

The Effects of Acute Consumption and Chronic Supplementation of Cocoa on Overweight and Obese Adults at Risk for Developing Diabetes

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Technical Abstract

The prevalence of obesity and diabetes is increasing in the United States and abroad and strategies are needed to prevent the progression from an at-risk state to the clinically diagnosed diseases. Flavanols in cocoa powder have been shown to reduce blood glucose concentrations, improve insulin sensitivity, and decrease gut permeability in animals and humans, but it is unknown if this occurs in adults with prediabetes. Therefore, we first hypothesized that an acute dose of cocoa would reduce postprandial glucose and enhance insulin and incretin hormone responses to a mixed meal challenge compared to a placebo. Second, we hypothesized that 15 g cocoa/day for 4-weeks would reduce gut permeability, attenuate endotoxin response to a high fat meal, improve insulin sensitivity, and improve measures of skeletal muscle substrate flexibility in a randomized, double blinded, placebo controlled parallel group design. To test the first hypothesis, 30 overweight or obese volunteers who were at-risk for diabetes completed two meal challenges using a randomized crossover design. Blood samples were collected hourly for 4 hours and were analyzed for glucose, insulin, C-peptide, glucagon-like peptide 1 (GLP-1), and gastric inhibitory peptide (GIP). Cocoa did not influence these measures. However, participants with the lowest fasting blood glucose concentrations were more likely to respond to the cocoa as hypothesized. To test our second hypothesis, 15 overweight or obese adults at risk for developing diabetes consumed either the cocoa or placebo treatments along with a controlled diet for one month. Overall, cocoa did not seem to influence insulin sensitivity, gut permeability, or endotoxin levels, although cocoa may influence skeletal muscle substrate metabolism. In

conclusion, the data for both studies suggests that cocoa did not exert substantial effects on the evaluated outcomes. However, the experiments did provide valuable information about incretin hormone levels in adults with impaired glucose tolerance. More research is needed to understand how cocoa can affect glucose homeostasis for overweight or obese adults.

General Audience Abstract

The purpose of this research project was to determine if cocoa powder could influence how we digest food and improve our health. For many people in the United States and abroad, controlling blood sugar levels is important for their well-being; uncontrolled spikes in blood sugar levels can eventually lead to type 2 diabetes and heart disease. Our first hypothesis was that one serving of cocoa could help reduce large spikes in blood sugar after a meal. Secondly, we hypothesized that consuming cocoa powder for one month could improve blood sugar and other health outcomes related to diabetes. To test the first hypothesis, 30 overweight or obese volunteers who were at-risk for diabetes completed two blood tests. At the beginning of the tests, they consumed a meal replacement shake mixed with cocoa powder or a placebo powder. The participants had their blood drawn every hour for four hours. A week later, they repeated the same test with the alternative powder. Blood samples were analyzed for sugar levels and four hormones that are important for controlling sugar spikes. We found that the cocoa made no difference in blood sugar levels. However, we noticed that some of our participants had greater sugar spikes than others. Those who had large sugar spikes had deficient levels of certain hormones. Although our experiments showed that cocoa did not affect these hormones, other studies found that compounds similar to cocoa could increase these hormone levels. Future directions could include using a different meal or studying more adults deficient in these hormones. For the second hypothesis, 15 overweight or obese adults at risk for developing diabetes completed the study. They consumed cocoa powder or placebo powder beverages along with a controlled diet provided to them for one month (they did not know which treatment they received). We tested several outcomes related to digestion and metabolism including leakiness of the gut, ability to control blood sugar levels, and the ability of skeletal muscle to burn sugars and

fats. Overall, cocoa did not seem to influence any of our outcomes. In conclusion, my experiments did not demonstrate any substantial effects of cocoa on blood sugar levels. However, the experiments did provide valuable information about hormone levels in overweight and obese adults. More research is needed to understand how cocoa can affect health outcomes for overweight or obese adults.

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Attributions

Chapter 2. The review of the literature is adapted from a publication in the Journal of Nutritional Biochemistry with multiple co-authors. Graduate students Thomas Rowley and Andrew Smithson assisted in the tables summarizing the current literature. Professors Jeffery Tessem, Matthew Hulver, Dongmin Liu, Brenda Davy, and Kevin Davy provided input throughout the document. Professor Andrew Neilson provided guidance throughout the writing and editing process.

Chapter 3. Professors Brenda Davy, Matthew Hulver, Kevin Davy and Andrew Neilson designed the study. Kevin Davy and Andrew Neilson provided equipment and materials to support the project and assisted in editing the manuscript.

Chapter 4. Andrew Smithson and Ryan McMillan assisted in sample analysis. Tanya Halliday completed food records and dietary analysis. Kristen Osterberg developed the standardized diet and assisted with clinical procedures. Professors Brenda Davy, Matthew Hulver, Kevin Davy, and Andrew Neilson designed the study. Kevin Davy and Andrew Neilson assisted in editing the manuscript.

Chapter 1. Introduction

The prevalence of obesity and diabetes is growing in the United States and abroad. In 2014 in the United States, there were approximately 29 million adults with diabetes, but there were also 86 million with prediabetes.¹ The prediabetic population is a large, growing populace that could benefit from strategies to prevent the progression from at-risk to fully developing the clinically diagnosed disease. Cocoa powder may be a useful agent for the prevention of developing diabetes.

The use of functional foods and dietary strategies to ameliorate or prevent diseases and weight gain is becoming increasingly popular. As such, it is important to understand the function of these foods in order to best utilize them for their desired purposes. There are a variety of ways to study dietary compounds in foods with many important considerations to take into account. First and foremost, it is important that the active compound is bioavailable, or can reach its desired target. Second, the food matrix within which the food is consumed can alter bioavailability. Foods (and pharmaceutical grade drugs) can have both immediate or acute effects, and also long-term or chronic effects. The resulting outcomes are likely different and should both be fully described.

The purpose of this dissertation research was to investigate both the acute and chronic physiological effects of cocoa powder in overweight and obese adults at risk for developing diabetes. Acute outcomes were related to digestion, including glycemic and hormonal responses, after consuming a meal with and without cocoa. The chronic outcomes, after 4-week supplementation with cocoa, were related to metabolic endotoxemia and insulin sensitivity. A review of the current literature and the results of these two studies and will be presented herein.

Chapter 2. Review of the Literature

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Mechanisms by Which Cocoa Flavanols Improve Metabolic Syndrome and Related Disorders

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License Agreement presented in Supporting Documents

Abstract

Dietary administration of cocoa flavanols may be an effective complementary strategy for alleviation or prevention of metabolic syndrome, particularly glucose intolerance. The complex flavanol composition of cocoa allows it to interact with a variety of molecules, thus allowing numerous opportunities to ameliorate metabolic diseases. These interactions likely occur primarily in the gastrointestinal tract, where native cocoa flavanol concentration is high. Flavanols may antagonize digestive enzymes and glucose transporters, causing a reduction in glucose excursion, which helps patients with metabolic disorders maintain glucose homeostasis. Unabsorbed flavanols, and ones that undergo enterohepatic recycling, will proceed to the colon where they can exert prebiotic effects on the gut microbiota. Interactions with the gut microbiota may improve gut barrier function, resulting in attenuated endotoxin absorption. Cocoa may also positively influence insulin signaling, possibly by relieving insulin-signaling pathways from oxidative stress and inflammation and/or via a heightened incretin response. The purpose of this review is to discuss the mechanisms that underlie these outcomes, critically review the current body of literature related to those mechanisms, explore the implications of these mechanisms for therapeutic utility, and identify emerging or needed areas of research that could advance our understanding of the mechanisms of action and therapeutic potential of cocoa flavanols.

1. Introduction

1.1 Metabolic Syndrome

Metabolic syndrome is a combination of conditions that increases an individual's risk for developing cardiovascular disease and type 2 diabetes mellitus (T2DM).^{2,3} The components of metabolic syndrome include abdominal obesity, dyslipidemia, elevated blood pressure, insulin resistance, glucose intolerance, β -cell loss, low-grade chronic inflammation, and a prothrombotic

state.²⁻⁴ The prevalence of obesity, cardiovascular disease, and diabetes has been increasing in the United States and worldwide for the past several decades. Approximately one in ten adults in the United States has diabetes, one in three has a cardiovascular disease, and one in three is obese.^{5,6} Many individuals with metabolic syndrome will progress to the full expression of these diseases. The prevalence of metabolic syndrome is now greater than 34% in the United States.⁷ Increasing attention has been directed towards finding novel strategies to prevent, slow the onset and/or progression of, and potentially reverse metabolic syndrome.⁸

1.2 Glucose Homeostasis

One component of the metabolic syndrome is derangement of glucose homeostasis, resulting in hyperglycemia and glucose intolerance. Glucose levels are controlled by the hormones insulin and glucagon. These two hormones are under tight regulation in order to maintain blood glucose levels between 4 and 7 mmol/L in normal individuals (glucose homeostasis).⁹

Sustaining adequate glucose levels is critical for protecting the brain, which utilizes glucose as its primary fuel source. Failure to maintain glucose homeostasis can lead to metabolic syndrome, adiposity, dyslipidemia, and glucose intolerance.¹⁰ Other problems related to high blood sugar are vision loss, kidney disease, neuropathy, atherosclerosis, and myocardial infarction.¹¹

Glucagon is a peptide hormone that increases blood glucose levels in response to hypoglycemia. Glucagon is stored in vesicles in the α -cells of the pancreas and is secreted in response to low blood glucose levels. Glucagon secretion may also be regulated by insulin secretion, fatty acids, amino acids, and the neurotransmitter gamma-aminobutyric acid (GABA).

Once released from the α cells of the pancreas, glucagon stimulates gluconeogenesis and glucose mobilization.¹²

Insulin is a peptide hormone and has powerful anabolic functions, promoting the storage of carbohydrates and fats. Insulin is produced in the β cells of the pancreas and is stored in granules until signaled for secretion. This signal comes directly from glucose in the blood stream, stimulating the granules to migrate, dock, and fuse to the cell membrane and release insulin into the circulation.⁹

Once in the blood stream, insulin promotes the uptake of glucose into the tissues of the body. Primary locations for glucose disposal are skeletal muscle, liver, and adipose tissues. Skeletal muscle and adipocytes utilize the insulin-sensitive glucose transporter 4 (GLUT4). Insulin also inhibits gluconeogenesis and promotes glycogen synthesis.⁹

When the insulin-signaling pathway becomes inhibited (in part due to inflammation²), a cyclical effect occurs where blood glucose levels remain elevated while the β cells are constantly stimulated. This causes the β cells to deteriorate and lose their ability to produce insulin, leading to “insulin-dependent” T2DM. When insulin is not secreted, the tissues cannot extract glucose from the blood stream and ketoacidosis occurs.

1.3 Endotoxemia and Inflammation

Metabolic endotoxemia can also contribute to the metabolic syndrome. Endotoxin, or lipopolysaccharide (LPS), is derived from the outer membrane of gram negative (-) bacteria and acts as a pro-inflammatory agent in the systemic circulation and in tissues. Several factors appear to modulate the concentration of LPS in the blood stream, including the gut microbial environment, high fat diet, and intestinal permeability.¹³ Low-grade, persistent inflammatory tone is observed with the metabolic syndrome, as well as obesity, and is thought to be a causative

mechanism. Circulating endotoxin induces this inflammation by binding to toll-like receptor 4 (TLR4) and initiating an inflammatory response.¹³ This persistent inflammation can disrupt energy homeostasis and the insulin-signaling pathway, leading to elevated blood glucose levels.

1.4 Flavanols and Metabolic Syndrome

Dietary flavanols offer an interesting potential complementary strategy that may improve this complex, multifaceted syndrome. First, flavanols may help reduce glucose excursion by slowing digestion and enhancing the incretin response. Second, flavanols may help reduce systemic endotoxin exposure via improvement in gut barrier function. While flavanols from a variety of dietary sources appear promising, cocoa flavanols represent an emerging approach for intervention in metabolic syndrome. Following an overview of polyphenols, this review will focus specifically on the flavanols found in cocoa. Cocoa bioavailability will be briefly reviewed, followed by a summary of the primary research utilizing cocoa and lastly, the hypothesized mechanisms by which cocoa flavanols improve metabolic syndrome will be discussed.

2. Cocoa Flavanols

2.1 Flavanols

Polyphenols are secondary metabolites found ubiquitously in plants. One prominent subclass of polyphenols is the flavonoids. The basic flavonoid skeleton consists of two benzene rings linked by a 3 carbon heterocyclic (*O*-containing) ring (Figure 1A). Flavonoids are further divided into subclasses based on the nature of the heterocyclic ring and substituents: flavanols, flavonols, flavones, flavanones, isoflavones, and anthocyanins.¹⁴ Flavanols are hydroxylated at C3 in the heterocyclic ring (Figure 1B), and are thus sometimes referred to as flavan-3-ols. This

hydroxyl group may be modified by an addition of a gallate group. Flavanols may exist as monomers, or as oligomers/polymers [with various degrees of polymerization (DP)] comprised of flavanol monomer residues (known as proanthocyanidins). Major dietary flavanol monomers include (+)-catechin (+C), (-)-catechin (-C), (-)-epicatechin (EC) (Figure 1C) and others. Cocoa is unique in that it is the only significant dietary source of -C. Procyanidins (PCs, as opposed to prodelphinidins) specifically refer to proanthocyanidins with predominantly catechin and epicatechin monomer residues.¹⁵ A representative cocoa procyanidin dimer is shown in Figure 2. Although largely beyond the scope of this review, PCs may also contain either A- or-B-type linkages.¹⁶ Cocoa, the focus of this review, contains PCs with B-type linkages.

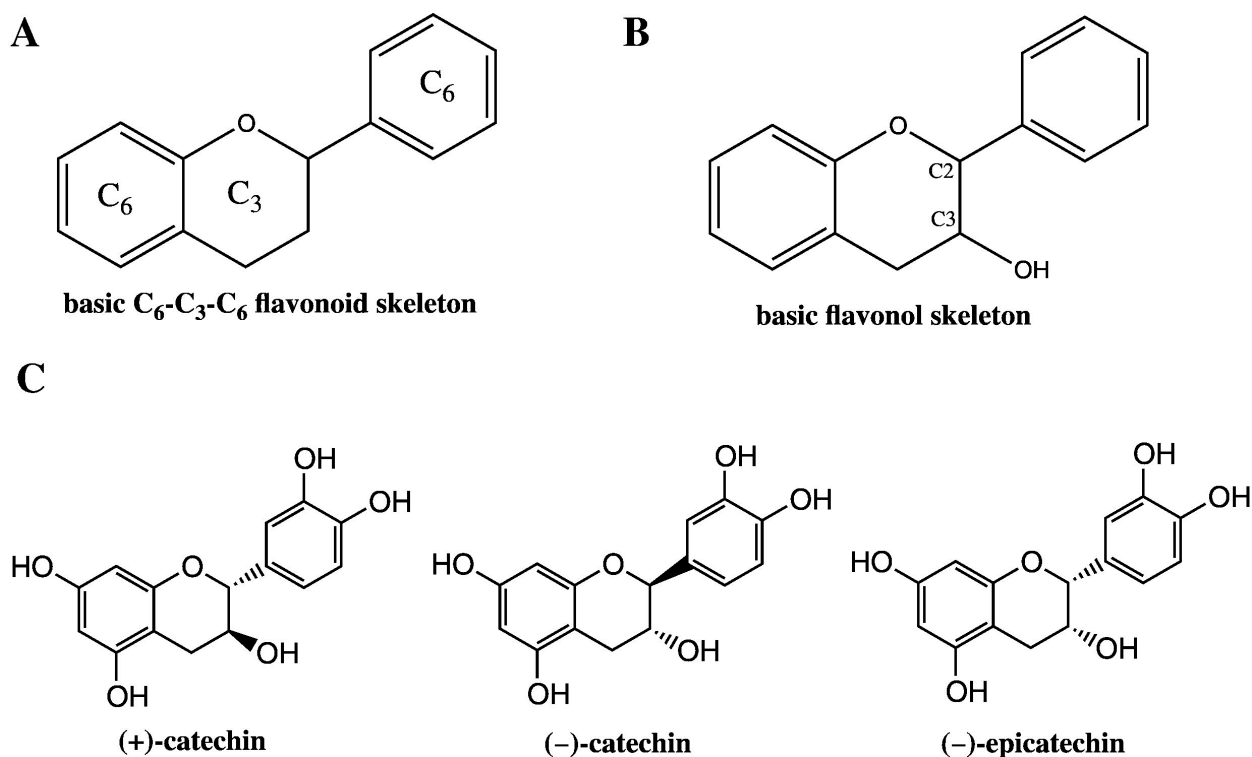
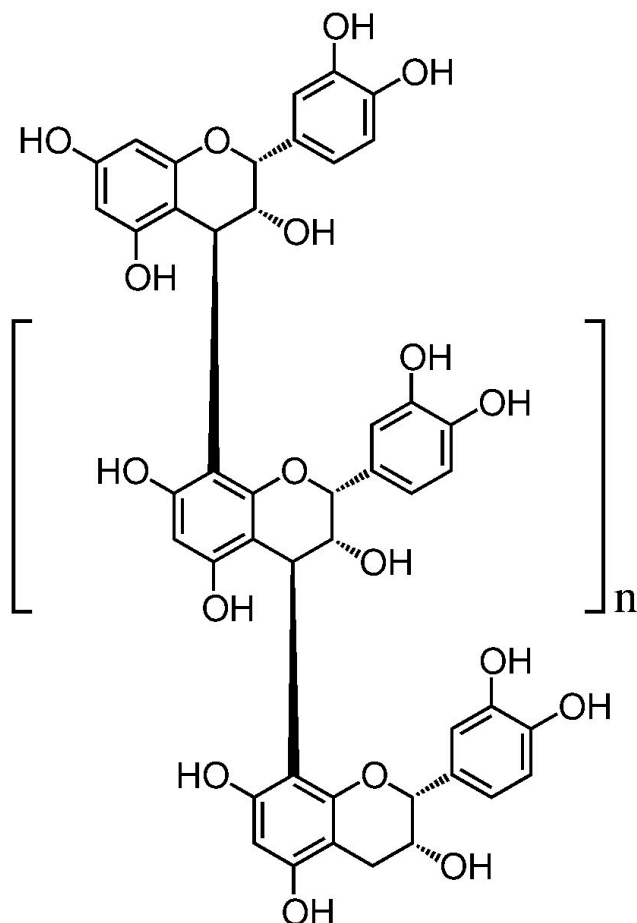


Figure 2.1. The basic 3-ring flavonoid skeleton (A), the C₃-hydroxylated flavanol skeleton (B) and structures of predominant flavanol monomers in cocoa (+) catechin, (-)-catechin and (-)-epicatechin (C).



procyanidin (PC) oligomers
[with (–)-epicatechin-(4β to 8)-(–)-epicatechin linkages]

Figure 2.2. Representative structure of cocoa B-type procyanidins.

2.2 Dietary Sources of Flavanols

Significant levels of flavanols are found in a variety of dietary plants including tea, apples, grapes, cocoa, berries, plums, apricots, and nuts.^{15,17-19} The flavanol content is higher in certain foods such as grapes, tea, and cocoa, compared to other plants, and thus the body of literature focuses on these products. Cocoa is generally regarded as the most concentrated dietary source of flavanols with the strongest antioxidant potential.^{8,20}

Although many potentially bioactive compounds are found in cocoa, many of the health benefits associated with its consumption are likely due to its high flavanol content. Cocoa is composed of flavanol monomers, oligomers, and polymers.²¹ The most common monomers found in cocoa are epicatechin (up to 35% of polyphenol content),^{22,23} as well as (±)-catechin. It is important to note that cocoa is one of the few foods with appreciable levels of (–)-catechin, which is produced by epimerization of (+)-catechin during fermentation. Cocoa contains PCs composed of up to 12 monomeric residues²⁴, although larger species likely exist but are not easily measured by common chromatographic methods. There can be great variability in cocoa phenol content from *Theobroma cacao* plants of different origins²² and the polyphenol content of cocoa powder is largely dependent on processing methods.

The impacts of tea and grape seed on metabolic syndrome have been extensively reviewed and analyzed.²⁵⁻²⁸ Furthermore, there is a large body of literature regarding the effects of cocoa on cardiovascular disease.²⁹⁻³¹ However, the potential link between cocoa and improvements to metabolic syndrome, and specifically glucose homeostasis and diabetes, is a newer, less-studied area, and warrants further investigation and a review of the current literature. Therefore, this review focuses specifically on the potential mechanisms by which cocoa flavanols improve metabolic syndrome, particularly glucose homeostasis and diabetes.

2.3 How Cocoa is Processed

The *Theobroma cacao* plant originated in South and Central America and can now be found in several African countries and Southeast Asia. The top cocoa bean producers in 2012 were the Ivory Coast, Ghana, and Indonesia, producing 36, 21, and 11% of the world's cocoa beans, respectively.³² Cocoa pods are harvested from the trees year round and a multistep processing method follows. The cocoa pods are opened (typically by a machete) to expose the

cocoa seeds and pulp. The seeds and pulp ferment for several days and then are dried. At this point, they are now called cocoa beans and are typically shipped as a raw material. The fermented and dried beans undergo a process called “winnowing” which separates the shell from the cocoa nib. The cocoa nibs are roasted and ground, creating cocoa liquor. Cocoa liquor usually contains about 55% cocoa butter. At this point, there is an optional alkalization agent added to the cocoa liquor to increase the pH of the product to improve organoleptic properties. The cocoa liquor is then hydraulically pressed, releasing liquid cocoa butter and leaving a “presscake.” The presscake is pulverized into a fine powder, producing cocoa powder that contains about 12% cocoa butter. In order to make chocolate, the cocoa powder is combined with cocoa butter and sugar in various ratios depending on the type of confection being made.^{18,33,34}

During this process, there are many variables that can affect the polyphenol content of the resulting cocoa powder or chocolate product. For example, beans from trees grown in different locations may have different polyphenols or different concentrations of polyphenols. The three varieties of cocoa trees are Criollo, Forastero, and Trinitario.^{34,35} The Forastero variety produces 95% of the world’s cocoa and is very draught and pest resistant. The Criollo variety, although not very hearty, produces very aromatic cocoa beans. The Trinitario variety is a crossbreed of the previous two and shows characteristics of both varieties. It is likely that genetics do not influence polyphenol content, but instead, the quality of the soil the trees are grown in has a greater impact on polyphenol content.³⁶

There are different methods used to process the beans and these differences can lead to unequal polyphenol content. The purpose of fermentation is to reduce the bitterness and astringency of the cocoa. Fermentation can drastically reduce the polyphenol content and alter the naturally occurring ratios of monomers to procyanidins.¹⁸ Cocoa nibs are roasted at a variety

of temperatures, and the temperature and duration of roasting can affect polyphenol content, as well.³⁴ When cocoa nibs are “Dutch” processed, an alkalizing agent is added in order to increase the pH of the cocoa to “smooth” the flavor. When this process is utilized, the polyphenols left in the chocolate product are reduced by approximately 90-95%.³⁷ As with any food product being studied, but especially with one that undergoes multiple processing steps, it is very important to report the exact composition of the product being used in each experiment.

2.4 Micronutrients and Fiber

Cocoa powder contains chemicals and nutrients other than polyphenols. Minerals observed in cocoa include calcium, copper, iron, magnesium, manganese, phosphorous, potassium, sodium, and zinc. The bioavailability of minerals found in chocolate products is reasonably high, since they can withstand the various processing steps.³⁸ Other nutrients in cocoa are methylxanthines (theobromine, theophylline, caffeine), organic acids, N-acyl ethanolamines, and sterols.^{35,37,38} It has been suggested that the health effects associated with cocoa may be a synergistic effect of all components, not just the polyphenols.³³

The methylxanthine in cocoa is predominantly theobromine and could be a prime candidate for improving health outcomes because of its stimulatory properties. Several studies investigated the effects of methylxanthine content in chocolate bars on cardiovascular outcomes.^{39,40} Most studies acknowledge that there may be synergistic effects from methylxanthine content, but overall there seems to be little to no effect on health outcomes due to methylxanthine.

Cocoa butter is often found in chocolate confections. Cocoa butter is predominantly composed of stearic acid, oleic acid, and palmitic acid.⁴¹ Cocoa butter does not have any polyphenol content, but it does have trace amounts of plant sterols that are beneficial for

cholesterol metabolism.³⁸ N-acylethanolamines, similar to anandamide, stimulate the cannabinoid system and it is hypothesized that these compounds are what make some people crave chocolate. However, the concentrations found in most cocoa powders and confections may not be enough to exert an effect.³⁴

Cocoa beans contain about 55% fat, 16% fiber, 10% protein, and 3% ash.⁴² However, cocoa powder typically contains even more fiber, approximately 40%. Hershey's unsweetened cocoa powder contains 2 g of dietary fiber in a 5 g serving. It is important to note that cocoa extract contains no fiber, and there is little fiber found in chocolate confections.

Overall, there are many chemicals other than flavan-3-ols in cocoa powder and chocolate products. It is critical to control for these compounds or at least take them into consideration when studying polyphenols in any plant product.

3. Bioavailability of Cocoa Flavanols

Understanding flavanol bioavailability is critical for identifying flavanol bioactivities.¹⁹ Bioavailability of cocoa flavanols from food is a multistep process including digestion and release of flavanol from its food matrix, solubilization and absorption into enterocytes, xenobiotic metabolism in the enterocytes, liver, and colon, and lastly, elimination.⁴³ Traditionally, bioavailability has been studied to determine if a drug will successfully reach its target and exert its desired effect. The bioavailability of phytochemicals must be conceptualized differently for several reasons. First, there are a wide variety of metabolites produced from phytochemicals and it is unclear whether or not the native phytochemical or one (or many) of its metabolites is exerting physiological effects. Second, it is unclear what targets the active

compounds are interacting with because the active site isn't necessarily well defined. For these reasons, we will use the definition provided by Neilson et al.:¹⁹

“Bioavailability is defined as the rate and extent to which the active ingredient or active moiety is absorbed from the ingested matrix and becomes available at the site of action.”

The term bio-accessibility can also be used to describe phytochemicals. If the chemical can pass through the undisturbed aqueous layer and reach the enterocytes, it is termed bio-accessible.

3.1. Digestion

Digestion occurs in the mouth, stomach, and small intestine. In order to be bio-accessible, polyphenols must first be released from any molecular interactions with the food matrix. This occurs by chewing, gastric acid, and gastric and intestinal proteases, lipases, and amylases.¹⁹ The wide variety of enzymes and the acidic environment may pose a threat to the integrity of certain molecules. However, cocoa flavan-3-ols are considered stable in both oral and gastric environments.

Potential PC instability during gastric transit has been suggested as a factor limiting bioavailability of orally administered flavanols. PCs could be hydrolyzed to form monomers (or partially hydrolyzed to form monomers and smaller PCs) in the low pH conditions of gastric juice. A study by Spencer *et al.*⁴⁴ reported that PC oligomers (up to DP 6) were degraded to monomeric flavanol residues when incubated in an acidic solution (pH ~ 2.0) for up to 3.5 h. However, there are conflicting reports on this phenomenon in both animals and humans.⁴⁵⁻⁵⁰ Tsang *et al.*⁴⁷ found that polyphenols from grape seed extract ((+)-catechin, (-)-epicatechin, procyanidins dimers, trimers, and tetramers) were in tact in the GI tract after an oral gavage in Sprague-Dawley rats. They concluded that there was neither a sizeable increase in monomers nor

a concomitant decrease in oligomers, suggesting that the oligomers were stable through gastric transit.⁴⁷

A clinical study by Rios *et al.*⁴⁵ suggested that procyanidins were intact after being ingested with a meal. After participants drank a 500 ml cocoa beverage, the pH of the stomach was elevated, keeping the cocoa powder protected from an extremely acidic environment (such as the environment utilized in the study conducted by Spencer *et al.*⁴⁴). Further, the *in vivo* study showed that the 500 ml beverage was emptied from the stomach in about 50 minutes, whereas the incubation study lasted up to 3.5 h.⁴⁵ Therefore, it appears that procyanidins, as well as monomeric flavan-3-ols, will be intact after gastric transit. There may be some depolymerization that occurs, but it is in such a low quantity that any increase in monomer concentration is negligible and gastric degradation is unlikely to limit flavanol bioavailability and bioactivity.^{15,47}

3.2. Absorption

Absorption involves solubilization of the molecule, after which it must be able to enter the undisturbed aqueous layer surrounding the cilia of the enterocytes. This is followed by either passive or facilitated diffusion into the enterocytes. Many researchers agree that cocoa flavanols of all sizes will appear in the intestines, but which will be absorbed and enter the blood stream is less apparent. Many studies utilize Caco-2 (human epithelial colorectal adenocarcinoma) cell culture models to study the effects of cocoa on absorption into the enterocytes. This model has proven to be an acceptable alternative for animal studies for the assessment of intestinal absorption.⁵¹

Polyphenols may enter the enterocytes through passive or facilitated diffusion by way of a monocarboxylic transporter (MCT). Polyphenols with low molecular weights may enter the

blood stream via paracellular diffusion, as well. Flavan-3-ols are highly hydroxylated, and therefore lipophobic, so they likely utilize facilitated diffusion to enter the enterocytes.¹⁹

The monomers, catechin and epicatechin, are relatively well compared to PCs.^{45,52,53} They first appear in the blood stream 30-60 min after ingestion³⁷ and reach peak plasma concentration at 2-3 h.⁴⁵ Epicatechin has been found in greater concentrations in human plasma than catechin. A study conducted by Holt *et al.*⁵⁴ found that there is a preferential absorption of epicatechin. Equal amounts of catechin and epicatechin were given to participants, and at 2 hours, there was 5.92 $\mu\text{mol/L}$ of epicatechin but only 0.16 $\mu\text{mol/L}$ of catechin found in the plasma.⁵⁴ Furthermore, the (+)-catechin is more bioavailable than (-)-catechin, which predominates in fermented cocoa.⁵⁵

Dimers, trimers, and tetramers are absorbed in their intact form, but at a much lower rate than monomers.¹⁵ Interestingly, Deprez *et al.*⁵⁶ showed that (+)-catechin and PC dimers and trimers had similar permeability coefficients as mannitol, an indicator of paracellular transport, in Caco-2 monolayers. Therefore, these smaller flavanols are likely entering the bloodstream via paracellular diffusion.^{51,56} Polymers larger than tetramers are generally not absorbed intact¹⁵ and proceed to the colon. Approximately 5-10% of polyphenols can be absorbed or broken down in the small intestine while the remaining 90-95% proceed to the colon.⁵⁷ Poor PC bioavailability therefore is likely a main factor that limits bioactivity in peripheral tissues, particularly for larger PCs. Their relatively low bioavailability indicates that the gut may be the primary location of action for cocoa PCs due to the high concentrations present there compared to levels in circulation.^{15,58} Concentrations of flavanols in the blood and tissues are typically less than 5 μM ,⁵⁹⁻⁶² which are at the lower end of concentrations typically used *in vitro* to assess bioactivity in cell models.⁶³ However, when the intestinal lumen or epithelial surface is the site of action

(such as inhibition of digestive enzymes or absorption transporters, modulation of gut barrier integrity, etc.), bioavailability is not a limiting factor.

3.3. Xenobiotic Metabolism

If a polyphenol enters an enterocyte via facilitated diffusion, or if it enters the blood stream via paracellular diffusion and travels to the liver, it is recognized as a foreign substance and will undergo xenobiotic metabolism, a process designed to eliminate potentially harmful substances from the body. This detoxification process has multiple steps, termed phase I (modification), phase II (conjugation) and phase III (excretion).

Phase I metabolism consists of oxidation and hydrolysis reactions. Oxidizing the compound prepares it for subsequent conjugation reactions. Flavan-3-ols typically do not undergo phase I metabolism since they are well oxidized and already have many hydroxyl groups.

