-18, pg/mL						
Placebo	303 \pm 13	302 \pm 11	290 \pm 12	325 \pm 13	339 \pm 15	Interaction: <0.001** Time: 0.45
Cocoa	300 \pm 12	279 \pm 11	286 \pm 12	267 \pm 9	250 \pm 9	Treatment: 0.001*

Data represented as mean \pm SEM; IL-6: interleukin-6; IL-1 β : interleukin-1-beta; IL-18: interleukin-18; *significant effect of cocoa intervention on IL-18 levels throughout 6-hour postprandial period, $P < 0.001$; **significant difference between cocoa and placebo interventions on IL-18 levels throughout 6-h postprandial period, $P < 0.001$

Figure 1: Serum IL-18 values over the 6-hour postprandial period following cocoa and placebo interventions



Data represented as mean \pm SEM; IL-18: interleukin-18; IL-18 serum levels in pg/mL; significant effect of cocoa intervention on IL-18 levels compared to placebo at 1h (p=0.03), 4h (p=0.001), and 6h (p<0.001) postprandial time points

Chapter 5: Discussion & Conclusion

Diabetic dyslipidemia and alterations in postprandial lipid metabolism have shown to contribute to the development and progression of ASCVD. Epidemiological evidence suggests that the consumption of flavanol-rich cocoa products is associated with a reduced risk of ASCVD, and several previously conducted clinical studies have shown cocoa to ameliorate lipid profiles through improvements in HDL-C, LDL-C, and/or TG. *The results of our study did not reveal a significant effect of cocoa on postprandial apoA1 or apoB levels, or on the apoB/apoA1 ratio following a high-fat fast-food-style breakfast in T2D participants.* These results are in agreement with two previously conducted short-term, regular-consumption studies examining the effect of cocoa on serum apoB and apoA1 concentrations in healthy lean (Neufingerl et al, 2013) and healthy overweight subjects (Lee et al, 2017). However, a study by Rostami et al (2015) showed that short-term, regular consumption of flavanol-rich dark chocolate significantly decreased apoB and increased apoA1 levels from baseline in participants with T2D and hypertension. Discrepancies between study results may be explained by differences in study duration (acute bolus vs. eight weeks regular consumption), study design (crossover vs. parallel), treatment dose (dark chocolate vs. cocoa powder of varying flavanol content), and sample size. Also, studies have shown serum levels of apoB to be higher and apoA1 to be lower in men compared to similar aged women in both healthy and CVD participants (Frondelius et al., 2017; Sharma et al., 2013). It is possible that differences in post-prandial apoB and apoA1 metabolism, and the response of these apolipoproteins to cocoa, exist between men and women. Thus, having a sample population with only four male participants (22% of total sample) may have affected our findings. It is recommended that future studies explore whether sex differences exist for

postprandial apoB and apoA1 metabolism and their response to flavanol-rich dietary interventions.

Our postprandial study did not show a significant effect of cocoa on plasma NEFA levels compared to placebo. This result is consistent with another postprandial study that examined the effect of flavanol-rich cocoa on NEFA concentrations following an oral fat load (Westphal & Luley, 2010). According to a study conducted by Jackson et al (2005), NEFA levels peak at the 7-hour postprandial time point following a high-fat mixed meal, thus it is possible that we would have seen a significant effect of cocoa on plasma NEFA levels had our study been of longer duration. Our study did show a significant effect of time ($p < 0.001$) on postprandial NEFA levels. As expected, NEFA levels were suppressed in the early postprandial phase, being the lowest at the 2-hour postprandial time point, and then showed a rise in levels back to baseline by the 6-hour postprandial time point. A similar effect of time was seen in other postprandial studies examining the effect of a high-fat mixed meal on plasma NEFA levels in healthy adults (Jackson et al., 2005; Teng et al., 2015).

Flavanol-rich cocoa products have shown to exhibit substantial anti-inflammatory effects in animal and in-vitro studies, but results of human studies have been conflicting. The results of our study did not show any significant effects of cocoa powder on postprandial IL-1 β or IL-6 levels in T2D participants following ingestion of a high-fat fast-food-style meal. These findings are consistent with previously conducted short-term, regular consumption studies in healthy (Mathur et al., 2002), overweight and obese (West et al., 2014), hypertensive (Muniyappa et al., 2008), and T2D (Monagas et al., 2009) participants. However, in contrast to our findings, short-term, regular cocoa consumption has shown to significantly reduce IL-1 β concentrations compared to placebo in moderately hypercholesterolemic subjects (Sarria et al., 2014) and IL-6

concentrations in obese (Stote et al., 2012) and hyperlipidemic T2D (Parsaeyan et al., 2014) subjects. Inconsistencies between study results may be elucidated by variances in study duration (acute bolus vs. daily, short-term consumption), study design (crossover vs. parallel), population studied, treatment dose (amount/composition of cocoa powder treatment used), and sample size.