Phase II metabolism includes conjugation reactions including sulfation, glucuronidation, and methylation. These reactions occur in the enterocytes and hepatocytes and flavan-3-ols can undergo multiple phase II reactions. Glucuronidated, sulfated, and methylated (epi) catechin has been observed in human blood and urine.^{64,65} There are more methylated conjugates than non-methylated conjugates found in plasma.⁶⁶

Phase III involves the efflux and elimination of the conjugates, which are released from the enterocytes or hepatocytes by transport proteins into the blood stream or back into the gastrointestinal tract (enterohepatic recycling⁴³). Flavan-3-ols have high affinity for efflux transporters including multidrug resistance-associated protein 1 (MRP1),⁶⁷ MRP2, and P-glycoprotein (P-gp).⁴³ This limits their ability to enter the blood stream because they are rapidly effluxed back into the GI tract.^{15,68}

3.4. Colon, Microbiome, and Excretion

Polyphenols are degraded in the colon by the gut microbiota and some of the resulting metabolites can then be absorbed into the blood stream. The conversion of (+)-catechin to (+)-epicatechin is a prerequisite step for microbial metabolism.⁶⁹ These monomers are typically metabolized to form 5-(3',4'-dihydroxyphenyl)- γ -valerolactone, 5-phenyl- γ -valerolactone and phenylpropionic acid.⁶⁹ The majority of cocoa PCs are degraded into many metabolites, including phenolic acids and phenylvalerolactones,^{15,70} and possibly others that have not been identified. As PCs increase in size, the ability of bacteria to metabolize them decreases.⁷¹ Gonthier *et al.*⁷² found that the yield of phenolic acids from monomers and dimers (10% and 7%) was much greater than those from trimers and polymers (0.7% and 0.5%). Bioavailability of large polymers is drastically decreased compared to monomers.⁷¹

It is important to acknowledge the microbial metabolites of polyphenols when considering the health effects of these compounds.^{57,73} Microbial metabolites of flavanols should be considered as potential contributors to the health effects of these compounds observed following oral administration,^{57,74} as they are extensively produced and comparatively more bioavailable^{75,76} than the native compounds themselves (particularly the PCs).^{74,75} Despite general recognition that these microbial metabolites are likely to contribute extensively to the activities observed during consumption of flavanols (and polyphenols in general),⁷⁷⁻⁷⁹ very little is known about the bioactivities of these compounds. In terms of glucose homeostasis, Fernandez-Millán *et al.*⁸⁰ showed that 3,4-dihydroxyphenylacetic acid, 2,3-dihydroxybenzoic acid and 3-hydroxyphenylpropionic acid potentially improve glucose-stimulated insulin secretion and resistance to oxidative stress in β -cells and rat islets. Carrasco-Pozo *et al.*⁸¹ recently demonstrated that 3,4 dihydroxyphenylacetic acid protected β -cells against impaired insulin

secretion, mitochondrial dysfunction and increased apoptosis induced by cholesterol. These metabolites are also known to have anti-inflammatory effects.^{74,82} Therefore, these microbial metabolites appear to have significant activities related to improving glucose homeostasis, but only a few of the dozens of compounds have been investigated, and the impact of these metabolites in most tissues critical to glucose homeostasis remain unstudied. Little to no published data exists regarding the potential impacts of these metabolites on skeletal muscle, adipose tissue, or liver physiology and metabolism. *In vitro* tissue culture experiments are needed in order to determine the impacts of microbial metabolites on pathways related to glucose homeostasis in these tissues. The activities of microbial metabolites may represent major mechanisms by which orally consumed cocoa flavanols exert their activities. Of all the mechanisms described in this review, this is the least investigated area and the area in which relevant data are most urgently needed. Therefore, it is possible that the potential activities of microbial metabolites are the area in which the greatest advances in knowledge stand to be gained.

Overall, absorption of flavanol-3-ols is likely to occur via paracellular or facilitated diffusion. Concentrations of flavanols in the blood and tissues are typically less than 0.005mM and may not be potent enough to be active.⁸³ However, metabolites of polyphenols can enter the circulation and it may be the metabolites that are exerting protective effects. Further studies are needed to determine the active targets for these metabolites.

4. Animal And Clinical Studies

4.1 Animal Studies

Many animal studies have been conducted to examine whether cocoa may reduce circulating endotoxin, oxidative stress and inflammation, and thus improve glucose control and other outcomes related to metabolic syndrome. These studies are summarized in **Table 2.1**. The studies listed are mostly chronic studies, lasting anywhere from one to 18 weeks,^{84,85} and there were only two acute studies.^{86,87} Many rodent models mimicking diabetes or pre-diabetes were utilized and many of the studies utilized high fat diets. Many of these studies reported improvements in glucose related outcomes (fasting glucose levels as well as glucose tolerance),^{23,86,88-94} while three studies reported no changes.^{85,95-97} One study reported changes in gut microbiome⁹⁸ and two studies reported attenuated endotoxin levels.^{23,85}

When evaluating these studies, it is important to note the experimental procedures by which cocoa was given to the animals. There was a wide range of doses used as well as a variety of dosing methods (discussed in more detail below). The dosing method may impact the mechanisms by which cocoa flavanols act *in vivo*. Some cocoa was available *ad libitum* by adding it into the chow or the drinking water. In this case, flavanols were co-consumed with macronutrients, thereby facilitating flavanol-mediated alteration of nutrient digestion. This cocoa supplement was often reported as a percentage of food (w/w) or water (w/v). Other studies supplemented the cocoa by means of an oral gavage, and these doses were often reported as a dose in mg/kg body weight. Oral gavage is often done in the fasted state, in which case flavanols would not be co-consumed with macronutrients, thereby precluding the opportunity for flavanol-mediated alteration of nutrient digestion. While each procedure had its advantages, it is important to note the differences between the two. When cocoa was provided *ad libitum*, the dose was dependent on food intake, which was sometimes not reported. Cocoa is extremely bitter, and high percentages of cocoa may have been unpalatable and therefore led to a reduced

food intake, possibly contributing to the observed positive outcomes. This is a potential mechanism of action in animal studies that is not likely translatable to humans. Further, studies comparing high-fat diets to normal diets (each with cocoa supplements)⁹¹ had significantly different food intakes, meaning different doses of cocoa were being ingested. Studies comparing normal animals to diabetic animals^{88,89} also had the same dilemma. In studies using diabetic rats, there were also significant differences in food intake, where the diabetic animals ate more and therefore were consuming more polyphenols.

Another aspect of study design to consider when evaluating the effective dose and potentially bioactive constituents of cocoa is the different types of cocoa product utilized. Animal studies have used cocoa liquor (liquefied cocoa mass), chocolate (cocoa liquor + sugar and possibly other ingredients), cocoa powder (cocoa liquor with most of the cocoa butter removed), cocoa extracts (prepared by distinct extraction procedures, containing various profiles of phenolic acids, flavanols, etc.), and pure compounds (catechins, epicatechin, etc.). These all have different amounts of fiber, lipids, carbohydrates, and polyphenols, all of which may possess beneficial activities that may have synergistic, or even antagonistic, effects with flavanols. While the majority of studies show efficacy, these confounding components make interpretation of the effective dose problematic. These non-flavanol components may act by mechanisms distinct from the flavanols. On the other hand, purified compounds alone are not representative of the complexity of cocoa products. While most studies show some efficacy of these various cocoa products, studies are still needed to isolate the activities of individual components. For example, the effect of flavanol vs. non-flavanol components could be elucidated by comparing the impact of cocoa vs. an equivalent dose of heavily Dutched cocoa. When evaluating the potential translational benefits to humans, it should be understood that humans generally consume

chocolate, cocoa powder, and cocoa liquor (in solid form), and generally do not consume cocoa extracts or pure compounds (although cocoa extracts or products with added cocoa extracts can be obtained in supplement form).

In summary, animal studies of the impacts of cocoa, chocolate, cocoa extracts or cocoa monomers on metabolic syndrome have been highly descriptive. These studies have suggested potential mechanisms, but do not definitively isolate or interrogate the proposed mechanisms.

Table 2.1. Animal studies related to the effects of dietary cocoa or cocoa flavanols on metabolic outcomes. Human equivalent doses were calculated by the equation provided by Reagan-Shaw et al.⁹⁹ using food intake and body weight data, if provided. Assumptions made for calculation are indicated in the footnotes.

Author, Year	Animal Model	Treatment/Delivery	Animal Dose (mg/kg body weight)	Human Equivalent Dose ^a (mg/day)	Acute/Chronic Design, Diet	Cocoa Treatment Outcomes
Matsui, 2005 ¹⁰⁰	Male Wistar rats	12.5 % (w/w) cocoa powder, in food	7,040 ^b	79,913 ^c	Chronic, 3 weeks, high fat diet	↓ final body weights, ↓ fatty acid synthesis
Ruzaidi, 2005 ⁸⁸	Male diabetic Wistar rats, (STZ ^d induced)	1, 2, 3% (w/w) cocoa extract ^e , in food	Diabetic rats ^f : 1% = 868 2% = 1,776 3% = 2580 Normal Rats: 1% = 433 2% = 860 3% = 1,200	Diabetic rats: 1% = 9,853 2% = 20,160 3% = 29,286 Normal rats: 1% = 4,919 2% = 9,762 3% = 13,622	Chronic, 4 weeks, normal diet	↓ glycemia, ↓ hypercholesteremia
Tomaru, 2007 ⁸⁹	Female, <i>db/db</i> mice (obese, diabetic)	0.5%, 1.0% (w/w) cacao liquor proanthocyanidin, in food	0.5% = 1,107 ^g 1.0% = 2,044	0.5% = 5,771 1.0% = 11,602	Chronic, 3 weeks, normal diet	↓ blood glucose in a dose dependent manner
Jalil, 2008 ¹⁰¹	Male <i>ob/db</i> Sprague-Dawley rats (STZ induced)	Cocoa extract, by oral gavage	600	6,811	Chronic, 4 weeks, high fat diet	↓ oxidative stress (8-isoprostane)
Jalil, 2009 ⁹⁰	Male <i>ob/db</i> Sprague-Dawley rats (STZ induced)	Cocoa extract, by oral gavage	600	6,811	Chronic, 4 weeks, high fat diet.	↑ glucose tolerance (OGTT- AUC), ↓ total cholesterol, ↓ triglycerides. No changes in insulin sensitivity
Perez-Berezo, 2011 ¹⁰²	Female Wistar rats	2%, 5%, or 10% (w/w) cocoa powder, in food	Unknown (food intake data not reported).	Unknown (food intake data not reported).	Chronic, 3 weeks, normal diet	↓ immune response (IgG1, IgG2, S-IgA) (5 and 10% treatments)
Si, 2011 ⁹⁵	Male <i>db/db</i> mice	0.25% epicatechin, in drinking water	150	851	Chronic, 15 weeks, normal diet.	↓ inflammatory markers (CRP, IL1B), ↓ oxidative stress (GSH, SOD), ↑ lifespan. no change in glycemia

Massot-Cladera, 2012 ⁹⁸	Female Wistar rats	10% (w/w) cocoa powder ^h , in food	Unknown (food intake data not reported).	Unknown (food intake data not reported).	Chronic, 6 weeks, normal diet.	Altered gut microbiome (↓ Bacteroides, Staphylococcus, Clostridium)
Yamashita, 2012 ¹⁰³	Male C57BL/6 mice	0.5, 2.0% (w/w) cacao liquor procyanidins ⁱ , in food	Normal diet ^l : 0.5% = 588 2.0% = 2,344 High fat diet: 0.5% = 310 2.0% = 1,532	Normal diet: 0.5% = 3,337 2.0% = 13,304 High fat diet: 0.5% = 1,759 2.0% = 8,695	Chronic, 13 weeks, control or high fat diet	↓ fasting glucose (2.0% treatment); ↑ glucose tolerance (OGTT AUC). ↑ translocation of GLUT4, AMPK phosphorylation, UCP expression
Yamashita, 2012 ⁸⁴	Male C57BL/6 mice	0.5%, 1% (w/w) cacao liquor procyanidins, in food	Unknown (food intake data not reported).	Unknown (food intake data not reported).	Chronic, 1 week, normal diet	↑ glucose tolerance in a dose dependent manner (OGTT-AUC)
Yamashita, 2012 ⁸⁴	Male ICR ^k mice	Cocoa liquor procyanidins, by oral gavage	50 or 250	283 or 1,418	Acute	↑ glucose tolerance (OGTT-AUC) (250 mg/kg dose)
de Oliveira, 2013 ⁹⁶	Male Wistar STZ-induced diabetic rats	Cocoa liquor ^l , by oral gavage	3,600 or 7,200	1,157 or 2,317	Chronic, 40 days, normal diet	↑ antioxidant capacity (ORAC, FRAP), no change in blood glucose levels
Yamashita, 2013 ⁸⁶	Male ICR mice	Procyanidins, by oral gavage	0.01	0.06	Acute	↑ plasma insulin; ↑ GLP-1 levels
Dorenko tt, 2014 ²³	Male C57L/l6 mice	Monomeric, oligomeric, and polymeric cocoa extract fractions, in food	25	142	Chronic, 12 weeks, high fat diet	Oligomeric fraction ↓ fasting blood glucose, ↑ glucose tolerance; ↑ insulin tolerance (OGTT); ↓ endotoxin
Gu, 2014 ⁹⁷	High fat-fed obese male C57BL/6J mice	8% (w/w) cocoa powder, in food	11,828 ^m	67,135	Chronic, 10 weeks, high fat diet	↓ weight gain, ↑ fecal lipid content, ↑ insulin sensitivity (HOMA-IR), ↓ inflammatory markers (IL-6, MCP-1), no change in blood glucose

Gu, 2014 ⁸⁵	Male C57BL/6J mice	8% (w/w) cocoa powder, in food	4,998 ⁿ	28,367	Chronic, 18 weeks, high fat diet	↓ inflammation (adipose tissue NF-κB expression), ↑ insulin sensitivity (HOMA-IR), ↑ gut barrier function (plasma GLP-2), ↓ plasma endotoxin
Gutierrez-Salmean, 2014 ¹⁰⁴	High fat-fed, obese, male Wistar rats	(-)-epicatechin, by gavage	1	11	Chronic, 2 weeks, low fat or high fat diet	↓ blood glucose, ↓ triglyceride levels, ↑ mitochondrial function (TFAM, mitofilin expression)
Gutierrez-Salmean, 2014 ⁹⁴	Male Wistar rats	(-)-epicatechin, by gavage	1	11	Chronic, 2 weeks, low fat or high fat diet	↓ fasting glucose ; ↑ glucose tolerance.
Matsumura, 2014 ⁸⁷	Male ICR mice	Flavanol fraction or (-)-epicatechin, by gavage	10	57	Acute	Flavanol fraction ↑ energy expenditure (REE), ↑ blood catecholamines
Osakabe, 2014 ⁴	Male Wistar rats	0.2% (w/w) flavanols, in food	78	890	Chronic, 4 weeks, high fat diet	↓ thermogenesis, ↓ lipolysis
Papadimitrou, 2014 ¹⁰⁵	Male SHR ^o rats, diabetic (STZ induced)	Cocoa powder, by gavage	24	272	Chronic, 16 weeks, normal diet	AMPK, ↓ NOX4 signaling
Watanabe, 2014 ⁹²	Male C57BL/J mice	Cocoa flavanols ^p by gavage	50	284	Chronic, 2 weeks, normal diet	↓ plasma glucose. ↓ resting energy requirements, mitogenesis
Fernandez-Millan, 2015 ⁹³	Male Zucker diabetic fatty rat	10% (w/w) cocoa powder, in food	8,311 ^q	94,345	Chronic, 9 weeks, normal diet	Prevented B cell mass loss, ↑ glucose tolerance (OGTT) insulin sensitivity (HOMA-IR), ↑ β cell function (HOMA-B), ↓ oxidative stress (carbonyl groups, TBARs)

^aBased on a 70 kg human.

^bUsed reported final body weight to calculate animal and human equivalent doses.

^cAuthor reported 50,000 mg/70 kg/day human equivalent dose.

^dStreptozotocin.

^e285.6 mg polyphenols/g extract.

^fAssumed body weights of rats were 0.30 kg for normal rats and 0.25 kg for diabetic rats, based on reported body weights, to calculate animal and

human equivalent doses.

^gAverage food intake and body weights during weeks 4-6 were used to calculate animal and human equivalent doses.

^hCocoa powder contains 10.62 mg/g polyphenols.

ⁱCocoa liquor procyanidin contained 69.8% polyphenols.

^jBased on body weights at the end of the experiment and total food intake averaged over the entire experiment.

^kInstitute of Cancer Research/Imprinting Control Region mouse.

^lTotal phenolics 2,845 mg/100 g dry weight.

^mBased on average weight at the start of the experiment (0.020 kg mouse) and does not account for weight gained during the experiment, since final weights not provided (only displayed in graph).

ⁿBased on average final weights (0.0471 kg mouse).

^oSpontaneously hypertensive rat.

^pFlavanol fraction was 72.4% w/w total polyphenols.

^qBased on final weight (0.2335 kg rat) and average food intake (19 g food/day) over 10 weeks.

4.2 Clinical Studies

There have been a variety of clinical trials assessing the effects of habitual cocoa intake on glycemic and insulinemic outcomes. These are summarized in **Table 2.2**. Many of the studies found cocoa to be beneficial for glucose control.¹⁰⁶⁻¹¹² Cocoa treatments were often provided in the form of chocolate bars¹⁰⁷⁻¹¹¹ or beverages.¹¹²⁻¹¹⁶ Chronic studies lasted from five days to three months^{112,115,116} but most lasted about two weeks.

In select studies, cocoa and cocoa flavanols improved insulin sensitivity and reduced blood glucose, insulin, and HbA1c in subjects with varying degrees of glucose homeostasis (normoglycemic, prediabetic, or T2DM) within 2-4 weeks.^{107-112,114,117} However, other studies showed no effect of cocoa on these outcomes.^{113,115,118,119} Despite its promising effects *in vitro* and in animal models, only five chronic studies of cocoa and glucose control have been performed in subjects with prediabetes or diabetes,^{107,109,110,115,118} as many of the studies were focused on cardiovascular or cardio-metabolic outcomes.^{107-110,116} In terms of diabetes biomarkers, most of these studies focused on insulin resistance/sensitivity; few focused on overall blood glucose control,^{110,118} which is a critical clinical outcome. Furthermore, only two of these five studies in pre-diabetic or diabetic subjects studies lasted >15 d.^{110,118} Neither of these two longer studies examined pre-diabetes (both used subjects with existing T1/2DM).^{110,118} Therefore, the potential for cocoa to improve long-term glucose control has not been sufficiently studied. Additional studies lasting 1-3 months (or potentially longer) are needed. Furthermore, the potential impact of cocoa in individuals with pre-diabetes has not yet been evaluated. Clinical trials in individuals with pre-diabetes are thus needed in order to determine the potential utility of cocoa for improvement of long-term blood glucose control and prevention of T2DM in this population, where early prevention may significantly reduce or delay progression to T2DM.

Furthermore, additional studies are needed in individuals with T2DM in order to evaluate the potential for cocoa to ameliorate T2DM and slow progression to β -cell exhaustion and failure.

Interestingly, no significant glycemic improvements were observed in the two studies that utilized epicatechin only.^{116,118} This supports the idea that the larger PCs may be important, despite their relatively low bioavailability.²³ However, these studies^{116,118} only examined patients with pre-diabetes or T2DM, so health status may be an important mediator for interventions with epicatechin; these interventions may be more effective in healthier individuals.

Overall, the existing clinical trials support the premise that cocoa can improve glycemic outcomes in healthy, overweight, or hypertensive adults. While many of these findings seem promising, these studies do not provide insight into the mechanisms responsible.

Furthermore, many of these studies were related to cardiovascular disease (generally hypertension), not glucose homeostasis/diabetes. Further, as stated above, there have been no long-term studies examining the effects of cocoa consumption in an at-risk (pre-diabetic) population, and only 5 studies in individuals with diabetes (4 examined T2DM, while one study did not specify whether subjects were diagnosed with T1 or T2DM).¹¹⁰ Additional studies of the mechanisms specifically related to glucose homeostasis in these populations are greatly needed moving forward.

As with reported animal studies, human clinical studies of cocoa or chocolate have been largely descriptive. While it is considerably more difficult to perform elegant mechanistic studies in humans due to feasibility or ethical concerns, opportunities to move towards mechanistic studies in humans will be discussed later in this review.

Table 2.2. Clinical trials assessing the effect of dietary cocoa on metabolic outcomes, in chronological order.

Author, year	Subjects	Health Status	Treatment (daily dose)	Acute/Chronic (duration)	Outcomes
Nguyen, 1994 ¹²⁰	N = 10	Healthy	100 g chocolate bar, (45 g cocoa)	Acute	Lesser but prolonged increase in glucose and insulin.
Brand Miller, 2003 ¹⁰⁶	N = 10	Healthy	6 food pairs, one flavored with cocoa ^a	Acute	↑ insulin response (insulin index) but not glycemic differences with chocolate flavored products.
Basu 2015 ¹¹⁷	N=14	Obese, type 2 diabetic	Cocoa beverage (960 mg polyphenols, 480 mg flavanols ^b)	Acute	↑ postprandial insulin secretion , no improvements in blood glucose or insulin resistance (except 4 h post-meal)
Grassi, 2005 ¹⁰⁷	N = 15	Healthy	100 g chocolate bar, (500 mg polyphenols)	Chronic (15 days), crossover design	↑ insulin sensitivity (HOMA-IR, QUICKI), ↑ glucose tolerance (OGTT).
Grassi, 2005 ¹⁰⁸	N = 20	Hypertensive	100 g chocolate bar, (88 mg flavanols ^c)	Chronic (15 days), crossover design	↑ insulin sensitivity (HOMA-IR, QUICKI, ISI).
Muniyappa, 2008 ¹¹³	N = 20	Hypertensive	150 ml beverage, 2x/day. (900 mg flavanols ^d)	Chronic (2 weeks), crossover design	No effects on insulin sensitivity (QUICKI and clamp).
Grassi, 2008 ¹⁰⁹	N = 19	Hypertensive, impaired glucose tolerance	100 g chocolate bar, (1008 mg phenols)	Chronic (15 days), crossover design	↑ insulin sensitivity (HOMA-IR, QUICKI, SI), ↑ β cell function .
Davison, 2008 ¹¹²	N = 49	Overweight and obese (BMI > 25 kg/m ²)	150 ml cocoa beverage (2x/day), high flavanol (902 mg) and low flavanol (36 mg)	Chronic (12 weeks), randomized arm	↑ insulin sensitivity (HOMA2-IR) at 6 and 12 weeks.
Mellor, 2010 ¹¹⁸	N = 12	Type 2 diabetic	45 g chocolate (3 bars/day), (16.6 mg epicatechin ^e)	Chronic (8 weeks), crossover design	No change in glycemic control (HOMA-IR, HbA1c, fasting glucose). ↑ HDL cholesterol .

Almoosawi, 2012 ¹¹¹	N = 42	Healthy (BMI < 25 kg/m ²) compared to overweight (BMI > 25 kg/m ²)	20 g dark chocolate, (500 mg polyphenols)	Chronic (4 weeks), crossover design	Treatment prevented unfavorable changes in insulin sensitivity (QUICKI, HOMA-IR) seen in the placebo treatment.
Desideri, 2012 ¹¹⁴	N = 90	Mild cognitive impairment	Cocoa beverage, (990 mg, 520 mg, or 45 mg flavanols).	Chronic (8 weeks), randomized arm	High flavanol and intermediate flavanol treatments ↓ fasting glucose , ↑ insulin sensitivity (HOMA-IR) but not fasting insulin compared to the low flavanol group.
Stote, 2012 ¹¹⁵	N = 19	Adults at risk for insulin resistance	Cocoa beverage (2x/day), (30, 180, 400, or 900 mg flavanols)	Chronic (5 days), crossover design	No effects on glycemia (OGTT) or insulinemia (HOMA, QUICKI, ISI)
Stellingwerff, 2013 ¹¹⁹	N = 16	Trained cyclists	Dark chocolate, (240 mg polyphenols ^f)	Acute, crossover design	↑ Blood glucose and ↑ insulin
Haghighat, 2013 ¹¹⁰ (abstract only)	N = 69	Hypertensive diabetic adults	25 g dark chocolate, (450 mg polyphenols)	Chronic (8 weeks), randomized arm	↓ Fasting glucose , ↓ HbA1c
Ramirez-Sanchez, 2013 ¹¹⁶	N = 5	T2D/stage II and stage III heart failure patients (compared with healthy controls)	18 g cocoa powder in a beverage (2x/day), (100 mg epicatechin)	Chronic (3 months), parallel arm	No effects on glycemia/insulinemia . ↓ oxidative stress in mitochondria.

^aFoods used include Coco Pops (Kellogg's cereal), Betty Crocker chocolate fudge super moist cake and creamy deluxe Dark fudge frosting, Plain chocolate block (classic full cream milk chocolate from Nestle), Ultra chocolate classic ice cream from Sara Lee, and chocolate instant pudding (White Wings Foods).

^bPlacebo contained 110 mg polyphenols, <0.1 mg flavanols.

^cConsists of the flavanols: catechin, epicatechin, quercetin, kaempferol, and isorhamnetin.

^dPlacebo contained 14 mg flavanols.

^ePlacebo contained < 2 mg epicatechin.

^fPolyphenols included epicatechin, catechin, procyanidin B2, procyanidin B5, trimer C, and tetramer D

5. Potential Mechanisms of Action

There are numerous potential primary molecular mechanisms by which cocoa flavanols appear to prevent or ameliorate metabolic syndrome. It is critical to clearly define “primary molecular mechanism of action” and differentiate it from downstream effects. The primary molecular mechanism of action is the initial biological effect caused directly by the bioactive compound of interest and may then have numerous downstream consequences in various pathways. Most research on cocoa flavanols and other dietary bioactive compounds in animals or humans (including studies from our lab^{23,121}) has been primarily “descriptive” in nature: a compound or food is administered, and biomarkers or outcomes are observed. These descriptive studies demonstrate the effects of the intervention and suggest, but do not definitively identify, primary molecular mechanisms of action by which these effects are achieved.¹²² Such studies are extremely valuable for hypothesis generation regarding the primary molecular mechanism of action. However, mechanism-oriented research (beyond measuring biomarkers of disease) is needed to isolate and identify the primary molecular mechanisms of action.¹²³⁻¹²⁷ Studies that isolate and probe specific molecular interactions and biological pathways (such as knockout or “knock-in” mouse models, use of receptor agonists/antagonists, use of pathway inhibitors in cell assays, gene silencing by siRNA, etc.) are needed to elucidate primary molecular mechanisms.

Numerous studies have demonstrated the positive effects of cocoa and cocoa flavanols, including improved glucose homeostasis, body composition, etc. However, the key initial events in the cascades of biological processes regulating these outcomes remain to be identified. Several possibilities include: inhibition of digestive enzymes, inhibition of glucose transporters, reduced metabolic endotoxemia, and stimulation of the incretin response. In all probability, flavanols act

through various mechanisms simultaneously. The most well studied and promising potential mechanisms and their implications will be reviewed here.

5.1 Carbohydrate Digestion

Perhaps the most direct mechanism by which flavanols may improve glucose homeostasis is by slowing carbohydrate digestion and absorption in the gut. Several mechanistic possibilities include inhibiting digestive enzymes, inhibiting glucose transporters, and stimulating an incretin response. These mechanisms are likely a result of a single, or acute, dose of cocoa. Possible mechanisms are illustrated in **Figure 2.3**.

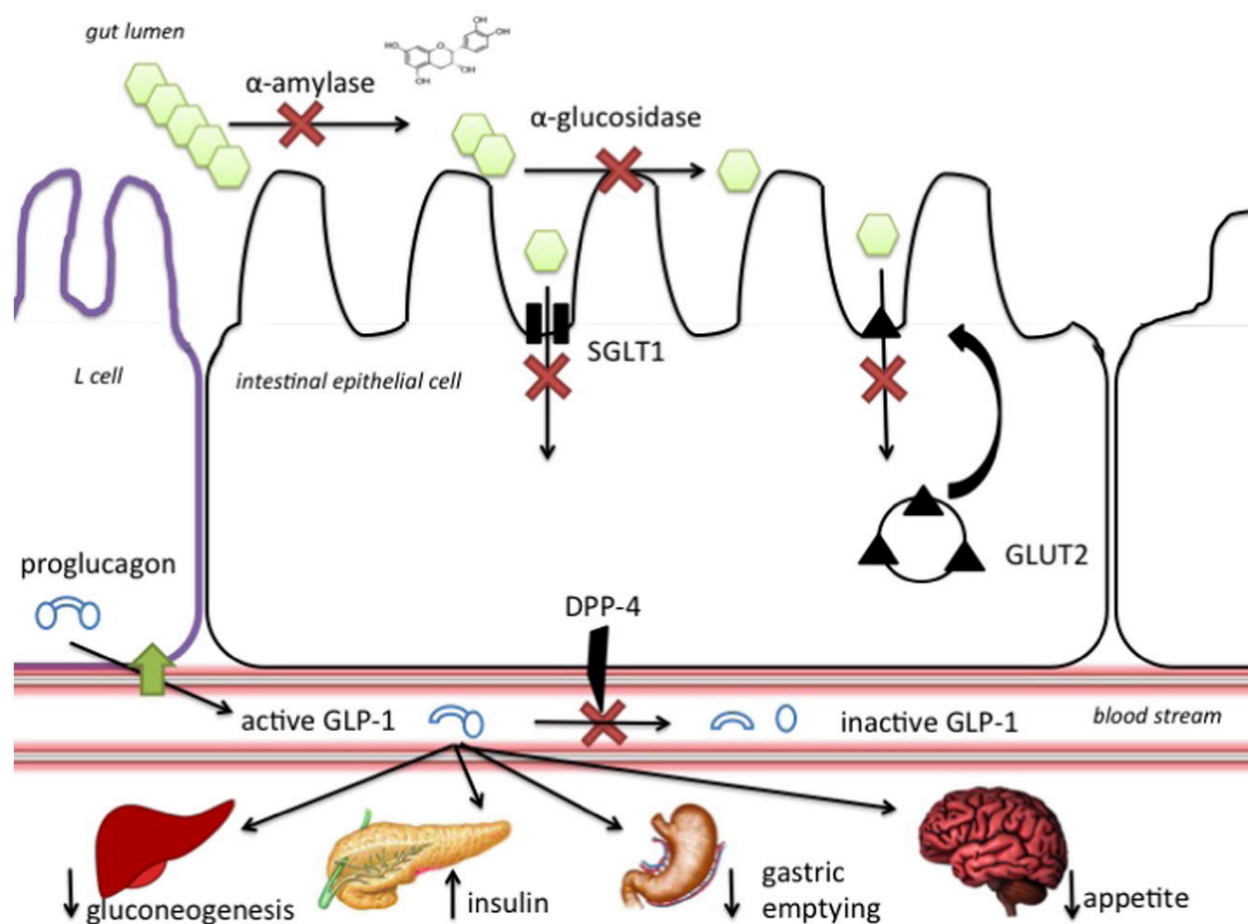


Figure 2.3. Hypothetical mechanisms by which cocoa flavanols may affect carbohydrate digestion. Mechanisms include inhibiting digestive enzymes α -amylase and α -glucosidase, inhibiting glucose transporters SGLT1 and GLUT2, promoting GLP-1 secretion and inhibiting DPP-4.

5.1a Inhibition of Digestive Enzymes

Cocoa can slow the rate and extent of macronutrient digestion by non-covalently binding to and antagonizing digestive enzymes. The complex ring structure with abundant hydroxyl groups allows cocoa to bind to proteins, particularly digestive enzymes. Cocoa flavanols interact with digestive enzymes by a variety of primary inhibition mechanisms.¹²⁸

Cocoa may inhibit α -amylase,¹²⁹ an enzyme that breaks down starch into glucose oligomers. There is evidence to suggest that polyphenols bind to this enzyme, reducing its activity.¹³⁰ Yilmazer-Musa *et al.*¹³⁰ found that grape seed extract (GSE) (including catechin, epicatechin, and PCs) with 86% total phenolics by weight was just as efficient as the drug acarbose at inhibiting α -amylase. Acarbose, the positive control, had a median inhibitory concentration (IC₅₀) of 6.9 μ g/ml compared to GSE with an IC₅₀ of 8.7 μ g/ml. On the other hand, white tea, which contains predominantly monomeric flavanols and only 34% total phenolics by weight, had an IC₅₀ of 378 μ g/ml. While total flavanol concentration plays a role in the observed IC₅₀ values, it also appears that the more complex the structure, the greater its ability to inhibit digestive enzymes. Thus, flavanols may reduce digestion of starches, thereby lowering glucose absorption via inhibiting this enzyme in the diabetic population. Interestingly, α -amylase expression is higher in individuals with T2DM than healthy individuals.^{63,131}

Glucosidase inhibitors are well studied and commercially available, but unwanted side effects such as diarrhea, gas, and cramping have been reported for these drugs.^{130,132} Acarbose is one such synthetic glucosidase inhibitor. Acarbose has reportedly been effective in reducing weight gain and comorbidities related to metabolic syndrome, such as diabetes and cardiovascular disease.⁶³ Flavanols may also inhibit α -glucosidase, which cleaves small oligosaccharides at the 1,4 linked alpha glucose residues, resulting in monomeric sugars that are

ready for absorption. This is another key enzyme involved in carbohydrate digestion. When these enzymes are inhibited, the breakdown of carbohydrates is slowed, resulting in an attenuated elevation of blood glucose after a meal.¹³³ Yamashita *et al.*⁸⁴ found that a 0.01% cocoa liquor procyanidin extract inhibited α -glucosidase activity *in vitro*; however, this result was not observed in an *in vivo* model using 250 mg/kg cocoa liquor PCs. In the study conducted by Yilmazer-Musa *et al.*,¹³⁰ acarbose also inhibited α -glucosidase, but the IC₅₀ values were 13 times lower compared to acarbose's inhibitory effect on α -amylase. Notably, both GSE (IC₅₀ = 1.2 μ g/ml) and white tea extract (IC₅₀ = 2.5 μ g/ml) were more potent α -glucosidase inhibitors than acarbose (IC₅₀ = 90 μ g/ml).

The structure of flavanols affects the affinity to which they can bind to these proteins. A study by Barrett *et al.*¹³³ compared flavanols from grape, cranberry, pomegranate, and cocoa to determine how well each can inhibit α -amylase. It should be noted that the cocoa used in this study primarily consisted of monomers and dimers, a composition that may not be reflective of most cocoa powders. It was found that all compounds had an effect, but cocoa flavanols (containing the smallest mean degree of polymerization used in the experiment) had the least inhibitory effect on either enzyme. More complex polyphenols, such as ones found in cranberries and pomegranates, were more successful at inhibiting the breakdown of carbohydrates.¹³³ Andujar and Gu state that the greater the degree of polymerization, the more potently the polyphenol can inhibit digestive enzymes.^{22,129} In addition, a study conducted by Gu *et al.*¹²⁹ found that cocoa potently inhibits pancreatic amylase, pancreatic lipase, and phospholipase A2. This group also examined the effects of processing methods on inhibitory capability. They found that the least processed cocoa, termed “lavado” (an unfermented cocoa which has the greatest

concentration and largest cocoa PCs), had the strongest inhibitory effect on these pancreatic enzymes. Inhibition of lipases will be reviewed in Section 5.5.

It has been established that cocoa flavanols can inhibit digestive enzymes, but the extent to which this inhibition affects postprandial glucose excursions is unclear. It is also unclear if these effects are observable *in vivo*. Reducing rapid increases in blood glucose after a meal is important for patients with metabolic disorders, since it helps them maintain glucose homeostasis. Cocoa flavanols may be as effective at inhibiting digestive enzymes as some pharmaceuticals and therefore deserve further consideration.

5.1b Inhibition of Glucose Transporters

Cocoa polyphenols not only inhibit certain digestive enzymes, but they may also inhibit glucose transporters. Similar to digestive enzyme inhibition, the primary molecular mechanism of action may be non-specific flavanol-protein interactions, or competitive inhibition at the transport active site. Inhibiting glucose transporters in the intestine could attenuate glucose excursion after a meal. Intestinal transporters that may be inhibited include glucose transporter 2 (GLUT2) and sodium/glucose cotransporter 1 (SGLT1).^{134,135}

GLUT2 is found on both the apical and basolateral surfaces of enterocytes. GLUT2 vesicles store the transporters within the cell and fuse with the cell membrane and facilitate transport of glucose (similar to insulin-stimulated GLUT4) upon increased glucose load. In diabetic patients, the control of this vesicle is lost and increased amounts of GLUT2 transporters are always found on the cell surface, contributing to elevated blood glucose levels. Kwon *et al.*¹³⁴ found that *in vitro* GLUT2-mediated glucose transport was inhibited by quercetin ($IC_{50} = 12.7 \mu M$), but not by epicatechin (no inhibition) or catechins (no inhibition). Further studies

examining the effects of PCs with varying degrees of polymerization are necessary to understand whether or not inhibition of transporters occurs in response to cocoa consumption.

SGLT1 is a Na^+ /glucose cotransporter, which permanently resides on the apical membrane of intestinal epithelial cells. T2DM patients exhibit increased expression of SGLT1 compared to healthy individuals, leading to decreased glucose control.⁶³ Monomeric (+)-catechin (0.5 mM) inhibited SGLT1 in a competitive mechanism in an *in vitro* study using *Xenopus* oocytes.¹³⁵ Polyphenols found in tea [(-)-epicatechin gallate and (-)-epigallocatechin gallate] also inhibited expression of SGLT1. The extent to which cocoa flavanols with large degrees of polymerization can inhibit this transporter is unknown.

Flavanol metabolites that reach the circulation may exert an inhibitory effect on glucose transporters in peripheral tissues. However, the concentration of metabolites in the circulation is relatively low (<3-5 μM) and is fleeting.^{59-62,134} Therefore, given the low bioavailability of cocoa flavanols and short half-lives of flavanol metabolites, inhibition of glucose transporters is likely a mechanism occurring exclusively in the gut. Again, this mechanism would be helpful for patients with metabolic disorders because it may reduce rapid glucose excursions after a meal, therefore promoting glucose homeostasis.

5.2 Hormonal Response to Meals

Cocoa flavanols also appear to modulate the secretion and activities of hormones critical for maintenance of glucose homeostasis, as explained below.

5.2a Stimulating the Incretin Response

The incretin response may be a key mechanism enhanced by cocoa. Incretins (GLP-1, GIP) are secreted from enteroendocrine cells after a meal. One of the roles of these hormones is to stimulate insulin secretion for glucose disposal.¹³⁶ Incretin hormones have other effects on the

pancreas, including increasing somatostatin secretion, decreasing glucagon secretion, and stimulating β -cell growth and neogenesis. Incretin hormones are not limited to stimulating the pancreas; incretin receptors are found in many tissues throughout the body, including the brain, liver, adipose, and skeletal muscle. Other incretin functions include suppressing appetite, delaying gastric emptying, and increasing glycogen synthesis.^{137,138} The incretin response is impaired in non-insulin dependent T2DM, possibly due to a lack of incretin secretion.^{137,139} The incretin response is greatly reduced when a glucose load is administered intraperitoneally compared to an oral glucose load.¹⁴⁰ This suggests that the gut is an important location for interventions targeting incretin levels, and therefore an interesting potential target for cocoa flavanols with poor bioavailability. It is possible that cocoa may enhance the incretin response by either stimulating incretin release or extending the half-life of incretin hormones.

5.2b Incretin Hormones

The incretin hormone glucagon-like peptide 1 (GLP-1) is released from epithelial endocrine L-cells found in the distal small intestine and colon. In response to either glucose or a mixed meal, proglucagon is cleaved and GLP-1 is released into the circulation.¹³⁶ The half-life of GLP-1 is about 2 minutes. GLP-1 exerts biological actions via its receptors, which are found on islet α and β cells in the pancreas, in the brain, and on vagal afferents.^{137,141} GLP-1 receptor agonists have been developed (i.e., Liraglutide, Novo Nordisk) and promote weight loss by suppressing hunger, reducing the duration of eating, and delaying gastric emptying.^{141,142}

Gonzalez-Albuin *et al.*^{143,144} showed an increase in GLP-1 concentration in healthy rats fed an oral glucose load (2 g/kg bw) 40 min after oral gavage of grape seed procyanidin extract (1 g/kg bw) compared to control. The increased concentration was not significantly different from the positive control treatment, 1 mg/kg bw of Vildagliptin (a DDP-4 inhibitor). Yamashita

*et al.*⁸⁶ also demonstrated increased GLP-1 secretion in mice 60 min after oral gavage of 10 µg/kg bw Cinnamtannin A2, a tetrameric cocoa procyanidin. This study was novel because it was performed in the absence of any macronutrients. Not only did it increase GLP-1 secretion, but insulin secretion and insulin action (measured by phosphorylation of insulin receptor substrate 1 (IRS-1) and insulin receptor (IRβ)) was increased, as well.⁸⁶ However, the impact of cocoa flavanols on incretin response in the presence of glucose is not yet known.

Gastric inhibitory peptide (also referred to as glucose-dependent insulintropic polypeptide) (GIP) is secreted from K cells in the proximal small intestine. The release of GIP is stimulated by the presence of nutrients, primarily fats, in the small intestine.¹⁴⁵ The *in vivo* half-life of GIP is approximately 5-7 minutes. When studying this peptide, it is important to distinguish between the cleaved, non-insulintropic metabolite [GIP (3-42)] versus the active hormone [GIP (1-42)].¹⁴⁵ Gonzalez-Abuin *et al.*,¹⁴⁴ found that GIP concentration was significantly reduced after a gavage of grapeseed procyanidin extract (1 g/kg bw) prior to an oral glucose load (2 g/kg bw). This response was similar to that of the positive control, Vildagliptin. However, clinical studies using solely pharmaceuticals (i.e. Sitagliptin) find that GIP concentration and area under the curve typically increases in healthy, non-diabetic males.¹⁴⁶ It is unclear why GLP-1 and GIP seem to respond differently in response to grape seed PCs. This is an area that warrants additional investigation, as research on flavanols has focused on GLP-1.

The primary molecular mechanism by which cocoa flavanols stimulate GLP-1 and GIP secretion likely occurs in the secretory cells, but remains unknown. It seems likely that consumption of cocoa polyphenols stimulates the release of GLP-1, but the effects of cocoa on GIP are less understood. It would be interesting to utilize a GLP-1 receptor knock-out model to see if cocoa can stimulate an incretin response via GIP. Further, a double incretin receptor

knock-out (DIRKO) model could be used to assess if an incretin response is an important mechanism utilized by cocoa to reduce glucose excursion in an acute fashion. Stimulating an incretin response is beneficial for patients with metabolic disorders because it assists in glucose disposal, slows gastric emptying, and reduces appetite.

5.2c DPP-4

Dipeptidyl peptidase IV (DPP-4) cleaves the penultimate proline or alanine residue in proteins.^{147,148} It is a transmembrane glycoprotein¹⁴⁹ found in nearly all human tissues and fluids.¹⁴⁷ Two DPP-4 targets are GLP-1 and GIP.^{136,145,148} These hormones are cleaved, and therefore inactivated, by DPP-4 almost immediately after they are secreted from their respective endocrine cells; consequently, the incretin hormones have short half-lives. DPP-4 levels in patients with type 2 diabetes, impaired glucose tolerance, and/or obesity are not different than normal controls.^{150,151} DPP-4 inhibitors have been considered potential treatments for T2DM because extending the active lifespan of these hormones could prolong the beneficial effects that incretin hormones have on glucose control.¹⁴⁷ Indeed, DPP-4 inhibition has been shown to improve glycemic outcomes in diabetic models and delay the onset of diabetes in Zucker diabetic fatty rats.¹⁵² DPP-4 inhibitor drugs (commonly named gliptins) mimic many of the same actions as GLP-1 receptor agonists (stimulating insulin secretion, inhibiting glucagon secretion, etc.) but they do not exhibit the same improvements in weight loss.¹³⁷ This is likely because the resulting increase in incretin hormones is much less compared to activating the GLP-1 receptor directly.¹³⁷ Gliptins are currently employed as a second-line therapy for T2DM poorly controlled by metformin alone.¹⁵³⁻¹⁵⁵

It appears that inhibition of DPP-4 may be another primary molecular mechanism of action of cocoa flavanols. Gonzalez-Albuin *et al.*¹⁴⁷ examined the effects of grape seed

procyanidin extract on DPP-4 using several methods. First, they determined that the extract is able to achieve 70% inhibition of commercial DPP-4 at the highest dose reported, 200 mg/L. Next, using cultured Caco-2 cell epithelial monolayers, they found that 100 mg/L of grape seed extract incubated for 3 days resulted in 20% inhibition of DPP-4 (shorter incubation periods did not show significant changes in inhibition). This was associated with a significant reduction in DPP-4 gene expression, as well. The same group examined the effects of grape seed extract on DPP-4 in *in vivo* models.^{143,144} They found that an acute grape seed extract (1 g/kg bw) inhibits intestinal DPP-4 activity.¹⁴⁴

Ultimately, it appears that while plasma DPP-4 inhibition is possible, it is likely not the main mechanism that would result in improved glucose homeostasis,¹⁴⁷ gut DPP-4 inhibition is a more plausible mechanism. DPP-4 inhibition has not been studied using cocoa extract or cocoa powder and remains an area in need of further investigation.

5.2d β Cells

The well being of the β cell is critical for the maintenance of glucose levels. Patients with type 2 diabetes and other metabolic diseases present with inflammation and excess reactive oxygen species, which can damage β cells, further exacerbating metabolic instability. Individuals with metabolic disorders can also present with a decrease in antioxidant potential (i.e. glutathione levels), so a dietary antioxidant may be beneficial for the health of these patients.

Cocoa polyphenols have antioxidant properties and may help protect β cells from oxidative damage. In Caco-2 cells, cocoa polyphenols were able to inhibit apoptosis by preventing oxidative stress and activation of the JNK pathway.¹⁵⁶ Further, Martin *et al.*¹⁵⁷ showed that in Ins-1 cells, a pancreatic β cell line, cocoa flavanols protected against oxidative stress. Most recently, in a rodent model using Zucker diabetic fatty rats, a 9-week cocoa-enriched

diet (10 % (w/w) cocoa powder) prevented the apoptosis of β cells by reducing oxidative stress by reactive oxygen species.⁹³ Overall, it is unclear if the cocoa flavanols are acting as reducing agents in the gut (possibly on acrylamide), in the circulation, or in the pancreas directly. Further studies are needed to elucidate the exact location that these antioxidant effects are taking place in an in vivo model and at doses more comparable to human intake.

5.3 Metabolic Endotoxemia and Inflammation

Endotoxin, or lipopolysaccharide (LPS), is derived from the outer membrane of Gram-negative (–) bacteria. If the bacteria lyse, LPS can separate from the membrane and, if gut barrier function is poor, the LPS can enter the circulation via paracellular diffusion and activate pro-inflammatory pathways through molecular pattern recognition receptors in systemic circulation and in tissues. Several factors appear to modulate the concentration of LPS in circulation, including the gut microbial environment, high fat diet, and intestinal permeability.¹³ Chronic, low-grade, inflammation may contribute to the pathogenesis of obesity and metabolic syndrome. Circulating endotoxin binds to toll-like receptor 4 (TLR4), a molecular pattern recognition receptor, and initiates an inflammatory response.¹³ This chronic, endotoxin-derived inflammation can disrupt energy homeostasis and insulin signaling, leading to elevated blood glucose levels (**Figure 2.4**). If the bacteria lyse, LPS can separate from the membrane and, if gut barrier function is poor, the LPS can enter the circulation via paracellular diffusion.

Recent evidence has suggested that cocoa flavanols can aid in the attenuation of this metabolic endotoxemia,^{23,85} however, the underlying mechanisms are less explored. These changes are primarily contributed to the chronic consumption of cocoa. Possible intermediate mechanisms responsible for this effect of cocoa are modulation of the gut microbiome composition and function, improvements in gut barrier function, and improved insulin signaling.

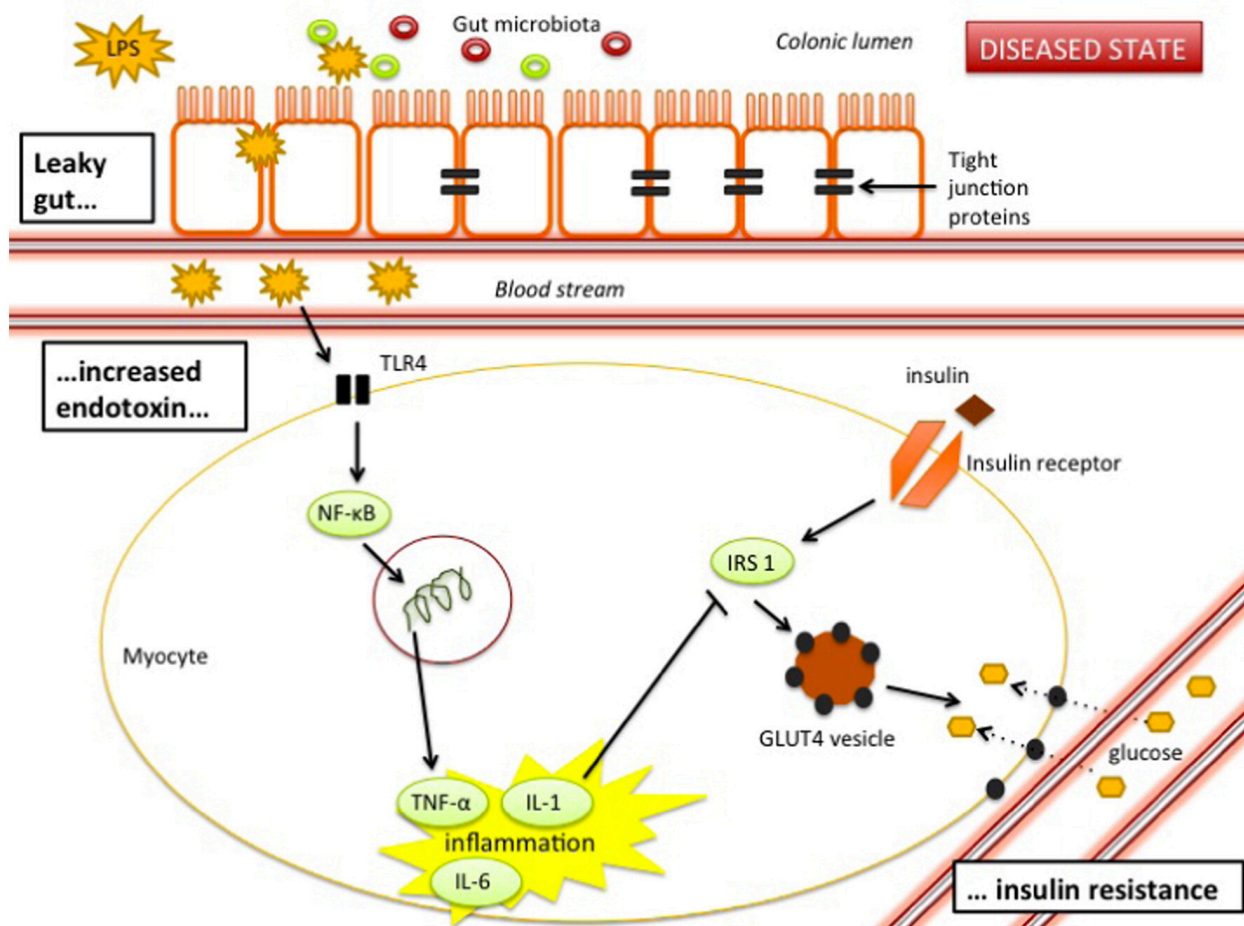


Figure 2.4. Suggested mechanism by which increased gut permeability and endotoxin levels lead to insulin resistance.

5.3a Gut Microbiota

Recently, the gut microbiome has become a very popular field of research. While once considered a “black box,” the commensal microbial communities of the human gastrointestinal tract are now known to be diverse, complex, and to have significant impacts on human health. It is believed that one’s diet plays a large role in the development and maintenance of the microbial community.^{13,158} Further, links have been drawn between the composition of one’s microbiome and their likelihood to present with obesity or metabolic disease.¹⁵⁸ Certain species are associated with harvesting nutrients and producing short chain fatty acids, improving the mucosa in the

colon, and improving gut barrier function, among many other outcomes.^{159,160} It is possible that cocoa may modulate levels and activities of certain species in the gut microbiome, although the primary mechanisms of action by which this is achieved remain poorly understood. Mechanistic studies are needed to understand the molecular interactions between flavanols and commensal bacteria, both on an individual cell and community level.

A large proportion of cocoa flavanols proceed to the colon where they interact with the gut microbiota. As discussed previously, the gut microbiota metabolize polyphenols. However, polyphenols also modulate the gut microbiome and exert prebiotic effects. A prebiotic is defined as a “non-digestible food ingredient that beneficially affects the host by selectively stimulating the growth and/or activity of one or a limited number of bacteria in the colon, and thus improves host health.”¹⁶¹ While prebiotics are commonly thought to be indigestible carbohydrates that are fermented by gut microbiota, flavanols can also fulfill this definition. Cocoa flavanols have shown prebiotic activity *in vitro*,⁶⁹ in rodents,⁹⁸ and in humans.¹⁶²

Tzounis *et al.*⁶⁹ found that incubation of (+)-catechin with fecal samples from healthy volunteers significantly increased the growth of *Clostridium coccoides*-*Eubacterium rectale* group, *Bifidobacterium spp.*, and *Escherichia coli*, as well as a significant inhibitory effect on the growth of the *Clostridium histolyticum* group. A rodent study showed decreases in *Bacteroides*, *Staphylococcus*, and *Clostridium* genera after a 6 week cocoa treatment (100 g cocoa/kg chow) compared to a reference group; this study utilized healthy animals and a normal chow diet.⁹⁸ It is important to note that the dietary fiber in cocoa could potentially elicit many of these observed benefits, and this study’s control group did not have matched soluble fiber content. Cocoa flavanols were also found to modulate human gut microbiota. After a 4-week cocoa treatment (494 mg flavanols/day), healthy volunteers had an increase in *Bifidobacterium* and

Lactobacillus, and a decrease in *Clostrum* compared to a low flavanol treatment (29 mg flavanols/day) group.¹⁶² Both treatments had equal amounts of dietary fiber.

It is evident that cocoa can exert prebiotic effects in both animals and humans, and improve gut barrier function. Both of these properties would be beneficial for patients with metabolic disorders. However, more research is necessary to understand if cocoa can exert prebiotic effects in an unhealthy or at-risk population and to what degree the naturally occurring fiber found in cocoa powder effects these results.

5.3b Tight Junction Proteins

The purpose of tight junction proteins is to ensure the integrity of epithelial tissues and act as a barrier to limit paracellular diffusion of water, ions, and other molecules. Occludin, claudin, and junction adhesion molecules (JAM) are important proteins found in tight junctions between epithelial cells.¹⁶³ The transmembrane proteins occludin and claudin attach to actin filaments within the cell via intracellular plaque proteins, such as zonula occludens (ZO).¹⁶⁴

Gut barrier function is important for human health. A high fat diet,¹³ alcohol,¹⁶⁵ and exercise¹⁶⁶ can increase gut permeability. Certain diseases such as Crohn's disease, inflammatory bowel disease, and Celiac's disease are associated with compromised integrity of the gut barrier.¹⁶⁷ In the perspective of metabolic syndrome and metabolic endotoxemia, a leaky gut allows endotoxin to enter the circulation via paracellular diffusion. Systemic endotoxin causes an inflammatory response that can disrupt insulin signaling and contribute to atherosclerosis and obesity.¹⁶⁸ Therefore, improving gut barrier function is an important target for preventing and/or resolving metabolic endotoxemia.

There has been evidence that the gut microbiome can affect gut barrier integrity.¹⁶⁹ The mucus layer in the GI tract is important for gut health¹⁷⁰ and certain bacterial species, such as

Akkermansia, reside in this layer.¹⁷¹ Increased mucus production by goblet cells is a prime environment for mucus-eating bacterium, such as *Akkermansia*, which has been shown to protect against metabolic syndrome.¹⁵⁹ Everard *et al.*¹⁷¹ showed that *Akkermansia* is beneficial to gut barrier function and normalizes metabolic endotoxemia. This species also improved glucose tolerance and decreased hepatic glucose production in mice with diet-induced obesity.¹⁷¹ Interestingly, *Akkermansia* is a gram (–) bacterium.

It is unclear if cocoa flavanols can increase *Akkermansia* populations, but flavanols have been shown to improve gut barrier function.^{121,172} While the mechanism by which this occurs remains unknown, this protective effect against gut permeability and therefore inflammation could be the mechanism for health-promoting effects of cocoa flavanols. This is a very intriguing research area that should be further explored.

There has been limited evidence to suggest that flavanol consumption has been correlated with improvements in tight junction protein expression and gut permeability. Goodrich *et al.*¹²¹ found 0.1% GSE in drinking water (100 mg/kg/day) increased occludin expression in the proximal colon in healthy rats compared to the control group. Another group found that GSE in a standard chow diet (250 mg/kg/day) increased ZO-1 and occludin expression and decreased intestinal permeability in the small intestine in healthy rats.¹⁷²

The primary molecular mechanisms behind the increased expression of tight junction proteins are unclear, but it may be related to prebiotic-induced changes in gut microbiota. Additionally, flavanols may interact directly with the epithelium to induce these changes. Future research is needed to determine if cocoa can protect against derangements in gut barrier function and inflammation caused by a high fat diet, and if these changes are associated with bacterial species such as *Akkermansia*.

5.3c Endotoxin-derived Inflammation

LPS is the primary ligand for TLR4, which is found on the cell surface of immune cells, skeletal muscle, and many other tissues.¹⁷³ LPS binding to TLR4 initiates an inflammatory cascade that leads to nuclear translocation of nuclear factor kappa B (NF- κ B), resulting in production of inflammatory cytokines.¹⁷³ Poor gut barrier function will lead to elevated plasma endotoxin levels and metabolic disease.¹³ Endotoxin-induced inflammation has been shown to disrupt energy homeostasis associated with metabolic syndrome.¹⁷⁴ Inflammation can hinder the normal processes of many tissues, including skeletal muscle, liver, adipose, brain, pancreas, and the endothelium of arteries. Inflammation can also disrupt insulin and leptin signaling; both of these hormones are involved with perceptions of satiety and fuel handling.¹⁷⁵

Several studies have explored the effects of cocoa on inflammation and its contribution to diseases.^{95,97,115,156} There are also studies investigating the effects of cocoa on metabolic endotoxemia.^{23,97} A study conducted by Gu *et al.*⁸⁵ examined the effects of an 18 week cocoa treatment (8% w/w cocoa powder in a high fat diet) in male mice on cytokine and endotoxin levels. It was found that the cocoa treatment was effective in reducing plasma LPS, TNF α , and IL-6 compared to the control high fat diet group. This study also showed that the cocoa treatment improved gut barrier function, resulting in 40.8% lower plasma endotoxin levels compared to the high fat diet group. Dorenkott *et al.*²³ also saw reductions in serum endotoxin levels, along with improvements in glycemic outcomes, in a similar study using a lower dose (25 mg/kg bw of cocoa extract monomeric and oligomeric fractions) for 12 weeks. Both studies utilized a C57Bl/6 mouse model on a high fat diet.

Overall, improving cocoa and other flavanols have the potential to improve gut barrier function, which may, in turn, alleviate metabolic endotoxemia. Further research is needed to

confirm these results, and a clinical study is warranted. It is unknown if reduced endotoxemia is due solely to alterations to gut microbiota and barrier function, of if flavanols can directly bind and inactivate LPS in the gut or blood, or modulate LPS-TLR4 binding and downstream signaling at the levels of skeletal muscle cells.

5.4 Insulin Signaling

It is increasingly recognized that chronic inflammation is associated with defective insulin signaling and insulin resistance. It has been shown that pro-inflammatory molecules inhibit the insulin-signaling pathway. For example, tumor necrosis factor- α (TNF- α) can induce the phosphorylation of the serine residues on IRS-1, which subsequently inhibits tyrosine auto-phosphorylation of the insulin receptor,¹⁷⁶ thereby impairing glucose disposal. Chronic hyperglycemia is toxic to pancreatic β -cells, causing impairments in insulin secretion and cell apoptosis, therefore further exacerbating elevated glucose levels.

Cordero-Herrera *et al.*^{177,178} studied the effects of epicatechin and cocoa extract at physiologically relevant doses on insulin signaling mechanisms in HepG2 cells. Both treatments successfully enhanced the activities of IR, IRS-1, IRS-2, PI3K/AKT pathway, and AMPK. However, it is unclear if the Phase-II and/or colonic metabolites would produce the same effects as the native polyphenols. Future studies may want to examine these outcomes with conjugated metabolites of epicatechin and other flavanols, since the metabolites would be most prevalent in circulation compared to native flavanols.

Yamashita *et al.*⁸⁴ showed that cocoa liquor extract provoked the translocation of GLUT4 to the plasma membrane in absence of insulin in L6 myotubes. This is an interesting finding for several reasons. Individuals with T2DM have a blunted GLUT4 translocation in response to insulin, despite the fact that they typically have normal amounts of GLUT4 expression in skeletal

muscle.^{25,29,179} If cocoa can promote the translocation of GLUT4, glucose disposal will be enhanced and blood glucose levels will normalize. Since this is an insulin-independent mechanism, this is especially useful for diabetic patients who may have deficits in insulin production. Future studies are warranted to see if these outcomes are reproducible *in vivo*.

Cocoa polyphenol extract was shown to inhibit insulin receptor kinase by direct binding, resulting in reduced lipid accumulation and differentiation in preadipocytes *in vitro*.¹⁸⁰ This is thought to be one mechanism by which cocoa flavanols may inhibit the onset of obesity.

In conclusion, cocoa may modulate insulin signaling in several ways. First, a heightened incretin response will promote insulin secretion. Second, if cocoa can improve gut barrier function, it will lead to a reduction in LPS and chronic inflammation, resulting in improved insulin signaling. Third, cocoa flavanols reduce insulin resistance by both insulin-dependent and insulin-independent mechanisms (including activation of the insulin signaling cascade in the absence of insulin). Glucose intolerance and insulin resistance are characteristic of metabolic syndrome. Dietary components aiding in either insulin secretion or insulin action would prove beneficial for patients with metabolic syndrome. However, the cellular mechanisms by which cocoa flavanols achieve these effects in glucose-disposing tissues remain unknown. Further research with pathway inhibitors, overexpression and gene silencing experiments are needed to move beyond identification of up-regulated/stimulated pathways and pinpoint the mechanistic targets that produce those effects (such as AMPK signaling, CAMK signaling, PI3K/Akt signaling, etc.). This, in turn, will enable therapeutic targeting of those primary mechanisms. In summary, potential mechanisms by which cocoa flavanols may improve glucose homeostasis are shown in **Figure 2.5**.