On the other hand, the results of our study showed cocoa powder significantly reduced IL-18 levels compared to placebo at one, four, and six hours following a high-fat fast-food-style breakfast in T2D participants. At the six-hour postprandial time point, serum IL-18 levels had decreased from baseline by 17% following the cocoa intervention, whereas they had increased by 12% following the placebo trial. The effect size observed ($d = -1.7$) is considered large, implying cocoa had a substantial, meaningful effect on serum IL-18 levels in this study. While reported studies on cocoa have not reported effects on IL-18, our results are in agreement with other studies examining how IL-18 concentrations are affected by various high-polyphenol foods or diets in healthy (Kelley et al., 2013; Landberg et al., 2011), metabolic syndrome (Esposito et al., 2014), T2D (Marfella et al., 2009; Nowotny et al., 2014), and high CVD risk (Trøseid et al., 2009) participants. Mechanisms of action responsible for the reductions of IL-18 by high-polyphenol diets or foods have yet to be elucidated. IL-18 is first expressed as an inactive precursor, pro-IL-18, which is activated to mature IL-18 by caspase-1 (Dinarello et al., 2013; Trøseid, Seljeflot, & Arnesen, 2010). Circulating levels of active IL-18 are neutralized by IL-18 binding protein (IL-18BP) in healthy adult humans and animals (Dinarello et al., 2013). The binding of IL-18 to IL-18BP inhibits the pro-inflammatory intracellular signaling capabilities of IL-18. An imbalance between free IL-18 and IL-18BP results in elevated levels of circulating free IL-18 (and its pro-inflammatory effects) and this imbalance has shown to worsen as disease severity escalates (Trøseid et al., 2010). The reduction of circulating IL-18 levels observed in our

study and in other studies using high-polyphenol dietary interventions may be due to the ability of these interventions to enhance the production or prevent the inhibition of IL-18BP expression. Future studies examining the mechanism of action responsible for the reduction of IL-18 levels by polyphenol-rich dietary interventions are recommended.

Our study has some limitations that should be considered when analyzing the results. The relatively small sample size in our exploratory study may have affected the statistical significance in certain inflammatory variables, though our study had adequate power for lipid and lipoprotein variables. Other limitations of this study were the absence of a non-diabetic control group or a low-fat test meal for comparisons; non-standardization of the diet during the study, especially the evening meal the night prior to study days; and the lack of assessment for habitual consumption of cocoa and other flavanol-rich foods prior to the study. Lastly, this was an acute intervention study and it is possible that a single dose of flavanol-rich cocoa powder may not exert an effect on all of the outcome variables assessed.

In conclusion, our study showed that ingestion of cocoa powder along with a high-fat fast-food-style breakfast significantly decreased postprandial serum IL-18 levels but had no significant effects on postprandial apoB, apoA1, NEFA, IL-6 or IL-1 β levels compared to placebo in a T2D population. Further studies examining the acute and long-term effects of flavanol-rich cocoa consumption on serum IL-18 are recommended to confirm our findings and determine whether cocoa plays a protective role against the development and progression of ASCVD.

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Curriculum Vitae

Rickelle Tallent
Rickelle.Tallent@gmail.com

Education:

Master of Science – Kinesiology, Exercise Physiology Expected graduation: Fall 2018
University of Nevada, Las Vegas

Dietetic Internship July 2014-February 2015
University of Nevada, Las Vegas
Southern Hills Hospital

Bachelor of Science - Nutrition Sciences May 2014
University of Nevada, Las Vegas
GPA: 3.8/4.0

Bachelor of Liberal Arts - Psychology May 2004
University of Nevada, Las Vegas
GPA: 3.7

Teaching Experience

Instructor

University of Nevada, Las Vegas Fall 2017 – Fall 2018

- Instructor for 2 Human Nutrition Courses (NUTR 121) per semester
 - ~90 students per section

Graduate Assistant

University of Nevada, Las Vegas Spring 2017

- 1 Nutrition Assessment Lab Section (NUTR 311)
 - Assisted with all lab set-ups and assessments
 - Graded all lab reports, lab data sheets, lab quizzes, and lab practical
 - Delivered 2 lab session lectures
 - Delivered 1 lecture during Nutrition Assessment lecture session
 - Delivered 1 exam for Nutrition Assessment lecture session
- 4 Human Nutrition Courses (NUTR 121)
 - Printed and graded all exams
 - Assisted students with content questions and course projects
 - Assisted UTA's with grading and any questions
 - Assisted UTA's with exam study sessions (10 sessions total)
 - Proctored make-up exams when needed
- 2 Introduction to Sports Nutrition Course (NUTR 340)
 - Assisted students with content questions and course projects
 - Assisted UTA's with grading and any questions