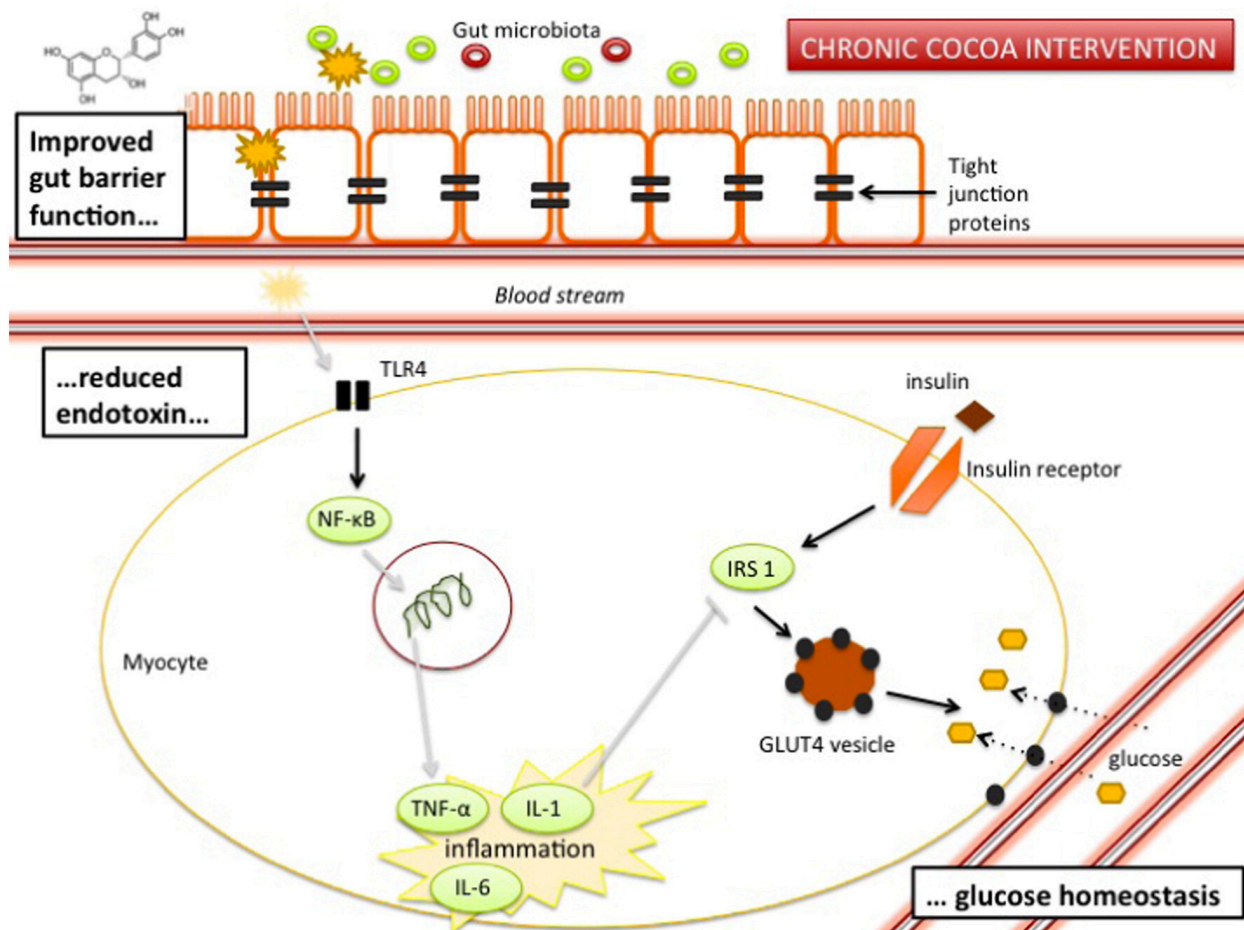


Figure 2.5. One mechanism by which a chronic cocoa supplement may improve glucose homeostasis. Cocoa may improve gut barrier function, leading to a reduction in serum endotoxin, minimizing inflammation, allowing for normalized glucose control.

5.5 Other Potential Mechanisms

The mechanisms addressed in this review are only a portion of the proposed mechanisms that are reported in the literature. Other mechanisms by which cocoa may affect health outcomes are important to acknowledge, in order to fully understand the potential effects that cocoa flavanols may have on glucose homeostasis.

This includes an antioxidant potential of cocoa flavanols that can be very beneficial to cardiovascular health, and has been extensively studied and reviewed elsewhere.¹⁸¹ Cocoa can impact nitric oxide production, endothelial function, and ultimately, atherosclerosis.

Cardiovascular health is an important facet of metabolic syndrome and must not be overlooked when developing drugs or designing studies to alleviate or assess this metabolic disorder.

Oxidative stress is present in obesity and metabolic syndrome. Reactive oxygen species can accumulate in metabolically active tissues and cause lipid peroxidation, damage β -cells, modulate the gut microbiota, and hinder cardiovascular function, insulin signaling, and mitochondrial function. Flavanols may protect against the effects of oxidative stress.⁴⁵

Gu *et al.*⁹⁷ suggested that inflammation can be reduced by cocoa flavanols via reducing lipid absorption. Along with the digestive enzymes already discussed, flavanols also inhibit digestive lipases, which results in increased lipid content in fecal matter. Further, this will reduce macrophage infiltration into adipocytes, lowering inflammatory tone.⁹⁷

Dyslipidemia is an important facet of metabolic syndrome. Many studies have examined the effects of chronic cocoa treatments on LDL cholesterol, HDL cholesterol, and triglycerides.^{107,108,113,182-186} Cocoa may be able to beneficially modulate cholesterol and triglyceride levels in metabolically unhealthy individuals.¹⁸⁷ Cocoa flavanols may improve blood glucose control indirectly, by modulating lipid digestion and thus reducing hyperlipidemia and its subsequent deleterious effects on glucose homeostasis. PCs are potent lipase inhibitors *in vitro*;^{129,188} they also reduce acute postprandial¹⁸⁸ and fasting plasma triglycerides⁸⁵ and increase fecal lipid excretion⁹⁷ in animals and humans. It has been well established that cocoa and PCs reduce blood triglycerides and lipid accumulation in viscera, liver, and β -cells in animal models.^{24,97,101,189,190} Prevention of lipid accumulation by cocoa PCs may indirectly improve glucose homeostasis by preserving metabolic flexibility and insulin sensitivity in skeletal muscle,^{191,192} insulin sensitivity in liver,^{193,194} and β cell viability and function.^{93,157,190,195} Clinical studies have shown that inhibition of lipid absorption and associated hyperlipidemia and fat

accumulation, can improve blood glucose control and insulin sensitivity in humans.¹⁹⁶⁻¹⁹⁸ Cocoa flavanols have not been evaluated for inhibition of lipid digestion and absorption in humans.

Finally, cocoa flavanols have been associated with an increase in lipolysis, fatty acid oxidation, and energy expenditure in animal models.^{87,91,92,199-202} Other suggested mechanisms involve the endocannabinoid system,¹⁷⁴ mitochondrial function,¹⁰⁴ anti-carcinogenic properties,²⁰³ and modulating immune function.³⁰

In summary, there are many possible primary and intermediate mechanisms that are outside the scope of this review, but they are still important to consider when evaluating the effects of cocoa on metabolic syndrome. It is likely that cocoa and cocoa flavanols exert pleiotropic effects on metabolism, which likely act synergistically to prevent or slow prediabetes and T2DM. However, it remains unknown which mechanisms and pathways are affected directly by cocoa, and which are modulated indirectly as downstream effects of improvements in the primary targets. In some cases, definitive identification of the primary molecular mechanism of action may be unnecessary. However, when moving forward to expensive, time-consuming clinical trials, knowledge of the most upstream targets will facilitate improved study design, identification of appropriate biomarkers to evaluate efficacy, and perhaps most importantly, define the biological contexts in which cocoa flavanols are likely to be effective.

6. Implications Of Potential Mechanisms

As detailed above, cocoa flavanols appear to possess important anti-diabetic activities. In some cases, these activities are similar to current pharmaceuticals for control of diabetes and obesity,²⁰⁴ such as acarbose,^{205,206} gliptins,^{153,207,208} and orlistat.¹⁹⁶⁻¹⁹⁸ Increased intake of cocoa flavanols may represent a viable dietary strategy to obtain the glucose-lowering benefits of these

pharmaceuticals without the deleterious side effects (oily stool, diarrhea, gas, bloating, etc.). However, the clinical utility of cocoa in preventing and ameliorating prediabetes and/or T2DM by exploiting these mechanisms remains largely unknown.

6.1 Implications of Bioavailability on Mechanism

Some of the proposed mechanisms suggest that cocoa flavanols may improve glucose control at least in part by acting locally in the gut lumen. This is critical due to the fact that flavanols, particularly the PCs (i.e the larger flavanols), have poor systemic bioavailability.²⁰⁹⁻²¹⁵ Reported oral bioavailability of flavanols is generally <10% for monomeric catechins^{216,217} (when phase-II metabolites are accounted for, bioavailability of monomers from catechins has been reported as high as 55%), much lower for small PCs (dimers, trimers), and essentially 0% for larger PCs.^{214,217-219}

Poor bioavailability likely limits flavanol activities in peripheral tissues compared to the gut. Therefore, cocoa flavanols are typically much more concentrated in the gut compared to peripheral tissues. The hypothesis of gut activity is strengthened by an intriguing study demonstrating that orally administered flavanols improved glucose tolerance in animals when glucose was administered orally, but not when glucose was administered by intraperitoneal injection.¹⁴⁷ Despite this evidence, the gut-located activities (inhibition of digestive enzymes, improved barrier to endotoxin, stimulation of GLP-1 secretion, etc.) of cocoa flavanols have not yet been rigorously tested nor targeted *in vivo* for inhibition or improvement of metabolic syndrome. Mechanistic animal and human clinical experiments are needed in order to demonstrate the ability of cocoa flavanols to act specifically by gut-mediated mechanisms. Demonstration that cocoa flavanols act through gut mechanisms is needed so that delivery and dosing strategies may be designed to specifically target these mechanism(s) and optimize

intervention efficacy, as well as identify behaviors and nutrition profiles that optimize the efficacy of these digestive effects.

6.2 Implications of Mechanism on Dose Distribution

Acute human studies demonstrate that consuming flavanols with a meal can lower postprandial hyperglycemia.²²⁰⁻²²⁴ Thus, co-consuming flavanols with meals may be a viable strategy for improving both acute and long-term blood glucose control, as well as reducing dyslipidemia. However, several of the proposed activities of cocoa flavanols (inhibiting carbohydrate/lipid digestion and improving the “incretin effect”) require the presence of flavanols in the lumen of the gut concurrent with macronutrients during digestion, similar to acarbose or orlistat. If co-consumption of flavanols with meals significantly improves acute glucose control and blood lipid profiles, it follows that chronic flavanol co-consumption with meals should maximize their activities compared to consumption at other times. Conversely, if acute effects require co-consumption with meals, consuming flavanols between meals may reduce their potential benefits; cocoa flavanols cannot inhibit macronutrient digestion if the two are not present at the required concentrations in the gut lumen simultaneously. However, it remains largely unknown whether consuming flavanols with meals (vs. other patterns) maximizes their efficacy, or if dose distribution does not affect efficacy.

Most animal studies,^{90,91,101,225} including those in our lab,^{23,121} administer flavanols incorporated into the diet (thus, flavanols and macronutrients are always co-consumed). Human interventions are not necessarily designed to recapitulate animal dosing patterns; rather, emphasis is simply placed on translating the effective dose from animals to humans. This may account for partial loss of efficacy during translational research. In at least four out of the reported effective chronic flavanol clinical interventions, dosing was synchronized with meals or

distributed widely throughout the day.^{107-111,226,227} Conversely, only one of the reported ineffective interventions was synchronized with a meal.^{113,115,228,229} The preliminary evidence therefore suggests that dosing strategies may matter in terms of flavanol efficacy. Consuming flavanols with meals, or evenly throughout the day, appears to maximize efficacy. Variations in design make it impossible to definitively assess the impact of dosing strategy from published studies.^{107-111,113,115,226-229} However, the impact of different flavanol dosing strategies on biomarkers of metabolic syndrome has not been rigorously tested. Studies are needed which examine the impact of dose distribution on efficacy.

6.3 Relationship Between Mechanism and Effective Dose

Animal and clinical studies alike have used drastically different doses of cocoa treatments, including doses that are likely not translatable to humans.^{23,93,104,114,115,230} Different mechanisms likely have distinct effective doses; since the mechanisms behind the beneficial health outcomes associated with cocoa have yet to be determined, it may be difficult to pinpoint an ideal dose before the mechanisms are defined. However, the “more is better” concept often used for phytochemical is inherently flawed, as many phytochemicals exhibit U-shaped dose response curves where lower doses are more effective, likely due to lower levels of detoxification pathway expression and different binding efficiencies for receptors and enzyme active sites, etc.^{231,232} (this is known as “hormesis”).^{233,234} Higher doses can result in reduced efficacy compared to lower doses, no effect, or even toxicity. The use of high doses can therefore mask potential efficacy of mechanisms that may be relevant to humans at translatable doses. Furthermore, non-translatable doses may modulate mechanisms that are not impacted at lower doses, thus suggesting potential mechanisms of action that are unlikely to be modulated

once translated to human dosing. Therefore, future studies should ideally be designed to examine the effects of lower, translatable doses of cocoa flavanols.

6.4 Relationship Between Flavanol Structure and Mechanism

Cocoa flavanols exist in a broad range of polymerization states. Different flavanols likely act through distinct mechanisms due to differences in structure and bioavailability. Animal studies have generally focused on whole cocoa or chocolate,^{93,96-98,100,105,225,235} extracts,^{84,88-91,236,237} and flavanol monomers (catechins).^{87,104,224,238} Little data exist on the bioactivities of larger flavanols (PCs), partly due to difficulty of isolation, complexity of analytical characterization, and lack of commercially available standards. However, recent data have suggested that the PCs may possess distinct (and in some cases, enhanced) activities for improvement of glucose homeostasis compared to flavanol monomers.

Recent data suggests that cocoa flavanols of different DP may possess distinct activities.^{23,86,128,239} Cocoa contains a wide distribution of flavanol DPs and the observed bioactivities are likely due to a variety of compounds acting through various mechanisms synergistically. Understanding the relationship between flavanol DP and bioactivities will facilitate an understanding of how cocoa composition impacts potential health benefits. Despite the cost and complexity associated with preparing or obtaining these larger flavanols, the influence of DP on flavanol bioactivity warrants further investigation. This is another emerging area with the potential to yield highly valuable, novel data to clarify the role of cocoa flavanols in metabolic syndrome. Efforts to isolate, purify, characterize and make these compounds available to other diabetes researchers will be central to this effort.

7. Conclusions

In conclusion, cocoa flavanols appear to alleviate metabolic syndrome, and specifically derangements in glucose homeostasis, by several intermediate mechanisms. First, cocoa may reduce glucose excursion after a meal by inhibiting digestive enzymes, inhibiting glucose transporters, and promoting an incretin response. These outcomes are most likely to be observed after an acute dose of cocoa and, since these mechanisms predominantly occur in the gut, the poor bioavailability of flavanols is not a limiting factor for these activities.

Second, chronic cocoa consumption may lead to beneficial changes in the gut microbiota, resulting in improved gut barrier function, reduced circulating endotoxin, and uninhibited insulin signaling mechanisms. PCs are stable through gastric and intestinal transit so they will reach the colon intact. Again, bioavailability is not a limiting factor.

Third, cocoa flavanols can act in peripheral tissues (improved β cell function and insulin sensitivity in skeletal muscle, etc.). These effects are limited by the poor bioavailability of many cocoa flavanols. Demonstration of the activities of flavanol microbial metabolites may be the missing link between oral flavanol consumption and activity in peripheral tissues.

It is likely that the potential benefits of cocoa consumption are mediated by all of these mechanisms to some extent. However, it remains unknown which, if any, of these mechanisms are primarily responsible for observed effects *in vivo*. Furthermore, the primary molecular mechanisms by which these intermediate mechanisms occur are generally unknown. Therefore, additional *in vivo* mechanistic studies are needed in order to isolate and assess individual primary and intermediate mechanisms of action.

There are many elements of this puzzle that are still unknown. First, it is unknown what acute effects cocoa may have on carbohydrate digestion in a population with existing prediabetes

or T2DM. So far, to our knowledge, the only acute studies (in both animal and clinical models) have examined healthy subjects or animals. Individuals with metabolic disorders will benefit greatly from a supplement to control glucose excursions, but it is unclear to what extent cocoa can be helpful in this population. Second, little is known regarding the impact of cocoa on human subjects with differing sub-states along the continuum of diabetes. Additionally, studies examining the impacts of cocoa and its mechanisms of action when administered in conjunction with common diabetes medications in subjects with T2DM (which is likely to occur in real-world clinical settings) are needed. Third, cocoa is metabolized in the colon by the microbiota into many metabolites and it is unknown what functions, if any, that these metabolites have on human health. Third, it is hypothesized that *Akkermansia* has beneficial effects on gut barrier function, but it is still unknown if cocoa can modulate this species, but this may be a worthwhile study to pursue. Lastly, it is unknown what doses of cocoa (for either acute or chronic outcomes) elicit the most beneficial outcomes related to metabolic syndrome.

In future studies, it is critical that all trials publish a full characterization of the cocoa utilized in the study, due to the impact of flavanols structure on potential mechanisms of action. Clinical studies should report the food matrix used in the treatment and animal studies should report food intake. Finally, utilizing acute and chronic study designs will be important to characterize the mechanisms of action of cocoa flavanols.

Insights into the mechanisms by which cocoa flavanols act and the sub-states of diabetes modulated by cocoa flavanols will refine the ability of clinicians to effectively use cocoa, in combination with diet, exercise, and medications, to effectively combat prediabetes and T2DM.

Chapter 3. The Effects of Acute Cocoa Consumption on Glucose, Insulin, and Incretin Hormones After a Mixed Meal Challenge in Adults at Risk for Type-2 Diabetes Mellitus

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Abstract

Prediabetes is an intermediate state between normal glucose homeostasis and type-2 diabetes. Strategies are needed to prevent progression from this “at-risk” state to overt disease. Cocoa is widely consumed and has a unique flavanol composition and may be a viable approach to improve glucose homeostasis. The purpose of this study was to test the hypothesis that cocoa consumption increases postprandial insulin and incretin concentrations and reduces postprandial glucose concentration. Thirty overweight or obese adults at risk for developing type 2 diabetes [54.9 ± 1.8 years old, body mass index (BMI) 33.4 ± 0.7 kg/m²] completed two meal challenges with a meal replacement shake blended with cocoa powder (10 g cocoa) or calorie-matched placebo (0 g cocoa) in a randomized crossover design. Blood samples were taken over 4 h post-meal. Our results suggest cocoa powder did not significantly influence postprandial glucose, insulin, C peptide, GLP-1, or GIP concentrations. Fasting glucose concentration was positively correlated with postprandial glycemic response ($R^2 = 0.1530$, $p = 0.0325$) and postprandial GIP concentration following cocoa ($R^2 = 0.2546$, $p = 0.0045$). Our results suggest that cocoa did not significantly influence postprandial glucose, insulin, and incretin concentrations. However, fasting glucose appeared to a significant correlate of the response to cocoa treatment. Funding was provided by The Hershey Company, Hershey, PA.

Introduction

Type 2 diabetes (T2DM) is major public health concern, currently affecting 29 million people in the United States.¹ Prediabetes is an intermediate state between normoglycemia and overt diabetes, and an estimated 86 million additional Americans have prediabetes.¹ The American Diabetes Association defines prediabetes as impaired fasting glucose (IFG) (fasting blood glucose concentration between 100-125 mg/dL), impaired glucose tolerance (IGT) [2 h

oral glucose tolerance test (OGTT) between 140-199 mg/dL], elevated glycosylated hemoglobin (HbA1c) (between 5.7-6.4%), or any combination of these results.²⁴⁰ The pathophysiology of these two subcategories of prediabetes is unique in that they have different sites of insulin resistance.²⁴¹ Insulin resistance is present in the liver in adults with IFG, leading to unregulated gluconeogenesis, and present in skeletal muscle in adults with IGT.²⁴¹ As these often develop independently, individuals with prediabetes may have either IFG or IGT, or both. Approximately 5-10% of adults with prediabetes will progress to T2DM annually.^{242,243} Gerstein *et al.*²⁴⁴ found the annualized incidence of diabetes in individuals with IGT was 4-6%, 6-9% in those with IFG, and 10-15% in those with and a combination of IFG and IGT. It is estimated that only 25% of adults with prediabetes have both IGT and IFG.²⁴⁵

Prediabetes represents an identifiable pre-disease state with elevated risk of developing T2DM and therefore also represents an opportunity to intervene in order to slow or prevent progression to T2DM. Both lifestyle modification and medications such as metformin have been shown to be effective in preventing the development of T2DM from a prediabetic state.²⁴⁶⁻²⁵⁰ Metformin reduced fasting blood glucose concentrations but was not as effective in reducing glucose concentrations following a glucose load compared with lifestyle intervention.²⁴⁶ Therefore, additional approaches are needed.

Dietary strategies therefore represent a critical complementary approach that may be employed to slow or prevent the progression from prediabetes to T2DM. Recently, there has been increasing interest in utilizing flavanol-rich foods such as cocoa for improvement of glycemic control. Flavanols are a subclass of flavonoids, or secondary metabolites widely distributed in plants. Cocoa typically contains three types of flavanols: catechins (37% of

polyphenol content), anthocyanidins (4%), and proanthocyanidins (58%).²² Cocoa powder can have up to 10% flavanols on a dry-weight basis.³⁸

While its potential mechanisms of action remain to be fully identified, cocoa and its constituent compounds appear to exert beneficial changes in digestive and metabolic processes.²⁵¹ *In vitro* and animal studies suggest that cocoa may inhibit α -glucosidase,^{37,252} α -amylase^{37,252} and lipase²⁵² and modulate gut incretin signaling via increased GLP-1 concentrations²⁴¹ and/or dipeptidyl peptidase 4 (DPP-4) inhibition.^{253,254} Thus, cocoa may attenuate postprandial blood glucose concentrations.

Previous human intervention studies have demonstrated the potential for cocoa to reduce fasting glucose¹¹¹ and reduce insulin resistance¹¹² in overweight and/or obese subjects. However, many of these studies have focused on cardiovascular health (reviewed here²⁵⁵), and there have been few studies^{109,115} investigating the anti-diabetic effects of cocoa on a prediabetic population. Furthermore, the majority of studies have been chronic feeding studies; therefore, it is largely unknown what acute effects cocoa may have on carbohydrate digestion and glycemic response in adults with prediabetes. So far, to our knowledge, acute studies have primarily examined healthy animals and subjects^{87,103,106,120,253} and one acute study examined obese, T2DM adults.²⁵⁶ The animal studies found that cocoa increased glucose tolerance,¹⁰³ increased plasma insulin and GLP-1,²⁵³ while the clinical studies found that cocoa increased postprandial insulin secretion, but did not change glycemic response.^{106,256} Whether cocoa consumption improves glucose homeostasis in individuals at risk of developing T2DM is unknown.

Individuals with prediabetes may benefit from the anti-diabetic activities of cocoa to assist in the maintenance of glucose homeostasis, but it is largely unknown to what extent cocoa can be beneficial for this population. It is also unknown what effects cocoa has on the incretin

response in humans. Therefore, the objective of this study was to determine the impact of acute cocoa consumption on incretin, insulin, and glycemic responses to a mixed meal in adults at risk of developing T2DM. We hypothesized that when co-consumed with a mixed meal, cocoa would enhance postprandial incretin and insulin response, and attenuate postprandial plasma glucose excursions compared to a placebo.

Methods

IRB Approval is in Appendix A. Detailed materials and methods are located in Appendix B. Informed Consent Form is in Appendix C. Supplementary information and additional data from this study are presented in Appendix D.

Participants. The CONSORT flow chart for this study is available in the **Supplementary Information Figure S3.1**. Eligible participants provided written informed consent. The protocol was approved by the Virginia Tech Institutional Review Board (IRB) (IRB# 13-755). The study was registered at ClinicalTrials.gov (NCT02203240).

Thirty-two overweight or obese (body mass index [BMI], 25-40 kg/m²) adults (40-75 years old) from the New River Valley, VA volunteered for the study. Participants were weight stable ($\pm$ 2.5 kg in previous 6 months), sedentary to recreationally active ($\leq$ 2 days/week, 20 min/day of low-intensity physical activity), and not taking any medications or supplements that would affect any study variables, including anti-inflammatory medications, fiber, and diabetes medications. Participants were free from chronic disease as determined by a health history questionnaire, lipid panel, and urinalysis. In addition, participants had at least one of the following criteria: fasting blood glucose 100-125 mg/dl, 2 h oral glucose tolerance test (OGTT)

blood glucose concentration 140-199 mg/dL, HbA1c 5.7-6.4%, or a score ≥ 5 on the American Diabetes Association Risk Assessment questionnaire.²⁵⁷ Subjects were excluded if any value (fasting, 2 h blood glucose, or HbA1c concentrations) met the criteria for T2DM. Full inclusion/exclusion criteria are presented in the **Supplementary Information Table S3.1**.

Cocoa and placebo treatments. Cocoa and placebo beverage dry mixes were individually pre-packaged in coded sachets, from single production lots obtained directly from The Hershey Company (Hershey, PA). The beverages were custom formulations produced by Hershey and designed to support human clinical trials. The contents were similar in color and flavor once blended with the vanilla-flavored meal replacement beverage. The cocoa treatment contained 864 mg total polyphenols, 50.4 mg epicatechin, and 531 mg total proanthocyanidins. The placebo treatment contained 247 mg total polyphenols, 0 mg epicatechin, and 0 mg total proanthocyanidins. Full characterization of polyphenols and nutrient content of cocoa and placebo packets are described in the **Supplementary Information Table S3.2**.

Meal challenge. A randomized, double-blind crossover design was utilized for the meal challenges to assess the impact of acute cocoa consumption on postprandial glucose and hormone responses. An investigator not involved in randomization or data collection was aware of the treatment codes. Participants completed both the cocoa and placebo treatments one week apart. Treatment order of the crossover design was determined by a computer-generated randomization scheme.

The test meal was 2-8 oz. servings of a meal replacement beverage providing a mixed macronutrient profile (**Supplementary Information Table S3.3**), supplemented with either the

cocoa beverage dry mix (supplying 10 g cocoa) or a calorie-matched placebo beverage mix (0 g cocoa) (**Supplementary Information Table S3.2**), thoroughly blended into the meal. The test beverage was initially 2-8 fl oz. servings of BoostTM Very Vanilla Original (Nestle HealthCare Nutrition, Inc., Florham Park, NJ). However, the Boost formula was changed in late 2014 and the study was completed with Equate Vanilla Nutritional Shake (Wal-Mart Stores Inc., Bentonville, AR). A total of $n = 16$ subjects completed the study with the Boost meal beverage in 2014, and a total of $n = 14$ subjects completed the study with the Equate meal beverage in 2016. These two beverages had no differences in glucose or insulin responses (AUC) (data shown in **Supplementary Information Figure S3.2**). After a baseline blood draw, participants were instructed to consume the test meal within 5 min. Subsequent blood samples were taken 1, 2, 3, and 4 h after the first sip of the test beverage. Blood for serum samples was allowed to clot in a glass tube (BD, Franklin Lakes, NJ) at room temperature while blood for plasma samples were collected in a glass tube with 12 mg K₃ EDTA (BD, Franklin Lakes, NJ) and placed on ice. Blood samples were centrifuged (1148 x g, 15 min, 4°C) and the resulting serum or plasma was frozen at -80° C until further analysis.

Measurements. Plasma glucose concentrations were analyzed immediately by the glucose oxidase method using a glucose auto-analyzer (Yellow Springs Instruments, Yellow Springs, OH, USA). Serum insulin, GLP-1, GIP, and C-peptide concentrations were determined using commercially available enzyme-linked immunosorbent assay (ELISA) kits (Human Insulin ELISA, Multi-species GLP-1 Total ELISA, Human GIP Total ELISA, Human C-peptide ELISA, Millipore, Billerica, MA, USA) per manufacturer's instructions. Total GLP-1 was measured as

opposed to active GLP-1 because of the short half-life of active GLP-1 and the timing of our blood draws. All analyses were performed in duplicate.

Data analysis. Pseudo-pharmacokinetic (PK) curves and parameters for blood biomarkers were constructed using PK plug-in functions for Microsoft Excel. Parameters determined include: fasting (baseline) concentration (C_0), maximal concentration (C_{MAX}), area under the PK curve (AUC), and excursion ($\Delta = C_{MAX} - C_0$). All delta (Δ) values were calculated as cocoa – placebo. One interim analysis was conducted after the cohort that received Boost was completed ($n = 16$).

Subjects were divided based on fasting blood glucose concentration and 2 h OGTT glucose concentration conducted during screening in order to determine if baseline subject fasting glucose or glucose tolerance significantly influenced the effects of cocoa on the outcomes. Participants with fasting glucose < 100 mg/dl were classified as having normal fasting glucose (NFG) ($n = 25$ NFG; $n = 5$ IFG). Participants who had 2 h OGTT plasma glucose values < 140 mg/dL were classified as having normal glucose tolerance (NGT) ($n = 24$ NGT; $n = 6$ IGT). Full comparisons of the subject characteristics divided by glucose tolerance and fasting glucose, respectively, are provided in the **Supplementary Information Tables S3.9 and 3.10**.

Statistics. Paired t-tests were conducted to determine any differences in values between the cocoa and placebo treatments for all subjects. Significance was defined *a priori* as $P < 0.05$. Two-way repeated measures ANOVA was used to determine the effects of phenotype and treatment on dependent variables of interest. Tukey's HSD was used for *post hoc* analyses. Pearson Product Moment correlations were used to assess relationships among dependent variables. Linear regression analyses were performed to assess relationships between dependent

variables (baseline subject characteristics or placebo response) and independent variables (postprandial responses to cocoa versus placebo treatments). Statistical analyses were performed on Prism v6 (GraphPad, La Jolla, CA) or IBM SPSS v22 (IBM Corp., Armonk, NY).

Results

Subject Characteristics. Subject characteristics are shown in **Table 3.1**. All enrolled participants ($N = 32$) completed the study, but only 30 were included in this analysis due to complications with administration of the test meal and blood sampling. There were no adverse events.

There were 2 participants who had isolated IFG, 3 who had isolated IGT, and 3 who had both IFG and IGT. Nineteen participants had impaired HbA1c. Ten qualified based on the risk assessment questionnaire alone. Therefore, all subjects exhibited risk for T2DM but there was a wide distribution of phenotypes and some subjects who were not considered prediabetic by the ADA guidelines.²⁴⁰

Table 3.1. Subject characteristics in the pooled sample^a

n	30
Female/male	19/11
Age (yr)	54.9 ± 1.8
Weight (kg)	93.4 ± 2.5
BMI (kg/m ²) ^b	33.4 ± 0.7
Serum triglycerides (mg/dL)	122 ± 10
Serum total cholesterol (mg/dL)	206 ± 8
ADA Risk Score ^c	5 ± 0
FBG ^d (mg/dL)	91.8 ± 1.7
2 h OGTT ^e (mg/dL)	115.2 ± 6.2
HbA1c ^f (%)	5.7 ± 0.1

^aValues are reported as mean $\pm$ SEM (except for n size and female/male)

^bbody mass index

^cScore on American Diabetes Association Risk Assessment Questionnaire

^dFasting blood glucose concentration

^e2 h oral glucose tolerance test: blood glucose concentration 2 h after
consuming 75 g glucose beverage

^fGlycosylated hemoglobin

Blood measures. The postprandial concentrations of the five measures (glucose, insulin, C Peptide, GLP-1, and GIP) during the meal challenge are shown in **Figure 3.1**. There were no significant differences between treatments in AUC, C_{MAX}, or excursion for any of the five blood measures (tabular data for all subjects presented in **Supplementary Information Tables S3.4-3.8**).

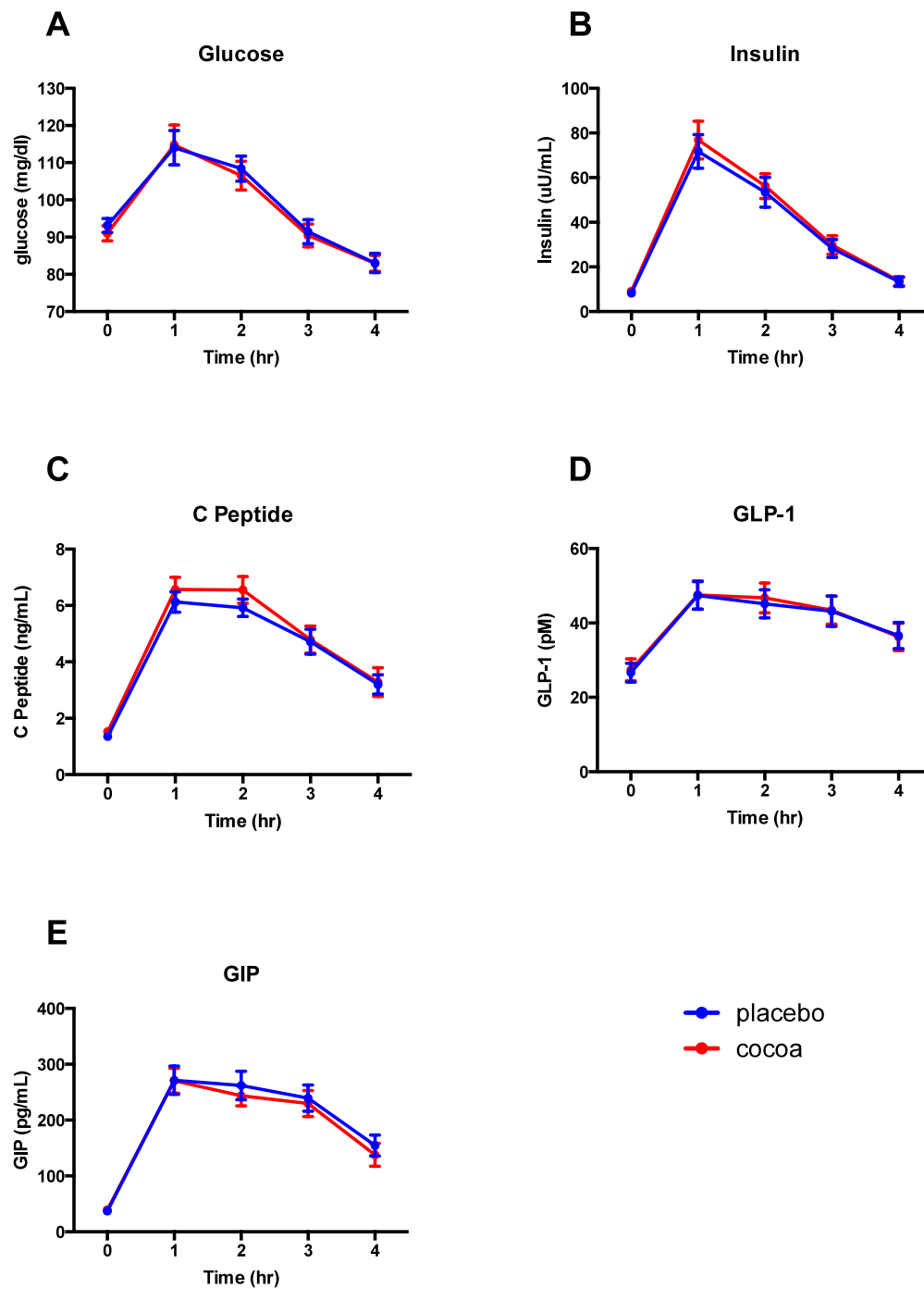


Figure 3.1. Mean ($\pm$ SEM) (A) plasma glucose concentration, (B) serum insulin concentration, (C) serum C Peptide concentration, (D) serum GLP-1 concentration, and (E) serum GIP concentration after consumption of the mixed meal with cocoa (•) or placebo (•). No significant differences between treatments at any individual time points were detected by paired t-tests, $P < 0.05$, $N = 30$.

Normal Glucose Tolerance vs. Impaired Glucose Tolerance. The calculated AUC for all measures are listed in **Table 3.2**. There were no significant two-way interactions. However, there was a significant main effect indicating that the IGT group had greater glucose AUC (regardless of treatment) compared to the NGT group ($p = 0.0016$). This confirmed that we had two distinct phenotypes (in terms of glucose postprandial AUC) in our pooled sample when separated by 2 h OGTT. However, the other biomarkers (insulin, C peptide, GLP-1 and GIP AUC) did not differ between IGT and NGT, although GLP-1 trended towards significance, suggesting that the IGT group had an overall smaller GLP-1 response compared to the NGT group ($p = 0.0729$). Mean values for fasting, AUC, excursion and C_{MAX} ($\pm$ SEM), delta values ($\pm$ SEM), and corresponding statistics for all subjects, NGT/IGT, and NFG/IFG are presented in the **Supplementary Information Tables S3.4-3.8**.

Table 3.2. Calculated area under the curve^a for each blood measure separated by glucose tolerance phenotype (NGT and IGT).^b

		Glucose (mg/dL/h)	Insulin (uU/mL/h)	C Peptide (ng/mL/h)	GLP-1 (pM/h)	GIP (pg/mL/h)
NGT (<i>n</i> = 24)	Placebo	384.3 $\pm$ 10.1	156.4 $\pm$ 16.2	19.0 $\pm$ 1.3	180.6 $\pm$ 15.1	861.7 $\pm$ 81.5
	Cocoa	377.1 $\pm$ 11.4	163.9 $\pm$ 17.6	20.0 $\pm$ 1.8	176.6 $\pm$ 15.5	801.1 $\pm$ 66.9
IGT (<i>n</i> = 6)	Placebo	455.8 $\pm$ 26.5	195.5 $\pm$ 36.2	19.1 $\pm$ 2.3	114.0 $\pm$ 22.7	899.3 $\pm$ 164.4
	Cocoa	471.7 $\pm$ 30.2	213.3 $\pm$ 41.7	21.0 $\pm$ 3.2	125.3 $\pm$ 20.6	925.2 $\pm$ 142.0
Two-way interaction	<i>p</i> value	0.2276	0.6831	0.6937	0.3502	0.4297
Main effects for phenotype	<i>p</i> value	0.0016**	0.2398	0.8725	0.0729	0.6142
Main effects for treatment	<i>p</i> value	0.6433	0.3249	0.2279	0.6549	0.7507

^aAll values are reported as the mean $\pm$ the standard error of the mean (SEM).

^bNGT: 2 h OGTT glucose < 140 mg/dL, IGT: 2 h OGTT glucose = 140-199 mg/dL

Normal Fasting Glucose vs. Impaired Fasting Glucose. Calculated AUC for each measure are shown in **Table 3.3**. There was a significant two-way interaction for GIP AUC ($p = 0.0118$), but no significant main effects. Glucose and GLP-1 two-way interactions approached significance ($p = 0.0812, 0.0732$, respectively). GIP AUC was significantly lower when the meal was consumed with cocoa compared to placebo for the NFG group (cocoa: 765.7 ± 59.3 pg/mL, placebo: 855.9 ± 74.8 pg/mL, $p = 0.0378$). In addition, the NFG GIP excursion and C_{MAX} approached significance ($p = 0.0524$ and $p = 0.0723$, respectively), indicating a smaller GIP postprandial response to cocoa compared to placebo (data presented in **Supplementary Information Table S3.8**). The IFG cohort had the opposite result; the meal consumed with cocoa tended to result in greater GIP excursion and C_{MAX} ($p = 0.0560$ and $p = 0.0559$, respectively) but not AUC ($p = 0.1717$). GLP-1 AUC tended to be lower for IFG compared to NFG with no significant effects from the cocoa treatment.

Table 3.3. Calculated area under the curve^a for each blood measure separated by fasting glucose phenotype (NFG and IFG).^b

		Glucose (mg/dL/h)	Insulin (uU/mL/h)	C Peptide (ng/mL/h)	GLP-1 (pM/h)	GIP* (pg/mL/h)
NFG (n = 25)	Placebo	393.4 $\pm$ 12.2	166.0 $\pm$ 15.6	19.5 $\pm$ 1.2	178.8 $\pm$ 14.4	855.9 $\pm$ 74.8
	Cocoa	384.9 $\pm$ 13.6	173.6 $\pm$ 17.8	20.6 $\pm$ 1.8	172.7 $\pm$ 14.2	765.7 $\pm$ 59.3
IFG (n = 5)	Placebo	424.8 $\pm$ 19.2	155.4 $\pm$ 46.9	16.9 $\pm$ 2.8	110.1 $\pm$ 30.2	935.8 $\pm$ 234.8
	Cocoa	451.7 $\pm$ 25.6	174.4 $\pm$ 47.7	18.1 $\pm$ 2.7	134.9 $\pm$ 40.2	1126.8 $\pm$ 157.3
Two-way interaction	p value	0.0812	0.6792	0.9691	0.0732	0.0118*
Main effects for phenotype	p value	0.1035	0.9046	0.4601	0.1351	0.1946
Main effects for treatment	p value	0.3526	0.3347	0.3504	0.2697	0.3422

^aAll values are reported as the mean $\pm$ (SEM).

^bNFG: fasting glucose < 100 mg/dL, IFG: fasting glucose = 100-125 mg/dL

Correlations. Fasting glucose concentration was positively correlated with delta glucose AUC ($R^2 = 0.1530$, $p = 0.0325$) (**Figure 3.2A**). Two h OGTT values were not correlated ($R^2 = 0.0561$, $p = 0.2320$) (**Figure 3.2B**).

Fasting glucose concentration was positively correlated with incretin response (GLP-1, $R^2 = 0.1466$, $p = 0.0368$; GIP, $R^2 = 0.2546$, $p = 0.0045$) (**Figures 3.2C and E**). However, 2 h OGTT response was not correlated with the incretin response (GLP-1, $R^2 = 0.0035$, $p = 0.7562$; GIP, $R^2 = 0.0016$, $p = 0.8334$) (**Figures 3.2D and F**).

Fasting incretin concentration was not correlated to incretin AUC (GLP-1: $R^2 = 0.004$, $p = 0.7331$; GIP: $R^2 = 0.009$, $p = 0.6178$) (**Figure 3.3A and C**). Placebo GIP AUC was associated with the magnitude of change in postprandial GIP AUC ($R^2 = 0.3001$, $p = 0.0017$) (**Figure 3.3D**), but there was no significant correlation with GLP-1 ($R^2 = 0.0678$, $p = 0.1645$) (**Figure 3.3B**).

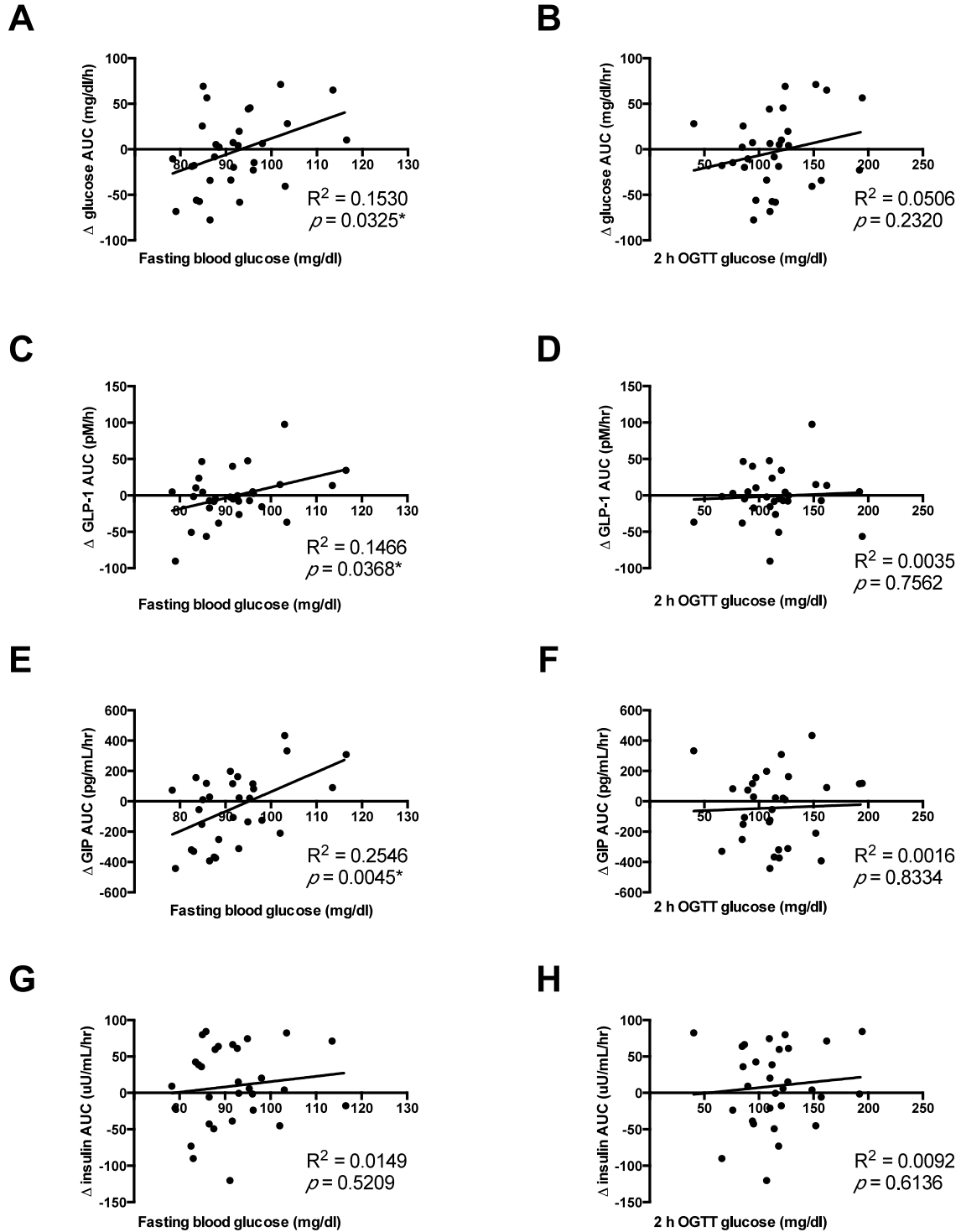


Figure 3.2. Correlations between baseline screening criteria (fasting blood glucose and 2 h OGTT) and delta (Δ) AUC values (cocoa – placebo) for the five blood measures. R^2 represents the coefficient of determination and p-values indicate the difference of the slope from 0. * indicates $p < 0.05$.

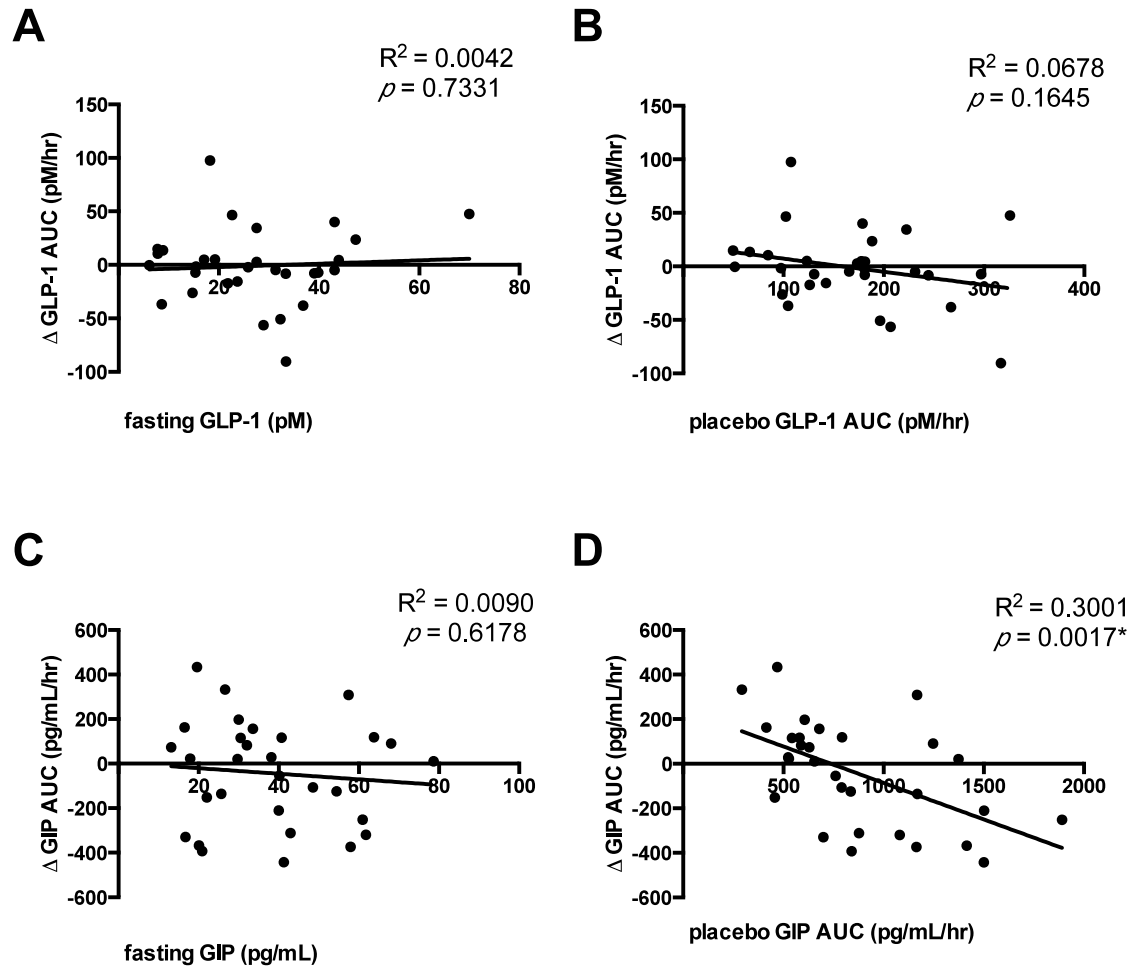


Figure 3.3. Figures A and C represent correlations between fasting incretin concentration [GLP-1 (A) and GIP (C)] and Δ AUC incretin response. Figures B and D represent the correlation between placebo incretin AUC and the delta incretin AUC. R^2 represents the coefficient of determination and p-values indicate the difference of the slope from 0. *indicates $p < 0.05$.

Discussion

The new finding of the present study was that consumption of cocoa powder did not improve glucose homeostasis following a mixed meal in subjects at risk for development of T2DM. The magnitudes of increase in insulin and incretin concentrations were not different following ingestion of cocoa powder or placebo. However, fasting glucose concentration was associated with increased postprandial glucose, GLP-1, and GIP AUC. It appears that individuals with lower fasting blood glucose in our study had the hypothesized glucose response to the cocoa

treatment (reduced postprandial glucose AUC). However, it was the individuals with higher fasting blood glucose that had the hypothesized incretin AUC (increased postprandial GLP-1 and GIP). In addition, cocoa was most effective at increasing GIP AUC in those with the lowest placebo GIP AUC, but this was not observed for GLP-1. This difference could either be due to differences in the incretin hormones in metabolically unhealthy individuals, or perhaps cocoa influences these hormones independently.

Our results suggest that the cocoa powder was most effective at reducing postprandial blood glucose excursions in overweight or obese adults with normal fasting blood glucose concentrations. Although adults with prediabetes would arguably benefit more from a dietary agent to control their blood sugar (in terms of preventing progression from prediabetes to T2DM), adults with normoglycemia could also benefit from an anti-glycemic agent to help prevent the development of prediabetes. However, these were only weak correlations and not all participants with normal fasting blood glucose experienced reductions in postprandial glucose concentration with the cocoa treatment. While participants with impaired fasting glucose values did not have the same reductions in postprandial glucose response, fasting glucose was positively correlated with GLP-1 and GIP response to the meal with cocoa. Due to the insulintropic effects of incretin hormones, an enhanced incretin response would be expected to lead to a reduction in postprandial glucose; however, this was not observed. Alternatively, when glucose tolerance was the independent variable, there were no correlations with incretin hormone response. This suggests that cocoa powder may have a greater influence on incretin hormone response for those with IFG compared to IGT.

Incretin hormone receptors are becoming increasingly popular pharmaceutical targets for diabetes medications. In addition to promoting insulin secretion, incretin hormones may also

slow gastric emptying and increase perceptions of satiety, which are benefits that may have both acute and chronic benefits to individuals at risk for developing T2DM. Most medications trigger an increased incretin effect either by acting as GLP-1 receptor agonists or inhibiting DPP-4. While there is some conflicting evidence, the most current literature indicates that the development of T2DM has dissimilar effects on incretin hormones. GLP-1 secretion appears to be reduced in T2DM^{258,259} but insulinotropic response is preserved,^{258,260,261} while postprandial GIP secretion is normal or increased^{259,262-264} but its function is impaired.^{260,261,265,266} Therefore, our initial hypothesis of increased incretin hormone response may actually not be ideal for all adults. In addition, changes in postprandial incretin secretion seem to develop after T2DM has already been established,^{265,267} which may help explain differences in the literature regarding incretin response in T2DM patients *versus* healthy controls. One study²⁶⁷ found no differences in incretin response in adults with recent onset of T2DM (3.2 ± 2.8 y) compared to healthy controls, but studies with patients with longer diabetes duration^{139,258} identified deficits in GLP-1 secretion. Because the participants in the present study did not have diabetes, the described impairments in incretin secretion and function are likely not remarkable in this cohort. However, there were variations in our *post hoc* analysis between GLP-1 and GIP so the interpretation of these hormones must be considered separately.

Our data suggests that participants with the smallest GIP response (postprandial placebo AUC), perhaps a “healthy” GIP response according to the current literature,²⁶² responded as hypothesized to the cocoa treatment. This demonstrates that cocoa may be most effective in enhancing postprandial GIP when there is an unimpaired GIP secretion. This is consistent with our results that indicate cocoa functioned as hypothesized to those with lower fasting blood

glucose concentration. This effect was not seen in GLP-1 where there were no changes from the cocoa, further highlighting the differences between these two hormones in this population.

Polyphenols have been reported to increase postprandial incretin hormone concentrations in healthy adults in some studies.²⁶⁸⁻²⁷¹ Our data encourage more research to identify ways to stimulate incretin hormone response in overweight and obese adults. Although our study was not powered to detect significant differences in incretin response for adults with IFG or IGT, this is a potential area warranting further investigation.

One acute treatment with cocoa may not be sufficient to attenuate glucose excursions in prediabetic adults, but perhaps chronic administration of cocoa or other dietary agents that can alter glucose excursions or incretin concentration could result in small, incremental improvements leading to a detectable improvement in glycemia over time. Further, our participants were relatively healthy, despite being overweight or obese and having some evidence of hyperglycemia in two thirds of participants. The effects of cocoa may become more apparent in adults with greater impairments or overt diabetes. Furthermore, as discussed above, the effects in normoglycemic individuals suggest that some effects of cocoa may be in preventing healthy subjects from progressing to prediabetes, which would only be observable in long-terms studies measuring onset of prediabetes in healthy subjects, a difficult task.

We selected meal-like beverages instead of glucose-only beverages in an effort to mimic a typical meal consumed by adults. As such, the glycemic response was likely smaller than that expected from a glucose-only beverage. Perhaps using a larger glycemic load would have improved our ability to detect an effect of cocoa powder ingestion on our outcomes. Future studies will be necessary to explore this possibility. We screened participants for inclusion with a glucose beverage, but then tested them with a mixed meal. As such, the mixed meal may have

not been a large enough stimulus to distinguish the effect of cocoa on the incretin response. Our NGT cohort had a rather small increase in blood glucose levels, only an average of about 20 mg/dL at the 1 h time point. It was clear that this meal was not, in terms of glucose load, very “challenging” to them. Another consequence of using a mixed meal is that the effect of the cocoa will be somewhat blunted compared to a simple sugar beverage challenge. Cocoa flavanols can bind to the proteins in the meal, thus reducing the amount of free flavanols to interact with digestive enzymes, glucose transporters, etc., resulting in fewer opportunities for cocoa to have an effect on glycemic response. Protein digestion will eventually free the flavanols, but the flavanol kinetics will be blunted.²⁷²

There are potential limitations to this study. First, our sample size was relatively small and comprised of mostly Caucasian individuals ($n = 28$) so our data may not apply to other racial/ethnic groups or younger adults (< 40 y) at risk for developing T2DM. Lastly, our measurements were limited to hourly blood samples. Other studies²⁷³⁻²⁷⁶ investigating glycemic, insulin, and incretin responses to a meal have used more frequent sampling (15 or 30 minute increments) in order to facilitate more precise excursion and AUC data, especially considering the short half-lives of the incretin hormones (2 minutes for GLP-1 and 5-7 minutes for GIP). One study with healthy adults found that glucose, insulin, GLP-1, and GIP all reached maximum values less than 1 h after consuming a mixed meal.²⁷³ Future studies should consider more frequent sampling intervals than 1 hour.

Future directions should aim to identify the incretin response to cocoa powder in a prediabetic population and should consider each hormone separately. Using a meal or beverage with a higher carbohydrate load and frequent blood sampling may provide the best opportunity to identify the incretin hormone response to cocoa powder.

Conclusions

In summary, the results of the present study suggest that cocoa powder did not significantly effect postprandial glucose homeostasis. Participants with lower fasting blood glucose had the hypothesized reduction in glucose AUC with cocoa compared to the placebo. In addition, participants with the lowest GIP AUC with the placebo beverage had the hypothesized enhanced GIP response with cocoa. Taken together, this suggests that therapies targeting incretin hormones, possibly via dietary agents such as cocoa, could be implicated for overweight or obese adults with normoglycemia and prediabetes. More research is needed to understand the various subtypes of prediabetes and their individual responses to cocoa.

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Chapter 4. Four Weeks of Dietary Cocoa Consumption Does Not Influence Insulin Sensitivity, Gut Permeability, or Metabolic Endotoxemia in Overweight and Obese Adults at Risk of Developing Diabetes

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Abstract

Cocoa flavanols have been shown to reduce blood glucose concentration, increase insulin sensitivity, and improve gut barrier function, all of which would be beneficial for individuals with metabolic syndrome. We hypothesized that 4 weeks of cocoa supplementation would increase insulin sensitivity and enhance skeletal muscle substrate flexibility and, if observed, that these changes would be associated with reduced gut permeability and circulating endotoxin concentrations. Fifteen overweight or obese adults were randomized to receive 15 g cocoa powder/day (or placebo) for 4 weeks while on a controlled, isocaloric diet (55% carbohydrates, 15% protein, 30% fat) in a parallel group design. The participants were 55.5 ± 2.5 years old and had a body mass index of 35.7 ± 2.5 kg/m². Insulin sensitivity, skeletal muscle substrate metabolism, gut permeability, and endotoxin response to a high fat meal were assessed before and after the intervention. Changes in insulin sensitivity, measured via intravenous glucose tolerance test (IVGTT), were not different [cocoa: 0.94 ± 1.12 , placebo: 0.69 ± 1.78 (μl)⁻¹*min⁻², $p > 0.05$]. Glucose oxidation increased in the fasted state (pre: 3.28 ± 0.55 , post: 5.54 ± 0.72 nmol/mg protein/h, $p < 0.05$) and total fatty acid oxidation increased in the fed state (pre: 8.43 ± 1.14 , post: 14.19 ± 1.71 nmol/mg protein/h, $p < 0.05$) for cocoa but not in the placebo group. There were no changes in gut permeability or endotoxin in response to the high fat meal ($p > 0.05$). Future research is necessary to further understand the effects of cocoa on metabolism in overweight and obese populations at risk for developing diabetes. Funding was provided by The Hershey Company, Hershey, PA.

Introduction

The prevalence of disease related to metabolic dysfunction results in tremendous economic and social burdens in the United States and worldwide. Approximately 35% of adults in the United States are estimated to have metabolic syndrome, a combination of cardiometabolic disease risk factors that increases one's risk for developing diabetes and cardiovascular disease.²⁷⁷ Treatments are needed to prevent progression to these diseases as well as strategies to reduce the prevalence of metabolic disease altogether. Recently, cocoa has been investigated for its ability to prevent and/or ameliorate obesity, diabetes, cardiovascular dysfunction, and inflammation.^{22,251,255,278}

Cocoa contains high levels of flavan-3-ols that have been shown to exhibit anti-diabetic and anti-obesity activities such as inhibition of carbohydrate and lipid digestion,^{37,252} improvement of insulin sensitivity in skeletal muscle *in vitro*,⁸⁴ and whole-body effects such as reduced inflammation,^{85,97} and improved insulin sensitivity and glucose tolerance^{93,104,279} in animal studies *in vivo*. Cocoa also contains other bioactive components, including fiber, theobromine, organic acids, and sterols which may also have anti-diabetic effects.^{35,38}

Metabolic endotoxemia is a component of metabolic disease and is indicated by elevated plasma lipopolysaccharide (LPS) concentrations.²⁸⁰ Endotoxin, or LPS, derived from the outer membrane of gram-negative bacteria acts as a pro-inflammatory agent in circulation and in tissues such as skeletal muscle.^{281,282} Low-grade, persistent inflammatory tone is observed with metabolic diseases and is thought to be a causative as well as symptomatic of metabolic syndrome.^{168,280} The insulin-signaling pathway can be impaired by low-grade inflammation,¹⁶⁸ and thus, glucose intolerance and insulin

resistance are characteristics of metabolic endotoxemia, as well. Flavan-3-ols are known to have anti-inflammatory effects^{283,284} and reduction of this chronic, low-grade inflammation may represent a mechanism by which dietary cocoa may ameliorate metabolic disease. In addition, recent evidence has suggested that cocoa procyanidins can attenuate metabolic endotoxemia, but the primary mechanisms are unclear.^{85,279}

Poor gut barrier function is also associated with metabolic endotoxemia because a leaky gut can allow endotoxin to enter the circulation.²⁸⁵ Cocoa flavan-3-ols can exert prebiotic effects in both animals and humans.^{162,286} This property of cocoa may be beneficial for patients with metabolic disease due to the association between a healthy microbiome and colon, and improvements in gut barrier function. There is also evidence to suggest that flavan-3-ols can reduce intestinal permeability,²⁸⁷ including permeability to endotoxin.^{288,289} However, it is unknown if cocoa can exert these effects in humans, particularly an at-risk population.

Cocoa flavan-3-ols have been reported to improve insulin sensitivity in adults that were healthy,²⁹⁰ hypertensive,¹⁰⁸ hypertensive and impaired glucose tolerant,¹⁰⁹ overweight and obese,^{112,291} and elderly with mild cognitive impairment.¹¹⁴ However, whether cocoa improves insulin sensitivity in adults at risk of developing type 2 diabetes is unknown. We hypothesized that 4 weeks of cocoa supplementation would increase insulin sensitivity and enhance skeletal muscle substrate flexibility and, if observed, that these changes would be associated with reduced gut permeability and circulating endotoxin concentrations.

Methods

IRB Approval is presented in Appendix A. Detailed materials and methods are presented in Appendix E. The Informed Consent Form is presented in Appendix F. Supplementary information is presented in Appendix G.