University of Nevada, Las Vegas

Fall 2016

- 2 Exercise Physiology Lab Sections (KIN 491)
 - Assisted in all lab set-up and assessments
 - Graded all lab data sheets
 - Delivered a lecture for one lab section (body composition)
- 4 Human Nutrition Courses (NUTR 121)
 - Printed and graded all exams
 - Assisted students with content questions and course projects
 - Assisted UTA's with grading and any questions
 - Assisted UTA's with exam study sessions (10 sessions total)
 - Proctored make-up exams when needed
- 1 Introduction to Sports Nutrition Course (NUTR 340)
 - Assisted students with content questions and course projects
 - Assisted UTA's with grading and any questions

Undergraduate Teaching Assistant

University of Nevada, Las Vegas

Spring 2012

- Human Physiology Lab (BIO 224)
 - Assisted with lab set-up, lectures, content questions and grading
 - Delivered one lecture

University of Nevada, Las Vegas

Fall 2010

- Human Nutrition (NUTR 121)
 - Graded all course projects and exams
 - Assisted students with course projects and content questions
 - Delivered one lecture

Research Experience

Poster Presentation

May 2018

Tallent, R.C., Basu, A. "Dietary Cocoa Improves Postprandial Lipids and Inflammation in Obese Adults with Type 2 Diabetes Following a High-Fat Breakfast." University Medical Hospital of Southern Nevada 2018 Research Empowerment Day Poster Session

Graduate Research Assistant

Spring 2017

- Title: Post-meal Walking Study
Purpose: To examine the effects of post-prandial walking on blood glucose levels in a pre-diabetic, older adult population.

Abstract Poster Presentations

Tallent, R.C., Woita, A.C., Aguilar, C.D., Young, J., Navalta, J.W., Bodell, N.G., Montes, J., Tanner, E.A., MacDonald, G.A., Thomas, C., Manning, J.W., Taylor, J. "Comparison of Mechanical Efficiencies from Steady State and Rapid Speed Rock Climbs." Annual Meeting of the Southwest American College of Sports Medicine, Costa Mesa, CA, 2016.

Case Study Poster Presentations

Tallent, R.C. "Refeeding Syndrome." Today's Dietitian Spring Symposium, Las Vegas, NV, 2014.

Professional Work Experience

Registered Dietitian

Anders&Dunaway Nutrition Consultants

Sept. 2015 – Sept. 2017

- Conduct nutritional assessment of patients of various ages, cultures and socioeconomic status.
- Provide appropriate nutritional education and counseling for several conditions and disease states, including but not limited to Pre-DM, DM (Type 1 and 2), HTN, hyperlipidemia, CKD, ESRD, pediatric obesity and wellness, pre and post-bariatric surgery, geriatric anorexia, malnutrition, gout, obesity and weight loss, and general health maintenance.
- Work with patients to devise and implement individual diet plans and goals, in accordance to the nutrition standards of care, to improve their health and increase vitality.
- Continuously follow-up with patients to reassess nutritional status, provide further education and counseling, and revise diet plan and/or goals when necessary.
- Maintain written documentation of all counseling sessions.
- Assist in mentoring medical students and dietetic interns during their rotation.
- Presented on cardiovascular health and heart healthy foods for approximately 60 Health Care Partners of Nevada members and physicians in Pahrump, NV
- Perform administrative duties, such as:
 - Enter patients and scan all documents into company computer system.
 - Send patient notes and documentation to their referring physician.
 - Fill out billing information, with ICD 10 codes, for each patient and send to company biller.
 - Assist receptionist and other dietitians with answering phones, scheduling patients and paper work when able to do so.
 - Help to maintain patient handouts.

Program Assistant

Dairy Council of Utah/Nevada

2010-Present

- Educate high-school athletes, as well as their parents, coaches and athletic directors, on chocolate milk and its role in post-exercise recovery.
- Promote dairy products by educating children and adults about their role in health at numerous events, such as state fairs, health fairs, health and wellness events, and food product launches.
- Act as liaison between Southern Nevada team members and management in Northern Nevada by coordinating events in Southern Nevada, managing team members at events, submitting team member hours and mileage after events, and assisting in new member training.

Awards

Recipient of Graduate Access Childcare Scholarship

2016-2018

University of Nevada, Las Vegas

Recipient of Outstanding Dietetics Student Award

2014

Academy of Nutrition and Dietetics

Recipient of B.F. Relin Scholarship 2013-2014
University of Nevada, Las Vegas

Professional Certifications

Registered Dietitian with Commission on Dietetic Registration April 2014 - Present
Licensed Dietitian with the State of Nevada May 2014 - Present

Professional Memberships

Academy of Nutrition and Dietetics, Nevada Dietetic Association 2013 - Present
Academy of Nutrition and Dietetics, Southern Nevada Dietetic Association 2013 – Present
UNLV Student Nutrition and Dietetic Association 2009-2014