Participants. Fifteen overweight or obese (body mass index [BMI], 25-40 kg/m²) adults (40-75 years old) from the New River Valley, VA completed the study. Participants were weight stable ($\pm$ 2.5 kg in previous 6 months), sedentary to recreationally active ($\leq$ 2 days/week of 20 min/day of low-intensity physical activity), and not taking any medications or supplements that would affect study variables. Participants were free from chronic disease as determined by a health history questionnaire, lipid panel, and urinalysis. Further, participants were defined as at-risk for developing diabetes by having at least one of the following criteria: fasting blood glucose concentration of 100-125 mg/dL, 2 h oral glucose tolerance test (OGTT) blood glucose concentration of 140-199 mg/dL, HbA1c of 5.7-6.4%, or a score $\geq$ 5 on the American Diabetes Association Risk Assessment questionnaire. See **Supplementary Information Table S4.1** for inclusion/exclusion criteria and **Figure S4.2** for CONSORT flow diagram. Eligible and interested participants provided written informed consent. Twenty participants enrolled and 15 completed the study. One participant discontinued the study because of lactose intolerance, two disliked the standardized diet, one had a family emergency, and another was uncomfortable continuing with the testing procedures. There were no adverse events. Approval from the Virginia Tech Institutional Review Board (IRB) was obtained

for all aspects of the study involving human subjects (IRB# 13-755). The study was registered at ClinicalTrials.gov (NCT02203240).

Experimental design. This study was a randomized, double-blinded, placebo-controlled trial conducted at Virginia Tech in Blacksburg, VA in Summer 2014 through Fall 2015. Testing procedures were conducted in 3 visits before and after the 4-week intervention (**Figure 4.1A**). The intervention included daily cocoa/placebo treatments and controlled feeding procedures. Subjects were randomized to receive the cocoa or placebo treatment with gender stratification. Both the participants and the investigators involved in data collection and analysis were blinded to the code.

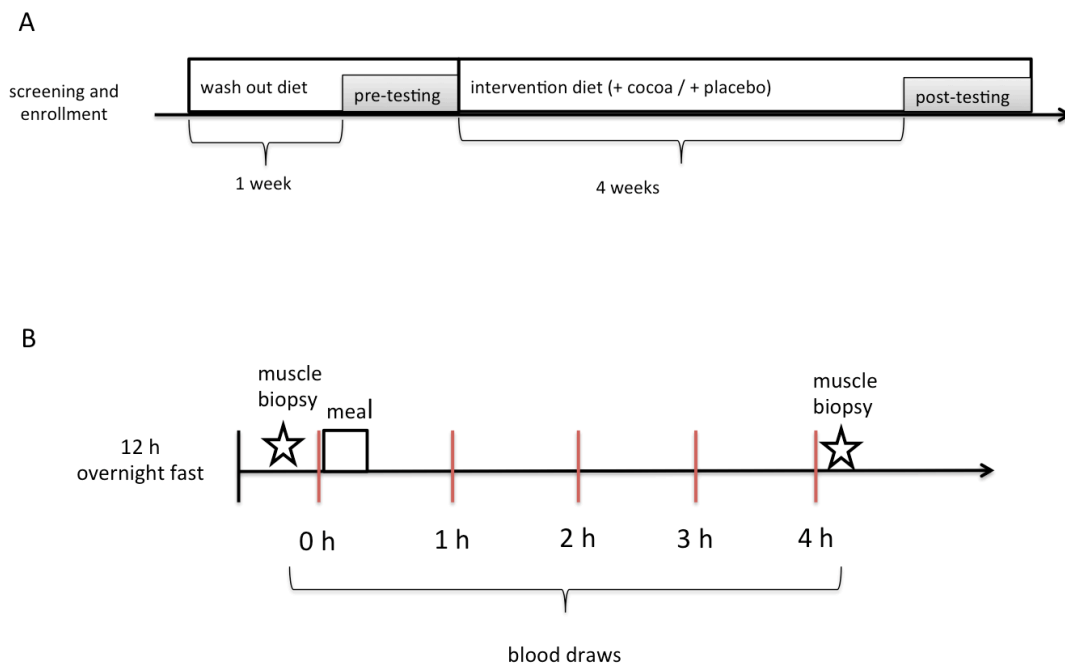


Figure 4.1 A) Study timeline. The study began with a 1-week wash out diet prior to the first day of testing. The wash out diet was continued throughout pre-testing. Three testing visits were completed over the course of 4-5 days. After the 4-week intervention diet, the three testing visits were completed again. The intervention diet was continued throughout post-testing. **B)** Testing procedures for the high fat challenge. Participants arrived in the laboratory between 6-9 A.M. after a 12-h overnight fast. They first underwent the muscle biopsy procedure. An intravenous catheter was inserted to obtain the baseline (fasted) blood draw. Participants then consumed the high fat meal within ten minutes. Participants remained seated and awake in the laboratory over the next four hours for the hourly blood samples. A second biopsy was completed after the 4 h blood sample.

Wash out diet. For one week prior and throughout baseline testing, subjects consumed their habitual diet but avoided consuming foods and beverages that contained high amounts of flavan-3-ols (i.e. cocoa, chocolate, apples, berries, wine, almonds, etc.) or other ingredients/foods that may have affected the study outcomes (i.e. yogurt, prebiotics, probiotics, etc.) (see **Supplementary Information Table S4.2** for full list).

Cocoa and Placebo Treatments. Cocoa and placebo beverage dry mixes were individually pre-packaged in coded sachets, from a single production lot and obtained directly from Hershey. The beverages were custom formulations produced by Hershey and designed to support human clinical trials. The daily cocoa treatment contained a total of 1296 mg total polyphenols, 76 mg epicatechin, and 797 mg total proanthocyanidins consumed in three separate doses throughout the day. The daily placebo treatment contained a total of 371 mg total polyphenols, 0 mg epicatechin, and 0 mg total proanthocyanidins. Full characterization and nutrition content of cocoa and placebo packets are described in **Table 4.1** (The Hershey Co., Hershey, PA). Participants consumed three treatment packets daily for four weeks. They were provided with a

shaker bottle and instructions to mix the contents of one sachet with hot or cold water and consume one sachet with each meal.

Table 4.1. Nutrient and polyphenol composition of cocoa and placebo dry mixes.^a

Analysis	Weight Basis (per 100 g)		Individual Sachet		Daily Intake (3 Sachets)	
	Cocoa	Placebo	Cocoa	Placebo	Cocoa	Placebo
Weight, g	100	100	18	17	54	51
Calories	309	354	56	60	167	180
Fat, g (calculated)	4	1	1	0	2	1
Sat fat, g	2	1	0	0	1	0
Trans fat, g	0	0	0	0	0	0
Cholesterol, mg	21	29	4	5	12	15
Sodium, mg	830	971	149	165	448	495
Carbohydrates, g	53	53	10	9	29	27
Dietary fiber, g	11	2	2	0	6	1
Sugar, g	35	47	6	8	19	24
Protein, g	29	32	5	5	16	16
Vitamin A, IU	15	20	3	3	8	10
Vitamin C, mg	5	6	1	1	2	3
Calcium, mg	866	1199	156	204	467	612
Iron, mg	12	0	2	0	6	0
Magnesium, mg	211	98	38	17	114	50
Potassium, mg	1613	1597	290	271	871	814
Caffeine, mg	65	0	12	0	35	0
Theobromine, mg	573	0.4	103	0	309	0
Total Polyphenols, mg	2400	727	432	124	1296	371
Catechin, mg	34.75	0	6	0	19	0
Epicatechin, mg	140	0	25	0	76	0
Total Proanthocyanidins (PAC), mg	1475	0	266	0	797	0
Total PAC DP ^b 1-10, mg	854.25	<0.001	154	<0.001	461	<0.001
PACs DP 1	190	<0.001	34	<0.001	103	<0.001
PACs DP 2	113	<0.001	20	<0.001	61	<0.001
PACs DP 3	75	<0.001	14	<0.001	41	<0.001
PACs DP 4	89	<0.001	16	<0.001	48	<0.001
PACs DP 5	91	<0.001	16	<0.001	49	<0.001
PACs DP 6	110	<0.001	20	<0.001	59	<0.001
PACs DP 7	133	<0.001	24	<0.001	72	<0.001
PACs DP 8	45	<0.001	8	<0.001	24	<0.001
PACs DP 9	58	<0.001	10	<0.001	31	<0.001
PACs DP 10	20	<0.001	4	<0.001	11	<0.001

^aValues provided by The Hershey Co., Hershey, PA.^bDP: degrees of polymerization

Controlled feeding. The controlled feeding portion of the study was conducted at the Metabolic Kitchen and Dining Laboratory at Virginia Tech. Subjects were provided with

all of their food during the intervention and were instructed not to consume anything else or add any additional ingredients (condiments, salt, pepper, etc.) to their food. Subjects were required to come to the laboratory a minimum of 3 days per week in order to have a supervised breakfast and treatment beverage, receive food, and track body weight. Subjects were weighed at each visit and were asked to report any intake of food or beverages not provided to them. Compliance was tracked by body weight and any uneaten items returned to the metabolic kitchen. Subjects remained on the diet throughout the post-testing period.

Subjects consumed an isocaloric, controlled diet (55% carbohydrate, 30% fat, 15% protein) during the 4-week intervention and post-testing. The energy and macronutrient content of the diets included the energy and macronutrients contained in the cocoa treatment (or placebo) products. The saturated fat (SFA) content of the controlled diet was <10% of total energy intake. Fiber intake was matched to typical U.S. intake, at 7.5 g/1000 kcal.²⁹² Sodium was restricted to 3,500 mg/d, which is similar to typical U.S. intake.²⁹³ Controlled diets were limited in polyphenol-rich foods (e.g., apples, tea, grapes, etc.). If subjects were habitual coffee drinkers, black coffee consumption was limited to 12 oz/d.

Energy requirements for each participant were estimated based on height, weight, age, gender and an activity factor based on self-reported physical activity levels.²⁹⁴ Caloric content of the diets ranged from 1,500-3,500 kcal/d. A 7-day rotation menu was planned using Nutrition Data System for Research (NDSR) software version 2013 (University of Minnesota, Minneapolis, MN) by a registered dietitian. A sample menu with nutrient breakdown is included in the **Supplementary Information Table S4.3**.

The individual macronutrients and saturated fat content of the diets were within ± 5 g of the target values for each day. The controlled diet was designed to maintain body weight and to reduce the variability in the individual participant's diets and its potential impact on the study outcomes. Additional food items in the form of 250 kcal modules (with a macronutrient composition matched to the overall diet) were provided to adjust kcal level and counter any trends in weight loss or gain during the intervention.

Study Procedures. The following procedures were performed before and after the 4-week intervention.

Body Weight, Height and Composition. Body weight was measured on a digital scale (Model 5002, Scale-Tronix, White Plains, NY) to the nearest ± 0.1 kg and height was measured on a stadiometer to the nearest ± 0.1 cm. Body composition was determined by dual-energy X-ray absorptiometry (Lunar iDXA enCORE v15) (General Electric Medical Systems Ultrasound & Primary Care Diagnostics, LLC, Madison, WI).

Intravenous Glucose Tolerance Test (IVGTT). Insulin sensitivity was determined using Bergman's minimal model (MINMOD Millennium software, version 6.02, Minmod, INC., Pasadena, CA) via a frequently sampled IVGTT.^{295,296} Subjects arrived at the laboratory between 5-9 A.M. after a 12 h overnight fast. An intravenous catheter was inserted into each antecubital vein (one arm was used for dextrose and insulin injections and the other for blood sampling). Baseline blood samples were taken prior to the dextrose injection (0.3 g/kg; 50% solution) (Hospira, Inc., Lake Forest, IL) (minute 0).

Venous samples (10 ml) were collected at minutes 1, 2, 3, 4, 5, 6, 7, 8, 10, 12, 14, 16, and 18. Insulin (0.025 U/kg) (Lilly USA, LLC, Indianapolis, IN) was injected at minute 20. Venous sampling continued at minutes 22, 23, 24, 25, 27, 30, 40, 50, 60, 70, 80, 90, 100, 120, 150, and 180. Glucose concentration was immediately analyzed by the glucose oxidase method on an automated glucose oxidase analyzer (Yellow Springs Instruments, Yellow Springs, OH). Insulin was later measured from serum using the Immulite 1000 immunoassay analyzer (Siemens, Deerfield, IL).

High Fat Meal Challenge and Muscle Biopsies. Subjects consumed a high fat meal containing 820 kcal, 52 g total carbohydrates, 24 g protein, and 58 g fat (for complete nutrient contents see **Supplementary Information Table S4.4**). Subjects consumed the meal within ten minutes and were provided an optional bottle of water. Blood samples were collected at baseline and 1, 2, 3, and 4 h after the first bite of the meal (**Figure 4.1B**).

During the high fat challenge, two skeletal muscle biopsies were conducted on alternate legs (see **Figure 4.1B**). Biopsies of the *vastus lateralis* were performed using a 5 mm modified Bergström needle (Cadence, Staunton, VA).²⁹⁷ The skin was cleaned with a providone-iodine swab (PDI Inc., Orangeburg, NY) and a sterile field was created. The skin and muscle was numbed with a local anesthetic (1% lidocaine) (Hospira Inc., Lake Forest, IL). A 0.25 in incision was made with a #11 scalpel (Feather Safety Razor Co. LTD., Kita-Ku, Osaka, Japan). Suction was applied to the Bergström needle in order to remove small samples of the muscle. Collected tissue was washed in 0.9% sterile saline to remove blood and tissue. Samples were weighed and either flash frozen in liquid

nitrogen or added to buffer containing 250mM sucrose, 1mM EDTA, 10mM Tris-HCL, and 2mM ATP and placed on ice for immediate analysis of substrate flexibility.

Substrate Flexibility. Pyruvate oxidation was used to assess the activity of pyruvate dehydrogenase (PDH), the enzyme that catalyzes the oxidation of pyruvate and the provision of glucose-derived acetyl CoA to the TCA cycle. Pyruvate oxidation was assessed by measuring ^{14}C -CO₂ production from the oxidation of labeled (1- ^{14}C) pyruvate. Total labeled (1- ^{14}C) palmitate (fatty acid) oxidation rates were determined by measuring and summing ^{14}C -CO₂ production and ^{14}C -labeled acid-soluble metabolites (ASM).²⁹⁸ Samples were analyzed as previously described.²⁹⁹ All samples were run in triplicate and data were normalized to total protein content and expressed in nmol/mg protein/h. Calculated metabolic flexibility is expressed as a ratio of pyruvate oxidation to pyruvate oxidation + free fatty acids. Oxidative efficiency is expressed as a ratio of total fatty acid oxidation (CO₂) to incomplete fatty acid oxidation (i.e., ASM).

Endotoxin Response to High Fat Meal Challenge. Serum endotoxin was measured in duplicate using Limulus Amebocyte Lysate (LAL) Pyrogen[®] 5000 assay kits (Lonza, Walkersville, MD). LAL assay plates, LAL reagent water, and pyrogen-free tubes (all from Lonza) were used. Serum was incubated in a hot water bath (70° C) for 15 min in order to minimize exogenous endotoxin contamination. Assays were performed using 100 µL serum diluted 40:1. Fluorescence was quantified using a BioTek Synergy 2 plate reader (Biotek, Winooski, VT), and data was processed using Gen 5 software (v1.08, BioTek). Pseudo-pharmacokinetic (PPK) curves and PPK parameters for serum endotoxin concentrations were constructed using PK plug-in functions for Microsoft Excel. PK parameters determined include: C₀ (fasting concentration), C_{MAX} (maximal

concentration observed), excursion ($C_{\text{MAX}} - C_0$), and AUC (area under the PK curve).

Gut Permeability. The four-sugar [40 g sucrose (N.F. grade), 1 g mannitol (U.S.P. grade), 1 g sucralose (N.F. grade), (Spectrum Chemicals, New Brunswick, NJ) and 5 g lactulose (U.S.P. grade) (Qualitest Pharmaceuticals, Huntsville, AL)] probe test was used to assess total gut permeability.³⁰⁰ After an overnight fast and urine evacuation, participants were asked to consume the sugar-probe beverage in 5 minutes or less. Participants were given two breakfast sandwiches (see **Supplementary Information Table S4.3** for nutrient content), which were the only items they were allowed to consume during the first 5 hours of the test in order to reduce confounding dietary factors (i.e. high sucrose content). This meal was selected for its ease of distribution and low sucrose content. Participants were also provided with one 16 oz bottle of water, which they were instructed to finish by the first hour of the test to promote urination during the first collection period. Participants were given two urine collection containers with 5 g of 10% thymol in methanol (w/v) (an antimicrobial preservative). Participants were instructed to collect all of their urine from the time the beverage was consumed (0 h) until 5 h in the first container, in order to capture gastric and small intestinal permeability. The second container was filled between 6-24 h to assess colonic permeability. Urine containers were returned after 24 h. Participants were asked to avoid consuming artificial sweeteners, caffeine, and alcohol during this test. The volume of urine in each container was measured and aliquots from each container were stored at -80°C until further analysis. Urinary sugars were measured as previously described.^{301,302} Retention times are displayed in **Supplementary Information Table S4.5** and extraction and quantification

methods are described in the **Supplementary Information**. Urinary sugar concentrations were converted to total sugar excreted using urine volume. Excretion was calculated as a % of total sugar dose recovered in urine for 0-5 and 6-24 h samples. Increased values indicate increased permeability. Gastroduodenal permeability was defined as sucrose/mannitol ratio (SMR) (0-5 h),^{303,304} as mannitol is a constant measure of epithelial surface area.³⁰⁰ Small intestinal permeability was defined as the calculated lactulose/mannitol ratio (LMR) for 0-5 samples.³⁰¹ Finally, colonic permeability was defined as both the 6-24 h LMR and 6-24 h sucralose/mannitol ratio.^{304,305}

Statistics. Multiple-sample repeated measure analysis of variance with a between-subject factors approach was used to test the effect of group, time, and group x time effects. Three-way mixed ANOVA was used to assess any interactions between treatment, high fat meal condition, and time for measures of substrate metabolism. Analyses of covariance were conducted with the baseline values set as the covariate. Changes over time, delta (Δ) values, are equal to post-intervention minus pre-intervention. Significance was defined *a priori* at the $P < 0.05$ level. Statistical analyses were performed on Prism v6 (GraphPad, La Jolla, CA) or IBM SPSS (IBM Corp., Armonk, NY).

Results

Subject characteristics. Fifteen participants completed the study (**Table 4.2**). Participants were an average of 55.5 ± 2.5 years old and had a BMI of 35.7 ± 2.5 kg/m². Six of the participants ($n = 3$ per group) qualified based on the ADA Risk Assessment

Questionnaire alone. The remainder of the participants ($n = 9$) were classified as prediabetic by the ADA criteria.²⁴⁰

Table 4.2. Baseline subject characteristics.

Parameter	All subjects	Cocoa	Placebo	<i>p</i> -value ^a
n	15	7	8	-
Physiological parameters				
Female/male	5/10	3/4	2/6	-
Age (yr)	55.5 ± 2.5	52.1 ± 3.8	58.5 ± 3.1	0.21
Weight (kg)	98.4 ± 3.5	98.3 ± 6.2	98.5 ± 4.0	0.97
BMI (kg/m ²)	35.7 ± 2.5	33.4 ± 1.7	37.6 ± 4.5	0.42
Serum triglycerides (mg/dL)	120 ± 12	124 ± 14	117 ± 20	0.79
Serum total cholesterol (mg/dL)	193 ± 9	203 ± 7	185 ± 15	0.30
ADA Risk Score ^b	6 ± 0	5 ± 0	6 ± 0	0.07
FBG ^c (mg/dL)	90.3 ± 2.4	84.6 ± 2.8	95.3 ± 3.0	0.02*
2 h OGTT ^d (mg/dL)	99.6 ± 7.4	99.0 ± 10.7	100.1 ± 11.0	0.94
HbA1c (%)	5.6 ± 0.1	5.6 ± 0.2	5.6 ± 0.1	0.85
Self-reported habitual nutrient intake				
Energy (kcal)	2275 ± 144	2270 ± 264	2279 ± 160	0.98
Fat (%)	37.7 ± 1.5	33.5 ± 2.0	41.4 ± 1.3	0.005*
SFA (%)	12.0 ± 0.4	11.7 ± 0.6	12.2 ± 0.4	0.57
Carbohydrates (%)	44.5 ± 1.7	49.1 ± 2.1	40.4 ± 1.5	0.004*
Protein (%)	15.8 ± 0.9	15.6 ± 0.8	16.0 ± 1.5	0.79
Alcohol (g)	2.0 ± 0.6	1.8 ± 1.2	2.2 ± 0.6	0.80

^at-test comparing cocoa cohort to placebo cohort. * $P < 0.05$

^bScore on ADA diabetes risk assessment questionnaire

^cFBG: fasting blood glucose concentration

^d2 h OGTT: blood glucose concentration 2 h after consuming 75 g glucose beverage

Values are reported as the mean ± SEM.

Body composition. Following the intervention, there was a significant reduction in body weight in the placebo group (pre: 96.8 ± 3.7 kg; post: 96.1 ± 3.7 kg) ($p = 0.0350$). Body weight did not change ($p > 0.05$) in the cocoa group (**Figure 4.2**). As a result of the intervention, the placebo group had a significant reduction in fat mass (pre: 38.4 ± 1.3 kg; post: 37.2 ± 1.5 kg) ($p = 0.0365$) and the reduction in lean mass approached significance (pre: 55.9 ± 3.3 kg; post: 54.9 ± 3.2 kg) ($p = 0.0749$). There were no changes in fat or

lean mass for the cocoa group as a result of the intervention. There were no differences in percent body fat for either group.

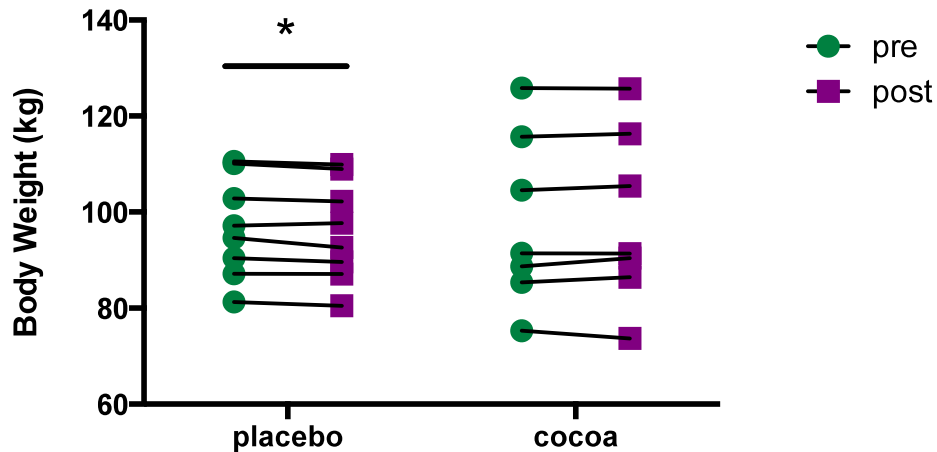


Figure 4.2. Individual changes in body weight of participants in each treatment group before (●) and after (■) the intervention. A paired t-test determined a significant difference in the placebo group from pre to post intervention ($p = 0.0350$).

Insulin Sensitivity. Two participants in the placebo group were unable to complete baseline or follow up IVGTT's due to difficulties obtaining sufficient blood throughout the duration of the test. Fasting glucose concentration was higher in the placebo group and approached significance at pre-testing (93.2 ± 3.4 vs. 84.8 ± 1.9 mg/dL, $p = 0.0605$) (Table 4.3). There were no significant differences in any of the IVGTT-related variables between the two groups following the intervention and no differences as a result of the intervention (Table 4.3).

Table 4.3. Intravenous glucose tolerance test (IVGTT)-related variables before and after the intervention.

Treatment		Fasting glucose (mg/dL)	Fasting Insulin (uU/mL)	AIRg ($\mu\text{U}\cdot\text{l}^{-1}\cdot\text{min}$)^a	DI^b	SI [($\mu\text{U}/\text{l}$)⁻¹ $\cdot\text{min}^{-2}$]^c	Sg (min^{-1})^d	β Cell Function ($\mu\text{U}/\text{mM}$)	Insulin Resistance ($\text{mM}\cdot\mu\text{U}/\text{l}^2$)
Placebo	Pre ^e	93.2 $\pm$ 3.4	24.1 $\pm$ 10.6	477 $\pm$ 135	2138 $\pm$ 782	5.74 $\pm$ 2.24	0.02 $\pm$ 0.00	370 $\pm$ 164	6.03 $\pm$ 2.70
	Post ^e	92.8 $\pm$ 4.7	32.7 $\pm$ 15.1	519 $\pm$ 118	2359 $\pm$ 653	6.09 $\pm$ 2.29	0.02 $\pm$ 0.00	470 $\pm$ 272	6.93 $\pm$ 3.32
	Δ^f	-0.35 $\pm$ 3.0	8.6 $\pm$ 6.5	8 $\pm$ 60	228 $\pm$ 1143	0.69 $\pm$ 1.78	0.00 $\pm$ 0.00	170 $\pm$ 123	1.17 $\pm$ 1.02
Cocoa	Pre ^g	84.8 $\pm$ 1.9	9.0 $\pm$ 1.5	643 $\pm$ 189	2200 $\pm$ 514	4.10 $\pm$ 0.72	0.02 $\pm$ 0.00	164 $\pm$ 25	1.86 $\pm$ 0.33
	Post ^g	83.3 $\pm$ 3.7	9.4 $\pm$ 1.6	622 $\pm$ 181	2214 $\pm$ 376	5.04 $\pm$ 1.33	0.02 $\pm$ 0.00	185 $\pm$ 30	1.96 $\pm$ 0.37
	Δ^f	-1.5 $\pm$ 2.9	0.4 $\pm$ 1.0	-21 $\pm$ 52	14 $\pm$ 325	0.94 $\pm$ 1.12	0.00 $\pm$ 0.00	21 $\pm$ 29	0.10 $\pm$ 0.21

^aAIRg = acute insulin response to glucose^bDI = disposition index^cSI = insulin sensitivity^dSg = glucose sensitivity^ePlacebo group, $n = 7$ ^fPlacebo group, $n = 6$; cocoa group, $n = 7$ ^gCocoa group, $n = 7$ Data are reported as the mean $\pm$ SEM.There were no significant differences between groups and no changes as a result of the intervention ($P < 0.05$).

Skeletal muscle substrate metabolism. There were two significant three-way interactions (incomplete fatty acid oxidation, $p = 0.009$ and total fatty acid oxidation, $p = 0.008$) (**Table 4.4, Figure 4.4**). Analyses of covariance (ANCOVA) with pre-testing values as the covariate and post-testing values as the dependent variable were not significant. Independent t-tests were used to compare means of the fasted and fed measures for both treatment groups regardless of if there was a significant three-way or two-way interaction (**Figure 4.3**). A complete statistical analysis of selected measures of substrate flexibility is presented in the **Supplementary Information**.

Table 4.4. Measures of skeletal muscle substrate metabolism in the fasted and fed states before and after the intervention.

		Glucose Oxidation	Fatty Acid Oxidation (CO ₂)	Fatty Acid Oxidation (ASM)	Total Fatty Acid Oxidation	Metabolic Flexibility	Oxidative Efficiency	Pyruvate Oxidation
Placebo								
Pre	Fasted	5.66 ± 0.81	0.44 ± 0.08	7.20 ± 0.63	7.68 ± 0.64	1.45 ± 0.12	0.06 ± 0.01	349.68 ± 34.31
	Fed	10.99 ± 2.15 [#]	1.20 ± 0.37	17.58 ± 2.22 [#]	18.81 ± 2.45 [#]	2.08 ± 0.31	0.07 ± 0.01	477.51 ± 54.99
	Meal effect	99.50 ± 26.17	151.81 ± 31.88	182.12 ± 49.13 [#]	146.91 ± 29.57 [#]	42.10 ± 13.73	9.40 ± 15.55	42.15 ± 15.93
Post	Fasted	5.15 ± 0.55	0.68 ± 0.19	10.42 ± 1.03	11.10 ± 1.17	1.55 ± 0.17	0.06 ± 0.01	327.68 ± 41.27
	Fed	6.63 ± 0.85	0.78 ± 0.08	13.49 ± 1.23	14.27 ± 1.24	1.62 ± 0.10	0.06 ± 0.01	360.81 ± 43.53
	Meal effect	28.02 ± 8.11	49.53 ± 23.49	41.21 ± 23.83	41.24 ± 23.82	9.98 ± 10.41	10.23 ± 13.89	15.41 ± 15.59
Cocoa								
Pre	Fasted	3.28 ± 0.55	0.40 ± 0.08	6.96 ± 0.81	7.30 ± 0.88	1.49 ± 0.20	0.06 ± 0.01	331.52 ± 41.46
	Fed	4.16 ± 0.63	0.60 ± 0.05	7.94 ± 1.11	8.43 ± 1.14	2.21 ± 0.42	0.09 ± 0.01	296.49 ± 36.54
	Meal effect	34.75 ± 12.43	92.94 ± 47.22	21.71 ± 22.51	23.65 ± 22.10	45.64 ± 14.11	100.03 ± 77.47	-10.21 ± 2.93
Post	Fasted	5.54 ± 0.72	0.46 ± 0.07	8.68 ± 0.45	9.15 ± 0.46	1.30 ± 0.08	0.05 ± 0.01	388.89 ± 52.25
	Fed	5.60 ± 0.87	0.63 ± 0.09	13.56 ± 1.69	14.19 ± 1.71	1.54 ± 0.24	0.05 ± 0.01	302.75 ± 23.86
	Meal effect	23.86 ± 41.42	52.41 ± 29.79	65.67 ± 33.81	64.54 ± 33.30	17.78 ± 14.95	-1.97 ± 19.49	-15.74 ± 10.43
Three-way interaction		0.133	0.093	0.009*	0.008*	0.871	0.329	0.598
Δ meal effect^a		-4.16 ± 33.31	2.88 ± 6.30	24.45 ± 9.98	23.31 ± 9.48	7.80 ± 4.54	-12.20 ± 5.60	-31.15 ± -5.16

*Indicates a significant treatment x meal x time effect ($P < 0.05$)

Indicates a significant treatment x time effect ($P < 0.05$).

^a Δ meal effect is the post intervention meal effect calculated as cocoa – placebo.

Meal effect is calculated as: [(fed-fasted)/fasted] * 100

All units are nmol/mg protein/h, data are expressed as mean ± SEM.

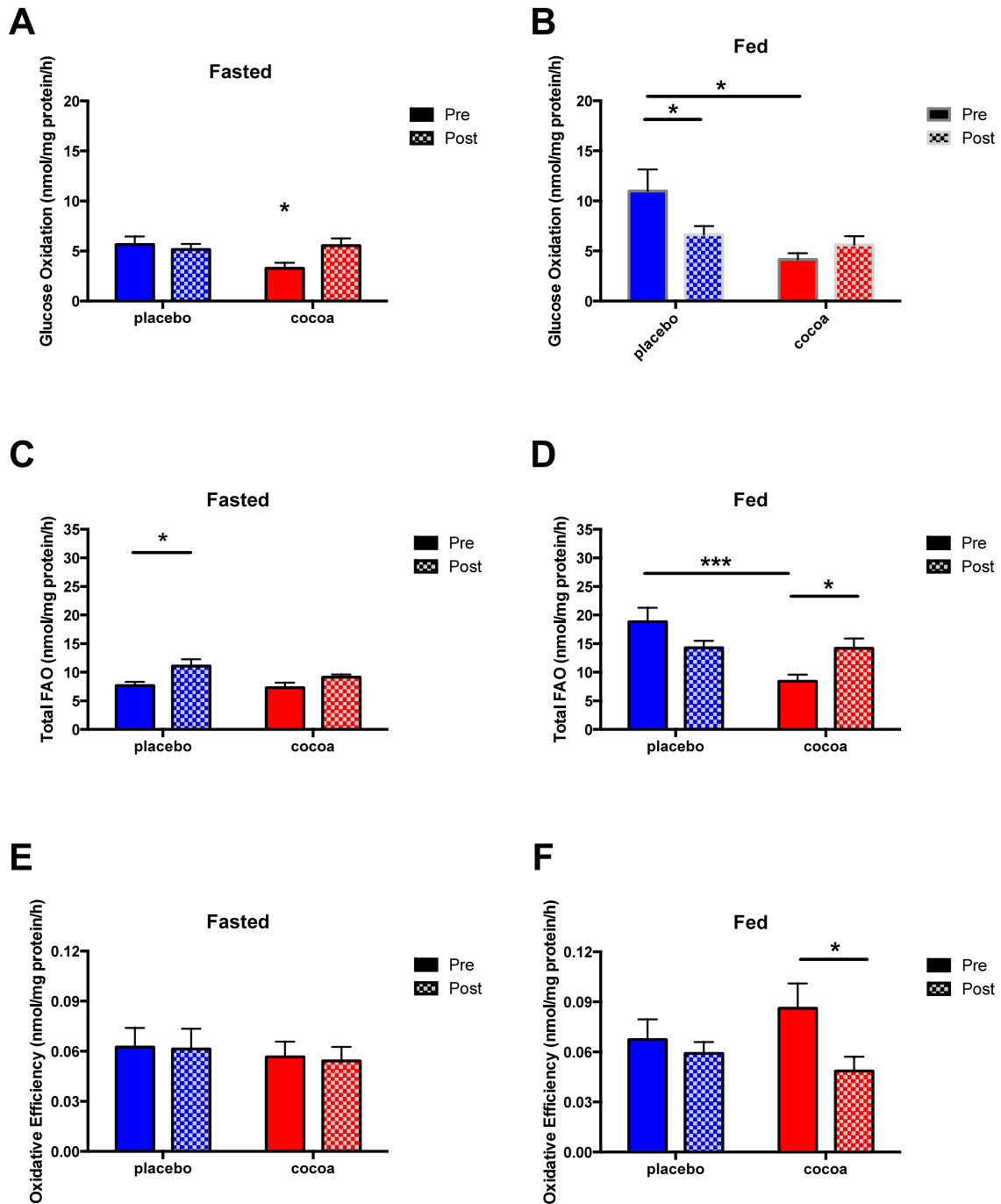


Figure 4.3. Mean $\pm$ SEM of substrate metabolism before (pre) and after (post) the intervention. Figures A, C, and E represent metabolism while fasted, and figures B, D, and F represent metabolism 4 hours after a high fat meal. Differences in dependent variables were assessed by independent t-tests (* $P < 0.05$, *** $P < 0.001$).

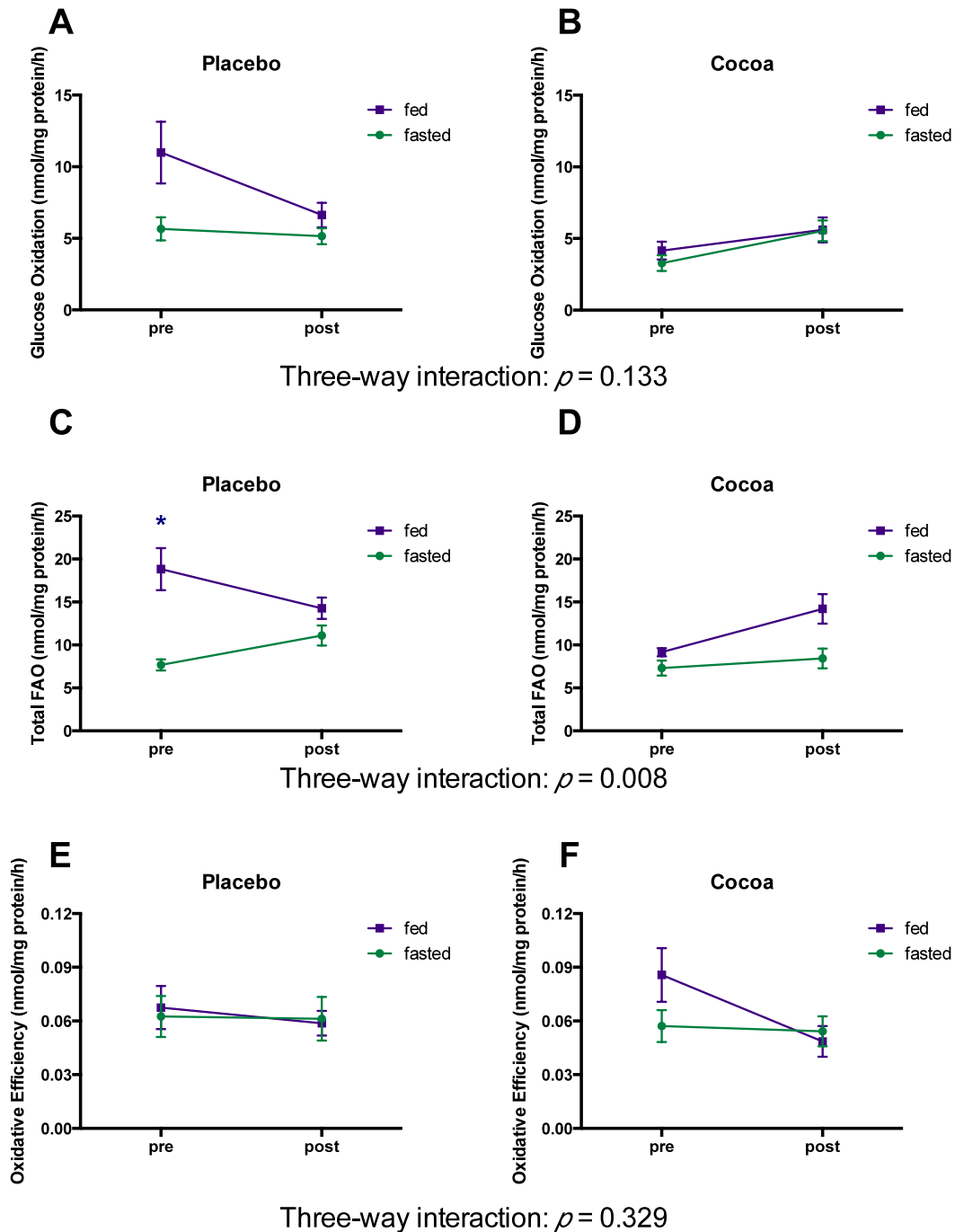


Figure 4.4. The mean ($\pm$ SEM) values for select muscle substrate metabolism measures. Figures A (placebo) and B (cocoa) show glucose oxidation during high fat meal conditions (fasted and fed) before and after the interventions (pre and post). Figures C (placebo) and D (cocoa) represent total fatty acid oxidation (FAO) and figures E (placebo) and F (cocoa) represent oxidative efficiency. Three-way interactions (treatment, meal condition, time) were determined via a 3-way mixed ANOVA. *Indicates statistically significant simple main effects ($P < 0.05$).

Gut Permeability. There were no significant differences in gut permeability between treatment groups before or after the intervention. The change in lactulose:mannitol ratio (6-24 h) approached significance ($p = 0.0814$) indicating an increase in permeability at post-testing compared to pre-testing for both treatment groups (**Figure 4.5**).

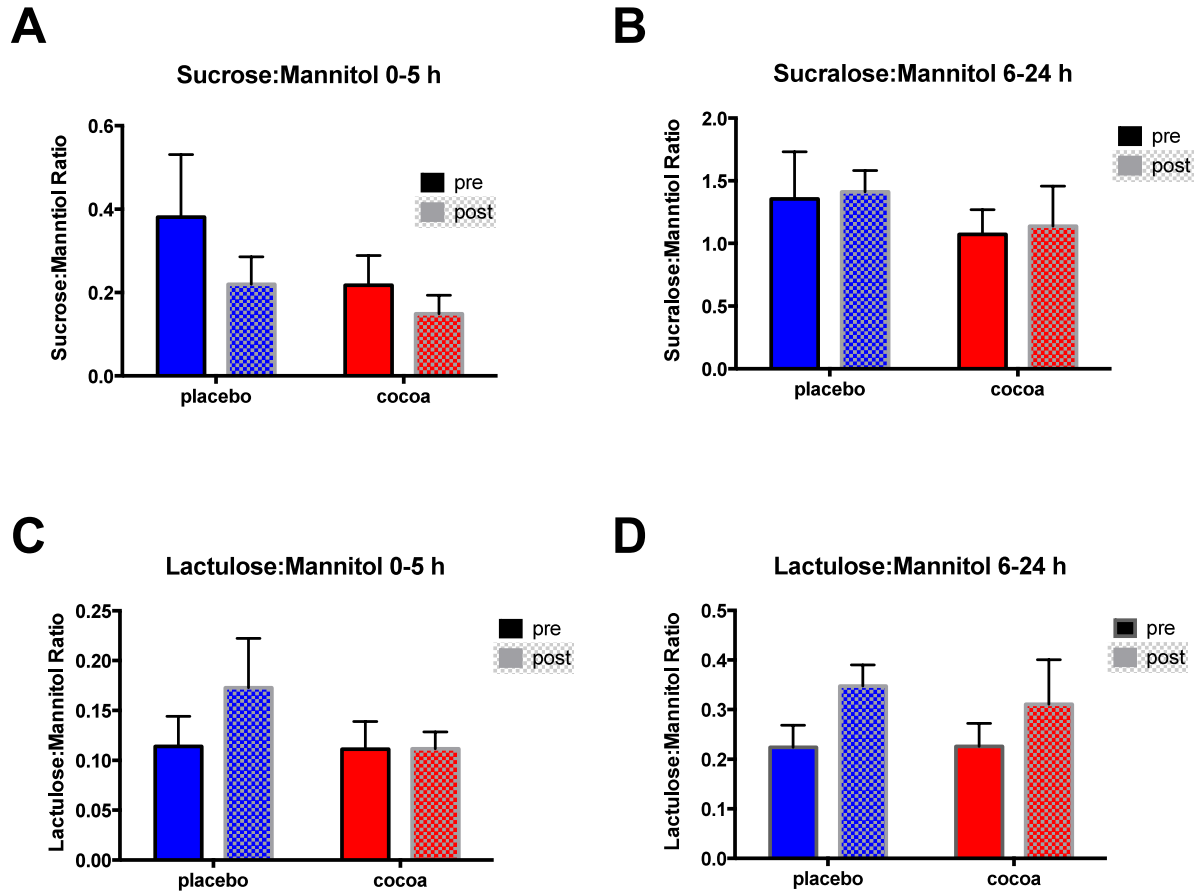
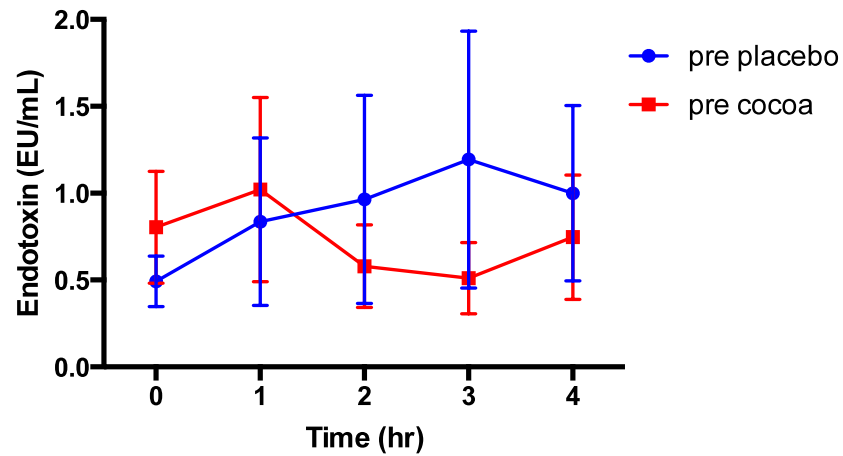


Figure 4.5. Mean ($\pm$ SEM) ratios of excreted sugars during the four-sugar probe gut permeability test. After consumption of the four-sugar probe drink at time 0 h, urine was collected into one container for 0-5 h and a second container from 6-24 h. Figure (A) depicts the sucrose:mannitol ratio from hours 0-5, (B) depicts the sucralose:mannitol ratio from hours 6-24, (C) depicts the lactulose:mannitol ratio from hours 0-5, and (D) depicts the lactulose:mannitol ratio from hours 6-24. After a 2-way repeated measures analysis of variance, there were no significant differences or interactions ($P < 0.05$).

Endotoxin. Endotoxin concentrations were not significantly different between groups at baseline. There were no significant differences in the endotoxin response (AUC, excursion, and C_{MAX}) to the high fat meal between the groups before or after the intervention (Figure 4.6, 4.7).

A

Pre-Intervention Endotoxin Response to Meal Challenge



B

Post-Intervention Endotoxin Response to Meal Challenge

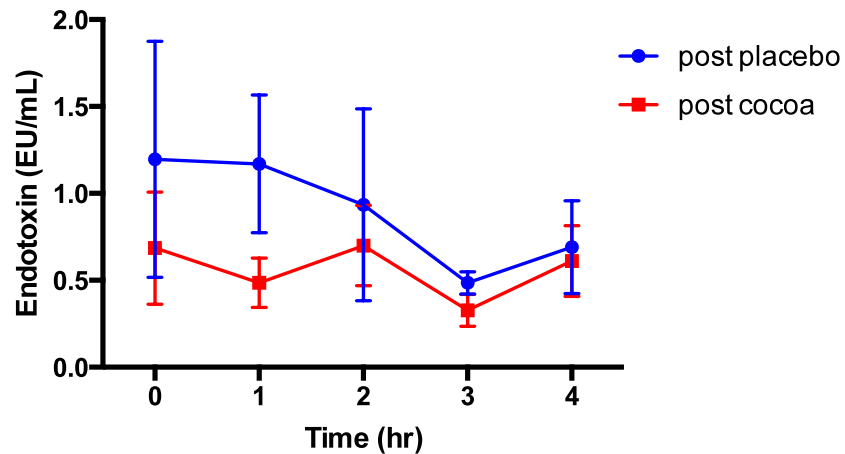


Figure 4.6. The mean ($\pm$ SEM) concentration of serum endotoxin in response to a high fat meal (A) before and (B) after the intervention with placebo ($\bullet$) or cocoa ($\blacksquare$). There were no significant differences between the groups at individual time points ($P < 0.05$).

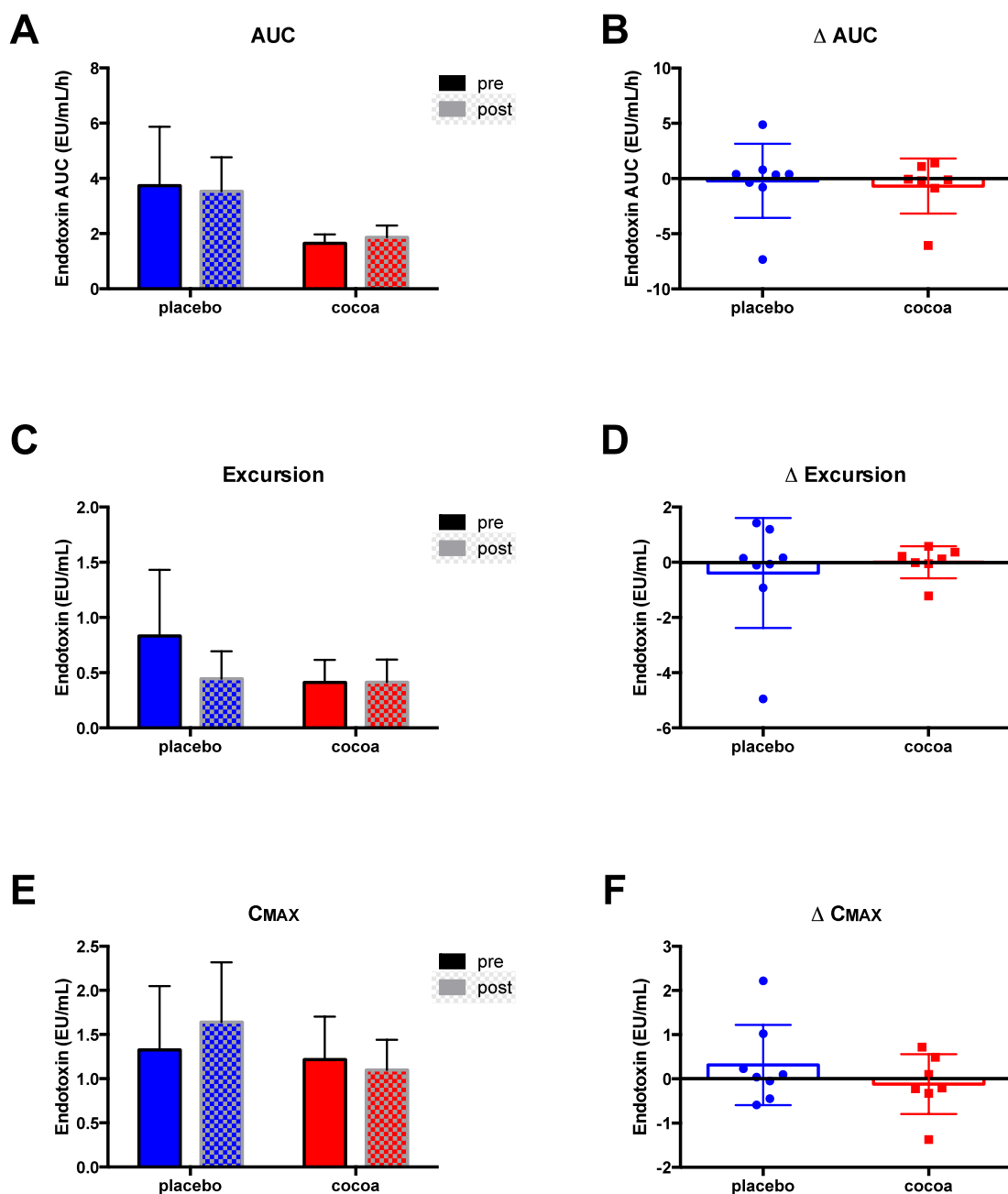


Figure 4.7. Data are presented as the mean ($\pm$ SEM) of the calculated endotoxin (A) AUC, (C) excursion, and (E) C_{MAX} values before and after the intervention. Figures B, D, and E depict the delta values (post-pre) for placebo ($\bullet$) and cocoa ($\blacksquare$). No significant differences were detected via two-way ANOVA or t-tests ($P < 0.05$).

Discussion

The main finding from this pilot study is that 4 weeks of cocoa consumption did not improve insulin sensitivity in overweight and obese adults at risk for developing diabetes. There were some indications that cocoa increased postprandial skeletal muscle substrate metabolism,. This is the first clinical study to investigate the effects of cocoa on substrate metabolism, postprandial endotoxin, and gut permeability. In addition, this is the first long-term cocoa supplementation study to use an IVGTT to assess insulin sensitivity.

The results of some^{107-109,112,114} but not all^{113,115,116} suggest that cocoa consumption improves insulin sensitivity and/or resistance. Improvements in glucose homeostasis have been reported in overweight and obese adults as well as adults with impaired glucose tolerance. Davison *et al.*¹¹² utilized 150 ml cocoa beverages (902 mg flavanols/d) for 6 weeks and reported a reduction in insulin resistance measured by homeostatic model assessment of insulin resistance (HOMA1-IR) in 49 patients using a randomized, placebo controlled trial. Grassi *et al.*¹⁰⁹ provided 100 g flavanol-rich chocolate bars (1008 mg phenols/d) for 15 days to 19 subjects and also found a decrease in insulin resistance and an increase in insulin sensitivity via HOMA2-IR and quantitative insulin sensitivity check index (QUICKI), respectively, using a crossover design. These studies used dosages similar to ours (1296 mg total polyphenols/d) and had larger sample sizes. Although HOMA, QUICKI, and IVGTT are all validated measures to assess insulin resistance, sensitivity and beta cell function, HOMA and QUICKI assess basal glucose and insulin levels while the IVGTT represents dynamic insulin secretion and sensitivity.³⁰⁶ Therefore, caution should be taken when directly comparing these

outcomes. Factors that may contribute to the variability in previous studies include timing of dosages, the food matrix and macronutrient content of the cocoa treatment, and the cardiometabolic health of participants.

Our data suggest that cocoa supplementation influences some aspects of skeletal muscle substrate metabolism. As a result of the cocoa supplementation, glucose oxidation increased in the fasted state and total fatty acid oxidation increased in the fed state compared to the placebo. However, oxidative efficiency, the ratio of complete to incomplete fatty acid oxidation, decreased as a result of the intervention for the cocoa group. Enhanced total fatty acid oxidation with decreased oxidative efficiency suggests an increase in incomplete fatty acid oxidation, a characteristic of metabolic dysfunction.³⁰⁷ The mechanisms behind these changes are uncertain given the negative findings from other outcomes in this study, so future research may consider measuring inflammatory markers related to metabolic dysfunction, such as nuclear factor kappa B (NF-KB), interleukin 6 (IL-6), monocyte chemoattractant protein 1 (MCP1).³⁰⁸

There were several strengths of our study that should be emphasized. We utilized a randomized, placebo controlled design and provided all foods consumed during the intervention period to the participants. In addition, a variety of high-quality measures were used to test for a wide range of metabolic outcomes. We used a practical dose of cocoa powder (3 doses of 5 g/d) that could easily be consumed and is within the range of dosages used by other clinical studies. In addition, our treatment was low in calories and could be recommended to adults with prediabetes, although it was relatively high in sodium.

There were some limitations of our study that should be considered. First, because this was a pilot study, our sample size was relatively small. As such, inclusion of a larger sample may have produced a different outcome. Second, we studied a group of Caucasian, middle-aged and older adults. Cocoa supplementation may produce a different outcome in other racial/ethnic groups or younger individuals at risk for diabetes. Third, we did not assess compliance to our instructions to our participants to maintain their sedentary to recreationally active levels of physical activity. Therefore, it is possible that the weight loss observed in the placebo group was due to increases in physical activity. However, we believe that it is unlikely that only the placebo group was non-compliant and increased their physical activity levels.

Conclusions

In conclusion, the major findings from this pilot study is that a one-month intervention with cocoa powder in overweight and obese adults at risk for developing diabetes does not improve insulin sensitivity but does impact some aspects of *in vitro* skeletal muscle metabolism. Future studies will be needed to better understand the effects of cocoa supplementation on metabolism. Important components of future studies will include controlling for dietary polyphenol intake, studying a larger sample size, and using robust measures of insulin sensitivity.

Chapter 5. Conclusions

This dissertation research examined the potential acute and chronic physiological effects of cocoa on overweight and obese adults at risk for developing diabetes. Previous research suggested that cocoa might reduce blood glucose concentration, increase insulin sensitivity, and improve gut barrier function, all of which would be beneficial for an individual with prediabetes. However, there was a gap in the literature examining cocoa's effects on these outcomes in a prediabetic population. This population is important to study since the prevalence of prediabetes is high and individuals are at increased risk for developing T2DM and other diseases.

The data presented here suggest that acute cocoa consumption had no effect on glycemic, insulin, or incretin hormone responses to a mixed meal. It is possible that a glucose challenge, as opposed to a mixed meal, may have provided a better opportunity to observe the hypothesized effects of cocoa on glycemic control for the present population. Adults with normal fasting blood glucose were more likely to respond to the cocoa and meal challenge as hypothesized for both glucose excursion and GIP AUC. It is possible that cocoa is not potent enough to elicit vast reductions in postprandial glycemia in adults with normal or hyperglycemia. Therefore, instead of treating a disease, cocoa powder will likely be most effective at preventing the development of diabetes and other diseases.

Future research using a meal with a carbohydrate load that elicits significant increases in postprandial glucose is needed to fully characterize cocoa's acute effects on the prediabetic population. In addition, comparing the effects of cocoa to the effects of digestive enzyme inhibitors, such as acarbose, could concretely determine cocoa's anti-nutritional potential *in vivo*. In this case, a mixed meal with complex carbohydrates would be needed in order to compare cocoa to acarbose, due to its inhibitory effects on carbohydrate digestion. A glucose beverage

would be appropriate to use for studies comparing cocoa to GLP-1 receptor agonists and DPP-4 inhibitors to further characterize the mechanisms by which cocoa acutely influences incretin response. The ability for cocoa to inhibit digestive enzymes has been well established using *in vitro* models, so there is strong likelihood this type of study would yield positive results. There is less known about cocoa's interactions with L cells and DPP-4, so these mechanisms should be considered exploratory.

Chronic cocoa consumption had no significant effects on insulin sensitivity, gut permeability, or endotoxin response to a high fat meal. While there were some significant effects on substrate metabolism, it is unclear to what extent or by which mechanisms cocoa might influence the skeletal muscle. Future studies should be designed to identify individual mechanisms before attempting to detect downstream effects. For example, cocoa flavanols have low bioavailability, its procyanidins are metabolized by the gut microbiota, and there is fiber found in cocoa powder. Therefore, it is likely that cocoa will modulate the gastrointestinal tract. These effects (i.e., gut barrier function, gut microbiota populations, mucus production, immune function, enteric nervous system function, etc.) need to be fully explored before including the consequences of improved gut health, such as reducing circulating endotoxin, as primary outcomes. For example, a chronic study comparing cocoa powder, Dutched cocoa powder, cocoa extract, and a placebo could determine if changes in the colon come from procyanidins, fiber, or perhaps there is an additive effect. If any of these do appear to positively influence the gut, then additional studies examining downstream effects of improved gut health would be warranted.

In conclusion, neither acute consumption nor chronic supplementation with cocoa influenced glucose homeostasis in overweight and obese adults in the present studies. However, cocoa may still have the potential to assist in the prevention of prediabetes and T2DM.

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Appendix A



Office of Research Compliance

Institutional Review Board
North End Center, Suite 4120, Virginia Tech
300 Turner Street NW
Blacksburg, Virginia 24061
540/231-4606 Fax 540/231-0959
email irb@vt.edu
website <http://www.irb.vt.edu>

MEMORANDUM

DATE: September 5, 2013
TO: Kevin Davy, Andrew P Neilson, Karen M Strat, Matthew Wade Hulver, John F Pownall Jr, Brenda Davy
FROM: Virginia Tech Institutional Review Board (FWA00000572, expires April 25, 2018)
PROTOCOL TITLE: Cocoa and Metabolic Health in Prediabetes
IRB NUMBER: 13-755

On September 5, 2013, the Virginia Tech IRB Chair, David M Moore, approved the interim application for the above-mentioned research protocol under 45 CFR 46.118.

This Interim approval only provides permission to being the initial planning required in developing study procedures and forms under this protocol and does not provide permission to begin human subject related activities. This Interim approval is being provided based on confirmation received from you that study procedures involving human subjects will not be initiated until regular (i.e., non-Interim) IRB approval is obtained.

Failure to obtain VT IRB approval prior to conducting human subject activities may result in serious sanctions such as the destruction of data, termination of research and loss of privilege to conduct research at Virginia Tech.

PROTOCOL INFORMATION:

Approved As: **Interim**
Protocol Approval Date: **September 5, 2013**
Protocol Expiration Date: **N/A**
Continuing Review Due Date*: **N/A**

*Date a Continuing Review application is due to the IRB office if human subject activities covered under this protocol, including data analysis, are to continue beyond the Protocol Expiration Date.

FEDERALLY FUNDED RESEARCH REQUIREMENTS:

Per federal regulations, 45 CFR 46.103(f), the IRB is required to compare all federally funded grant proposals/work statements to the IRB protocol(s) which cover the human research activities included in the proposal / work statement before funds are released. Note that this requirement does not apply to Exempt and Interim IRB protocols, or grants for which VT is not the primary awardee.

The table on the following page indicates whether grant proposals are related to this IRB protocol, and which of the listed proposals, if any, have been compared to this IRB protocol, if required.

Invent the Future

VIRGINIA POLYTECHNIC INSTITUTE AND STATE UNIVERSITY
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MEMORANDUM

DATE: October 1, 2013
TO: Kevin Davy, Andrew P Neilson, Karen M Strat, Matthew Wade Hulver, John F Pownall Jr, Brenda Davy
FROM: Virginia Tech Institutional Review Board (FWA00000572, expires April 25, 2018)
PROTOCOL TITLE: Cocoa and Metabolic Health in Prediabetes
IRB NUMBER: 13-755

Effective September 9, 2013, the Virginia Tech Institution Review Board (IRB), at a convened meeting, approved the New Application request for the above-mentioned research protocol.

This approval provides permission to begin the human subject activities outlined in the IRB-approved protocol and supporting documents.

Plans to deviate from the approved protocol and/or supporting documents must be submitted to the IRB as an amendment request and approved by the IRB prior to the implementation of any changes, regardless of how minor, except where necessary to eliminate apparent immediate hazards to the subjects. Report within 5 business days to the IRB any injuries or other unanticipated or adverse events involving risks or harms to human research subjects or others.

All investigators (listed above) are required to comply with the researcher requirements outlined at:

<http://www.irb.vt.edu/pages/responsibilities.htm>

(Please review responsibilities before the commencement of your research.)

PROTOCOL INFORMATION:

Approved As: **Full Review**
Protocol Approval Date: **September 9, 2013**
Protocol Expiration Date: **September 8, 2014**
Continuing Review Due Date*: **August 25, 2014**

*Date a Continuing Review application is due to the IRB office if human subject activities covered under this protocol, including data analysis, are to continue beyond the Protocol Expiration Date.

FEDERALLY FUNDED RESEARCH REQUIREMENTS:

Per federal regulations, 45 CFR 46.103(f), the IRB is required to compare all federally funded grant proposals/work statements to the IRB protocol(s) which cover the human research activities included in the proposal / work statement before funds are released. Note that this requirement does not apply to Exempt and Interim IRB protocols, or grants for which VT is not the primary awardee.

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MEMORANDUM

DATE: May 13, 2014
TO: Kevin Davy, Andrew P Neilson, Karen M Strat, Matthew Wade Hulver, John F Pownall Jr, Brenda Davy
FROM: Virginia Tech Institutional Review Board (FWA00000572, expires April 25, 2018)
PROTOCOL TITLE: Cocoa and Metabolic Health in Prediabetes
IRB NUMBER: 13-755

Effective May 12, 2014, the Virginia Tech Institution Review Board (IRB), at a convened meeting, approved the Amendment request for the above-mentioned research protocol.

This approval provides permission to begin the human subject activities outlined in the IRB-approved protocol and supporting documents.

Plans to deviate from the approved protocol and/or supporting documents must be submitted to the IRB as an amendment request and approved by the IRB prior to the implementation of any changes, regardless of how minor, except where necessary to eliminate apparent immediate hazards to the subjects. Report within 5 business days to the IRB any injuries or other unanticipated or adverse events involving risks or harms to human research subjects or others.

All investigators (listed above) are required to comply with the researcher requirements outlined at:

<http://www.irb.vt.edu/pages/responsibilities.htm>

(Please review responsibilities before the commencement of your research.)

PROTOCOL INFORMATION:

Approved As: **Full Review**
Protocol Approval Date: **September 9, 2013**
Protocol Expiration Date: **September 8, 2014**
Continuing Review Due Date*: **August 25, 2014**

*Date a Continuing Review application is due to the IRB office if human subject activities covered under this protocol, including data analysis, are to continue beyond the Protocol Expiration Date.

FEDERALLY FUNDED RESEARCH REQUIREMENTS:

Per federal regulations, 45 CFR 46.103(f), the IRB is required to compare all federally funded grant proposals/work statements to the IRB protocol(s) which cover the human research activities included in the proposal / work statement before funds are released. Note that this requirement does not apply to Exempt and Interim IRB protocols, or grants for which VT is not the primary awardee.

The table on the following page indicates whether grant proposals are related to this IRB protocol, and which of the listed proposals, if any, have been compared to this IRB protocol, if required.

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MEMORANDUM

DATE: July 10, 2014
TO: Kevin Davy, Andrew P Neilson, Karen M Strat, Matthew Wade Hulver, John F Pownall Jr, Brenda Davy
FROM: Virginia Tech Institutional Review Board (FWA00000572, expires April 25, 2018)
PROTOCOL TITLE: Cocoa and Metabolic Health in Prediabetes
IRB NUMBER: 13-755

Effective July 10, 2014, the Virginia Tech Institution Review Board (IRB) Administrator, Carmen T Papenfuss, approved the Amendment request for the above-mentioned research protocol.

This approval provides permission to begin the human subject activities outlined in the IRB-approved protocol and supporting documents.

Plans to deviate from the approved protocol and/or supporting documents must be submitted to the IRB as an amendment request and approved by the IRB prior to the implementation of any changes, regardless of how minor, except where necessary to eliminate apparent immediate hazards to the subjects. Report within 5 business days to the IRB any injuries or other unanticipated or adverse events involving risks or harms to human research subjects or others.

All investigators (listed above) are required to comply with the researcher requirements outlined at:

<http://www.irb.vt.edu/pages/responsibilities.htm>

(Please review responsibilities before the commencement of your research.)

PROTOCOL INFORMATION:

Approved As: **Full Review**
Protocol Approval Date: **September 9, 2013**
Protocol Expiration Date: **September 8, 2014**
Continuing Review Due Date*: **August 25, 2014**

*Date a Continuing Review application is due to the IRB office if human subject activities covered under this protocol, including data analysis, are to continue beyond the Protocol Expiration Date.

FEDERALLY FUNDED RESEARCH REQUIREMENTS:

Per federal regulations, 45 CFR 46.103(f), the IRB is required to compare all federally funded grant proposals/work statements to the IRB protocol(s) which cover the human research activities included in the proposal / work statement before funds are released. Note that this requirement does not apply to Exempt and Interim IRB protocols, or grants for which VT is not the primary awardee.

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MEMORANDUM

DATE: September 9, 2014
TO: Kevin Davy, Andrew P Neilson, Karen M Strat, Matthew Wade Hulver, John F Pownall Jr, Brenda Davy
FROM: Virginia Tech Institutional Review Board (FWA00000572, expires April 25, 2018)
PROTOCOL TITLE: Cocoa and Metabolic Health in Prediabetes
IRB NUMBER: 13-755

Effective September 8, 2014, the Virginia Tech Institution Review Board (IRB), at a convened meeting, approved the Continuing Review request for the above-mentioned research protocol.

This approval provides permission to begin the human subject activities outlined in the IRB-approved protocol and supporting documents.

Plans to deviate from the approved protocol and/or supporting documents must be submitted to the IRB as an amendment request and approved by the IRB prior to the implementation of any changes, regardless of how minor, except where necessary to eliminate apparent immediate hazards to the subjects. Report within 5 business days to the IRB any injuries or other unanticipated or adverse events involving risks or harms to human research subjects or others.

All investigators (listed above) are required to comply with the researcher requirements outlined at:

<http://www.irb.vt.edu/pages/responsibilities.htm>

(Please review responsibilities before the commencement of your research.)

PROTOCOL INFORMATION:

Approved As: **Full Review**
Protocol Approval Date: **September 8, 2014**
Protocol Expiration Date: **September 7, 2015**
Continuing Review Due Date*: **July 27, 2015**

*Date a Continuing Review application is due to the IRB office if human subject activities covered under this protocol, including data analysis, are to continue beyond the Protocol Expiration Date.

FEDERALLY FUNDED RESEARCH REQUIREMENTS:

Per federal regulations, 45 CFR 46.103(f), the IRB is required to compare all federally funded grant proposals/work statements to the IRB protocol(s) which cover the human research activities included in the proposal / work statement before funds are released. Note that this requirement does not apply to Exempt and Interim IRB protocols, or grants for which VT is not the primary awardee.

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MEMORANDUM

DATE: March 19, 2015
TO: Kevin Davy, Andrew P Neilson, Karen M Strat, Matthew Wade Hulver, Brenda Davy, Kris Osterberg, Elaina Lynn Marinik, Michael Patrick O'Rourke, Cassie Marie Mitchell
FROM: Virginia Tech Institutional Review Board (FWA00000572, expires April 25, 2018)
PROTOCOL TITLE: Cocoa and Metabolic Health in Prediabetes
IRB NUMBER: 13-755

Effective March 19, 2015, the Virginia Tech Institutional Review Board (IRB), at a convened meeting, approved the Amendment request for the above-mentioned research protocol.

This approval provides permission to begin the human subject activities outlined in the IRB-approved protocol and supporting documents.

Plans to deviate from the approved protocol and/or supporting documents must be submitted to the IRB as an amendment request and approved by the IRB prior to the implementation of any changes, regardless of how minor, except where necessary to eliminate apparent immediate hazards to the subjects. Report within 5 business days to the IRB any injuries or other unanticipated or adverse events involving risks or harms to human research subjects or others.

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<http://www.irb.vt.edu/pages/responsibilities.htm>

(Please review responsibilities before the commencement of your research.)

PROTOCOL INFORMATION:

Approved As: **Full Review**
Protocol Approval Date: **September 8, 2014**
Protocol Expiration Date: **September 7, 2015**
Continuing Review Due Date*: **July 27, 2015**

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FEDERALLY FUNDED RESEARCH REQUIREMENTS:

Per federal regulations, 45 CFR 46.103(f), the IRB is required to compare all federally funded grant proposals/work statements to the IRB protocol(s) which cover the human research activities included in the proposal / work statement before funds are released. Note that this requirement does not apply to Exempt and Interim IRB protocols, or grants for which VT is not the primary awardee.

The table on the following page indicates whether grant proposals are related to this IRB protocol, and which of the listed proposals, if any, have been compared to this IRB protocol, if required.

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VIRGINIA POLYTECHNIC INSTITUTE AND STATE UNIVERSITY
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MEMORANDUM

DATE: June 11, 2015

TO: Kevin Davy, Andrew P Neilson, Karen M Strat, Matthew Wade Hulver, Brenda Davy, Kris Osterberg, Elaina Lynn Marinik, Michael Patrick O'Rourke, Cassie Marie Mitchell

FROM: Virginia Tech Institutional Review Board (FWA00000572, expires April 25, 2018)

PROTOCOL TITLE: Cocoa and Metabolic Health in Prediabetes

IRB NUMBER: 13-755

Effective June 8, 2015, the Virginia Tech Institution Review Board (IRB), at a convened meeting, approved the Amendment request for the above-mentioned research protocol.

This approval provides permission to begin the human subject activities outlined in the IRB-approved protocol and supporting documents.

Plans to deviate from the approved protocol and/or supporting documents must be submitted to the IRB as an amendment request and approved by the IRB prior to the implementation of any changes, regardless of how minor, except where necessary to eliminate apparent immediate hazards to the subjects. Report within 5 business days to the IRB any injuries or other unanticipated or adverse events involving risks or harms to human research subjects or others.

All investigators (listed above) are required to comply with the researcher requirements outlined at:

<http://www.irb.vt.edu/pages/responsibilities.htm>

(Please review responsibilities before the commencement of your research.)

PROTOCOL INFORMATION:

Approved As: **Full Review**
Protocol Approval Date: **September 8, 2014**
Protocol Expiration Date: **September 7, 2015**
Continuing Review Due Date*: **July 27, 2015**

*Date a Continuing Review application is due to the IRB office if human subject activities covered under this protocol, including data analysis, are to continue beyond the Protocol Expiration Date.

FEDERALLY FUNDED RESEARCH REQUIREMENTS:

Per federal regulations, 45 CFR 46.103(f), the IRB is required to compare all federally funded grant proposals/work statements to the IRB protocol(s) which cover the human research activities included in the proposal / work statement before funds are released. Note that this requirement does not apply to Exempt and Interim IRB protocols, or grants for which VT is not the primary awardee.

The table on the following page indicates whether grant proposals are related to this IRB protocol, and which of the listed proposals, if any, have been compared to this IRB protocol, if required.

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MEMORANDUM

DATE: August 10, 2015

TO: Kevin Davy, Andrew P Neilson, Karen M Strat, Matthew Wade Hulver, Brenda Davy, Kris Osterberg, Elaina Lynn Marinik, Michael Patrick O'Rourke, Cassie Marie Mitchell

FROM: Virginia Tech Institutional Review Board (FWA00000572, expires July 29, 2020)

PROTOCOL TITLE: Cocoa and Metabolic Health in Prediabetes

IRB NUMBER: 13-755

Effective August 10, 2015, the Virginia Tech Institution Review Board (IRB), at a convened meeting, approved the Continuing Review request for the above-mentioned research protocol.

This approval provides permission to begin the human subject activities outlined in the IRB-approved protocol and supporting documents.

Plans to deviate from the approved protocol and/or supporting documents must be submitted to the IRB as an amendment request and approved by the IRB prior to the implementation of any changes, regardless of how minor, except where necessary to eliminate apparent immediate hazards to the subjects. Report within 5 business days to the IRB any injuries or other unanticipated or adverse events involving risks or harms to human research subjects or others.

All investigators (listed above) are required to comply with the researcher requirements outlined at:

<http://www.irb.vt.edu/pages/responsibilities.htm>

(Please review responsibilities before the commencement of your research.)

PROTOCOL INFORMATION:

Approved As: **Full Review**
Protocol Approval Date: **September 8, 2015**
Protocol Expiration Date: **September 7, 2016**
Continuing Review Due Date*: **July 25, 2016**

*Date a Continuing Review application is due to the IRB office if human subject activities covered under this protocol, including data analysis, are to continue beyond the Protocol Expiration Date.

FEDERALLY FUNDED RESEARCH REQUIREMENTS:

Per federal regulations, 45 CFR 46.103(f), the IRB is required to compare all federally funded grant proposals/work statements to the IRB protocol(s) which cover the human research activities included in the proposal / work statement before funds are released. Note that this requirement does not apply to Exempt and Interim IRB protocols, or grants for which VT is not the primary awardee.

The table on the following page indicates whether grant proposals are related to this IRB protocol, and which of the listed proposals, if any, have been compared to this IRB protocol, if required.

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VIRGINIA POLYTECHNIC INSTITUTE AND STATE UNIVERSITY
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MEMORANDUM

DATE: October 19, 2015
TO: Kevin Davy, Andrew P Neilson, Karen M Strat, Matthew Wade Hulver, Brenda Davy, Elaina Lynn Marinik, Cassie Marie Mitchell, James B Grubb
FROM: Virginia Tech Institutional Review Board (FWA00000572, expires July 29, 2020)
PROTOCOL TITLE: Cocoa and Metabolic Health in Prediabetes
IRB NUMBER: 13-755

Effective October 16, 2015, the Virginia Tech Institutional Review Board (IRB) Chair, David M Moore, approved the Amendment request for the above-mentioned research protocol.

This approval provides permission to begin the human subject activities outlined in the IRB-approved protocol and supporting documents.

Plans to deviate from the approved protocol and/or supporting documents must be submitted to the IRB as an amendment request and approved by the IRB prior to the implementation of any changes, regardless of how minor, except where necessary to eliminate apparent immediate hazards to the subjects. Report within 5 business days to the IRB any injuries or other unanticipated or adverse events involving risks or harms to human research subjects or others.

All investigators (listed above) are required to comply with the researcher requirements outlined at:

<http://www.irb.vt.edu/pages/responsibilities.htm>

(Please review responsibilities before the commencement of your research.)

PROTOCOL INFORMATION:

Approved As: **Full Review**
Protocol Approval Date: **September 8, 2015**
Protocol Expiration Date: **September 7, 2016**
Continuing Review Due Date*: **July 25, 2016**

*Date a Continuing Review application is due to the IRB office if human subject activities covered under this protocol, including data analysis, are to continue beyond the Protocol Expiration Date.

FEDERALLY FUNDED RESEARCH REQUIREMENTS:

Per federal regulations, 45 CFR 46.103(f), the IRB is required to compare all federally funded grant proposals/work statements to the IRB protocol(s) which cover the human research activities included in the proposal / work statement before funds are released. Note that this requirement does not apply to Exempt and Interim IRB protocols, or grants for which VT is not the primary awardee.

The table on the following page indicates whether grant proposals are related to this IRB protocol, and which of the listed proposals, if any, have been compared to this IRB protocol, if required.

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Date*	OSP Number	Sponsor	Grant Comparison Conducted?
09/24/2013	13261005	Hershey Foods Corporation	Not required (Not federally funded)

* Date this proposal number was compared, assessed as not requiring comparison, or comparison information was revised.

If this IRB protocol is to cover any other grant proposals, please contact the IRB office (irbadmin@vt.edu) immediately.

Appendix B

Chapter 3: Acute Study Detailed Materials and Methods

Subjects, recruitment, screening and enrollment. Approval from the Virginia Tech Institutional Review Board (IRB) was obtained for all aspects of the study involving human subjects (IRB# 13-755). The study was registered at ClinicalTrials.gov (NCT02203240). Volunteers were recruited from the New River Valley, VA via posted and mailed fliers, web postings, and newspaper advertisements. Interested individuals completed an online questionnaire to assess eligibility. Inclusion/exclusion criteria are shown in Table S3.1. Eligible and interested prospective participants provided written and oral informed consent and completed a health history questionnaire, Godin Leisure-time questionnaire,³⁰⁹ an infection/inflammation questionnaire, and the American Diabetes Association Risk-Assessment questionnaire²⁵⁷ at an initial informational session. Body weight was measured on a digital scale (Model 5002, Scale-Tronix, White Plains, NY) to the nearest ± 0.1 kg and height was measured on a stadiometer to the nearest ± 0.1 cm. Subjects also received instructions on how to complete a 4-day food diary (3 consecutive week days and one weekend day) to estimate habitual energy and macronutrient content. Once completed, the record was reviewed by a registered dietitian and analyzed using The Nutrition Data Systems for Research software (NDS-R 2013 University of Minnesota, Minneapolis, MN).

On a different day, participants who were still eligible and interested arrived at the lab between 6-9 A.M. after a 12-h overnight fast to complete screening procedures. Subjects provided a urine sample for a urinalysis conducted at a commercial lab (Solstas

Lab Partners, Roanoke, VA) to test for white blood cell esterase and other markers of infection or disease and a pregnancy test (Kroger, Cincinnati, OH) for premenopausal women. Participants were asked to sit quietly for 10 min prior to assessing their blood pressure using an automated blood pressure monitor (Omron Healthcare Inc., Bannockburn, IL). A fasting serum blood sample was taken from the antecubital vein to assess cholesterol and triglycerides by a commercial laboratory (Solstas Lab Partners, Roanoke, VA) using standard enzymatic techniques. The sample was allowed to clot for 15 min at room temperature (21°C) before being centrifuged (1148 x g, 15 min, 4°C). A finger stick blood sample was obtained to measure HbA1c (HbA1c Now⁺) (PTS Diagnostics, Indianapolis, IN). A fasting plasma sample was taken from the antecubital vein to measure glucose concentration. The whole blood sample was collected in a vacutainer with 12 mg K₃ EDTA (BD, Franklin Lakes, NJ) and immediately stored on ice until centrifugation (1148 x g, 15 min, 4°C). Blood glucose concentration was measured by the glucose oxidase method using a glucose auto-analyzer (Yellow Springs Instruments, Yellow Springs, OH, USA). Participants then consumed a 75 g glucose beverage (Sun Dex, Fisher, Pittsburgh, PA) within the first five min of the 2-hour oral glucose tolerance test (OGTT). An antecubital plasma blood sample was taken 2 h after the first sip of the beverage and analyzed for glucose as described above. Subjects were asked to remain seated and awake during the OGTT. Body composition was analyzed via dual-energy x-ray absorptiometry (Lunar iDXA enCORE v15) (General Electric Medical Systems Ultrasound & Primary Care Diagnostics, LLC, Madison, WI). All subjects completed the study. Characteristics of subjects are shown in Table 3.1.

Cocoa and placebo treatments. Cocoa and placebo beverage dry mixes were individually pre-packaged in coded sachets, from single production lots and obtained directly from The Hershey Company. The beverages were custom formulations produced by Hershey and designed to support human clinical trials. Random samples indicated that the composition of each mix was consistent between sachets. Upon receipt, beverage mixes were refrigerated (4°C) until immediately prior to use. The mixes are designed to be shelf-stable at room temperature for 2-3 yr. Full characterization and nutrition content of cocoa and placebo packets are described in Table 3.3 (The Hershey Co., Hershey, PA).

Meal challenge. A randomized, double-blind crossover design was utilized for the meal challenges to assess the impact of cocoa on postprandial glucose and hormone responses. Treatment packets were coded and only The Hershey Company and an investigator not involved with data collection and analysis knew the code assignment. Order of the crossover design was determined by a randomization scheme created prior to data collection.

Participants completed both meal challenges (+ cocoa vs. + placebo) one week apart. For each meal challenge, subjects arrived at the Human Integrative Physiology Lab between 6-9 A.M. following a 12-h overnight fast. An intravenous catheter was inserted into the antecubital vein to facilitate blood collections. After a baseline blood draw, participants were instructed to consume the test meal within 5 min. The test meal was 2-8 oz. servings of a meal replacement beverage providing a mixed macronutrient profile (Table 4), supplemented with either a cocoa beverage mix (supplying 10 g cocoa) or a placebo beverage mix (0 g cocoa) (Table 3). The test beverage was initially 2-8 fl oz.

servings of BoostTM Very Vanilla Original (Nestle HealthCare Nutrition, Inc., Florham Park, NJ). However, the Boost formula was changed in late 2014 and the study was completed with Equate Vanilla Nutritional Shake (Wal-Mart Stores Inc., Bentonville, AR) due to lack of availability of the original meal replacement beverage. A total of $n=16$ subjects ($n=11$ normal glucose tolerance (NGT), $n=5$ impaired glucose tolerance (IGT)] completed the study with the Boost meal beverage, and a total of $n=14$ subjects [$n=13$ (NGT), $n=1$ (IGT)] completed the study with the Equate meal beverage. Subsequent blood samples were taken 1, 2, 3, and 4 h after the first sip of the test beverage. Plasma samples were kept on ice and serum samples were allowed to clot at room temperature for > 15 min. All blood samples were centrifuged ($1148 \times g$, 15 min, 4°C). Plasma samples were immediately analyzed for glucose concentration as described above. Plasma and serum samples were stored at -80°C until further analysis. In addition to the blood draws, the second cohort of participants ($n=14$ Equate beverage) completed Visual Analogue Scale (VAS) questionnaires about their perceptions of hunger, fullness, desire to eat, thirst, and nausea. The questionnaires were completed every thirty minutes during the meal challenge (9 times total).

Blood biomarkers. Measurements of serum insulin, GLP-1, GIP, C-peptide, and adiponectin were determined using commercially available enzyme-linked immunosorbent assay (ELISA) kits (Human Insulin ELISA, Multi-species GLP-1 Total ELISA, Human GIP Total ELISA, Human C-peptide ELISA, Millipore, Billerica, MA, USA). Samples were analyzed in duplicate. All assays were performed per manufacturer instructions.

Data analysis and statistics. Psuedo-pharmacokinetic (PK) curves and parameters for blood biomarkers were constructed using PK plug-in functions for Microsoft Excel. Parameters determined include: fasting concentration (C_0), maximal concentration (C_{MAX}), area under the PK curve (AUC), and excursion ($\Delta = C_{MAX} - C_0$). Paired t-tests (with matching from the cross over design) were conducted to determine any differences between the cocoa and placebo treatments for all subjects. Following initial analyses with all subjects pooled, subjects were divided based on their 2 h OGTT conducted during screening. Two-way repeated measures ANOVA was used to analyze the AUC, max, and excursion for the two phenotypes (NGT, IGT) and two treatments (cocoa, placebo). We assessed the effect of order from the crossover design and statistically controlled for its effect by including it as a predictor in the analysis of variance. If significant main effects were detected, Tukey's HSD *post hoc* test was performed to determine significant differences between treatment means. Significance was defined as $P < 0.05$ throughout. Statistical analyses were performed on Prism v6 (GraphPad, La Jolla, CA) or IBM SPSS (IBM Corp., Armonk, NY).

Appendix C

Informed Consent for Participants of Investigative Projects

Department of Human Nutrition, Foods and Exercise

Virginia Tech

TITLE: The Acute Effects of Dietary Cocoa on Glucose Levels After A Meal

INVESTIGATORS: Andrew Neilson, Ph.D.
Brenda Davy, Ph.D., R.D.
Kevin P. Davy, Ph.D.
Matthew W. Hulver, Ph.D.
Elaina Marinik, Ph.D.
Tanya Halliday, M.S., R.D.
Karen Strat, B.S.
James Grubb, R.N.

MEDICAL DIRECTOR: Jose M. Rivero, M.D.

PURPOSE:

Several factors, including age, excess body fat, a sedentary lifestyle, and family history, increase the risk of developing type 2 diabetes. Individuals at increased risk of type 2 diabetes tend to have higher glucose levels after eating a meal. Cocoa is rich in substances that, when eaten, reduce glucose levels in animals. However, it is not known whether these same benefits occur in humans. Therefore, the purpose of this study is to determine if cocoa reduces glucose levels after a meal. Thirty-six (36) men and women at risk for developing type 2 diabetes will be included in this study. Support for this study is provided by The Hershey Company.

You will not be eligible to participate in this study if any of the following apply:

- You are a smoker
- You have unstable heart disease (chest pain or heart failure) or diabetes
- You have untreated high blood pressure or high cholesterol
- You have health problems that would make it unsafe for you to participate
- You gained or lost more than 5 pounds in the last 6 months
- You take medications or supplements that would influence study variables
- You have taken an antibiotic in the past 3 months
- You exercise two or more times a week at level that causes you to breathe hard and sweat
- You have certain food allergies (e.g., dairy foods, gluten allergy)
- You are pregnant, nursing or plan on becoming pregnant

METHODS:

You are being asked to participate in a study where each participant consumes a beverage mixed with either a cocoa supplement or a placebo for a meal test. The meal test, called a "mixed meal challenge," involves drinking a beverage and having your blood drawn. Blood samples will be taken prior to and following consumption of the beverage. You will be randomized to consume either the cocoa or placebo beverage first. One week later, you will repeat the test with the other option.

If you agree to be involved in this study, you will first have to fill out a health history questionnaire. Your results may be discussed with the study medical director to determine if you can be a subject. You may be able to be a subject if you are between 40 and 75 years of age, your body mass index (a measure of obesity) must be between 25-40 kg/m² (indicating overweight or obese), and at least one of the following criteria:

1. You have a blood glucose level between 100 and 125 mg/dl after an overnight fast.
2. You have a blood glucose level between 140 and 200 mg/dl after consuming a glucose drink.
3. You have an HbA1c level between 5.7 and 6.4%.
4. You are considered at risk of developing type 2 diabetes by the American Diabetes Association risk assessment questionnaire.

If you don't have at least one of the four criteria or if you test above any of these ranges, you will not be able to participate.

There will be a total of 4 visits if you decide to participate in the study: two visits for screening, and two visits for the actual study tests. This will require approximately 12 hours of time commitment. All subject responsibilities are described below.

STUDY PROCEDURES:

Screening session 1: (1 hour)

- **Medical History:** You will be asked to complete a medical history questionnaire. This questionnaire is used to screen for health problems or reasons you should not participate in this study. Your height and weight will also be measured at this time.
- **Physical Activity Questionnaire:** You will be asked to complete a physical activity questionnaire about the type, intensity, and duration of physical activity you perform each week.
- **Infection/Inflammation Questionnaire:** You will be asked to complete a questionnaire about any recent illnesses or infections that you may have had in the past month.
- **American Diabetes Association Diabetes Risk Assessment Questionnaire:** You will be asked to complete a brief questionnaire about your health history and your family's health history to determine if you are at risk to developing diabetes.
- **Diet Records:** To get an idea of what and how much food you eat, you will be asked to record all of the food you eat for 4 days (3 weekdays and one weekend day). You will be taught how to properly fill out the diet records during this session.

Screening session 2: (2.5 hours)

- **Overnight Fast:** You will be asked to avoid eating or drinking anything except water and to avoid consuming any caffeine-containing beverages for 12 hours prior to this visit so that the test results will not be influenced by the food you eat or by the normal digestion process. You may drink only water during this time.
- **Urine Test:** You will be asked to urinate in a small cup that we provide to you. We will measure the amount of sodium and other electrolytes, glucose, protein, pH and whether there

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are blood cells present to determine whether it is safe for you to participate in the study

- **Pregnancy Test:** If you are female you will be required to have a pregnancy test. This will require you to collect 2-3 teaspoons of your urine. If you are pregnant or the test indicates that you are pregnant you will not be able to participate in this study. If you are a postmenopausal female who has not menstruated for at least 1 year then you do not have to complete this test.
- **Catheter and Blood Draw:** A small needle will be inserted into one of your arm's to draw blood (approximately 3 tablespoons). We will measure glucose, cholesterol, triglycerides, and other factors to determine your eligibility.
- **Oral Glucose Tolerance test (OGTT):** For this procedure, you will be asked to drink a very sweet sugar solution (75 grams of glucose). Blood samples will be taken both before and 2 hours after you drink this solution. The purpose of this procedure is to determine whether you have a normal glucose control after drinking this sweet tasting drink (oral glucose tolerance). This procedure will take approximately 2 hours of your time. If your glucose response indicates you may have diabetes you will not be able to continue participation in this study and you will be referred to your personal physician.
- **Hemoglobin A1c Test:** This is a measure of your average blood glucose level over the period of several months. For this test, we will make a small prick in your finger to produce a drop of blood. This blood will be applied to a testing kit to determine whether or not you are in the prediabetic range. If your results indicate your hemoglobin A1c is too high, you will not be able to continue participation in the study and you will be referred to your personal physician.
- **Blood Pressure:** You will be asked to rest quietly for 15 minutes. We will then measure your resting blood pressure using a stethoscope and standard blood pressure cuff or an automatic blood pressure monitor.
- **Body Weight and Composition:** These tests are to measure your body weight and body fat. Your body weight will be measured without shoes on a hospital scale and your height will be measured, as well. Then, you will lie on a hospital-type bed and a small amount of x-ray will be passed through your body to determine the amount of bone, muscle, and fat in your body. This unit is called a DEXA scan. This test takes approximately 15 minutes and there is no pain associated with the procedure.

Testing Day 1: (4.5 hours)

- **Overnight Fast:** You will be asked to avoid eating or drinking anything except water and to avoid consuming any caffeine-containing beverages for 12 hours prior to this visit so that the test results will not be influenced by the food you eat or by the normal digestion process. You may drink only water during this time.
- **Mixed Meal Challenge:** You will drink two servings (16 fl oz total) of a vanilla flavored meal replacement shake mixed with cocoa powder or a placebo.
- **Catheter and Blood Draw:** A small needle will be inserted in your arm to draw blood (approximately 1 tablespoons/draw). We will measure various hormones that influence your metabolism (how your body burns calories and produces body heat). Blood will be collected before the meal challenge and at 1, 2, 3, and 4 hours after the meal. The catheter will remain in

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your arm throughout the entire test.

- **Hunger Questionnaire:** Every 30 minutes (9 times total), you will be asked to indicate how hungry, thirsty, nauseated, and full you feel during the course of the mixed meal challenge.

Testing Day 2: (4.5 hours)

One week after testing day 1, we will repeat the same test and procedures with the other treatment (cocoa or placebo).

Take-Home Tests

- **Diet Records:** To get an idea of what and how much food you eat, you will be asked to record all of the food you eat for 4 days (3 weekdays and one weekend day prior to the mixed meal challenges).

SUMMARY OF SUBJECT RESPONSIBILITIES

- Provide an accurate history of any health problems or medications you use before the study begins.
- Inform the investigators of any discomfort or unusual feelings before, during or after any of the study sessions.
- Be on time and attend all of the scheduled experiments.
- Follow all participant instructions for each session.
- Follow physical activity instructions provided by the investigators.
- Inform the study investigators if you are pregnant or intend to become pregnant during the study.

RISKS OF PARTICIPATION

- **Catheter and Blood Draw:** Some pain or discomfort may be experienced when the catheter is inserted in the vein, but this should persist for only a short time. During the blood draws, you may have pain and/or bruising at the place on your arm where the blood is taken. In about 1 in 10, or 10% of the cases, a small amount of bleeding under the skin will cause bruising. The risk of a blood clot forming in the vein is about 1 in 200, while the risk of infection or significant blood loss is 1 in 1000. There is a small risk of the vein becoming inflamed and/or painful in the hours or days after the catheter is removed. If you feel faint during/after a blood draw, you should notify the study doctor or study staff immediately and lie down right away to avoid falling down. Having staff that are experienced in catheter placement and blood draws will minimize these risks.
- **Oral Glucose Tolerance:** Because this procedure requires the placement of a catheter in a vein in each arm, the risks here are identical to those stated above. In addition, there is a small risk of low blood sugar occurring during or after the test. If this happens, orange juice (with table sugar) or some other sugar containing food will be given to you.

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- DEXA Scan: The amount of radiation that you will receive in the DEXA exam is less than the amount permitted by the Food and Drug Administration (FDA) per year. The amount you will receive is equal to 1/20 of a chest x-ray. The more radiation you receive over the course of your lifetime, the more likely your risk increases in developing cancerous tumors. The radiation in this study is not expected to greatly increase these risks; however, the exact increase in such risk is not known.
- It is not possible to identify all potential risks in an experiential study. However, the study doctors and study staff will take all possible safeguards to minimize any known and potential risks to your well being. We believe the overall risks of participation are minimal. All of the procedures are well established and used routinely in the study investigators laboratory.
- Side effects are possible in any research study despite high standards of care, and could occur through no fault of your own or the study doctors or study staff.

BENEFITS OF PARTICIPATION

As a result of your participation, you will obtain health information related to your body composition, blood pressure, blood glucose, and cholesterol. However, you should not consider this a wellness or medical exam. You should discuss any concerns about your health information with your personal physician. Your participation will contribute to improving the understanding of how cocoa supplementation impacts health.

COMPENSATION

You will be compensated \$50 dollars each time you complete the mixed meal challenge. The total compensation you will receive for participation in this study is \$100.

CONFIDENTIALITY

The data from this study will be kept strictly confidential. No data will be released to anyone but those working on the project without your written permission. Data will be identified by a code, without anything to identify you by name. In the event that any of your tests indicate a problem, your results may be shared with the medical director, Dr. Rivero, and your personal physician.

FREEDOM TO WITHDRAW

You are free to withdraw from the study at any time for any reason. Simply inform the experimenters of your intention to cease participation. In addition, circumstances could arise which would lead to your exclusion from the study. For example, lack of compliance to instructions, failure to attend testing sessions, and illness could be reasons for the researchers to stop your participation in the study. All of the sessions and measurements are required components.

INJURY DURING PARTICIPATION IN THIS STUDY

Neither the researchers nor the University have money set aside to pay for medical treatment that would be necessary if you are injured as a result of your participation in this study. Any expenses that you incur, including emergencies and long term expenses would be your own responsibility. You should consider this limitation before you consider participating in this study.

APPROVAL OF RESEARCH

This research has been approved, as required, by the Institutional Review Board for Research Involving Human Subjects at Virginia Tech. You will receive a copy of this form to take with you.

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SUBJECT PERMISSION

I have read the informed consent and fully understand the procedures and conditions of the project. I have had all of my questions answered, and I hereby give my voluntary consent to be a participant in this research study. I agree to abide by the rules of the project. I understand that I may withdraw from the study at any time.

If you have questions, you may contact:

- Principal Investigator: Kevin Davy, Professor, Department of Human Nutrition, Foods and Exercise. (540) 231-3487; After hours: 540-230-0486
- Chairman, Institutional Review Board for Research Involving Human Subjects: David Moore, Associate Vice President for Research (540) 231-4991

Name of Subject (please print) _____

Signature of Subject _____ Date _____

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Appendix D

Chapter 3: Acute Study Supplementary Information and Additional Data.

CONSORT 2010 Flow Diagram

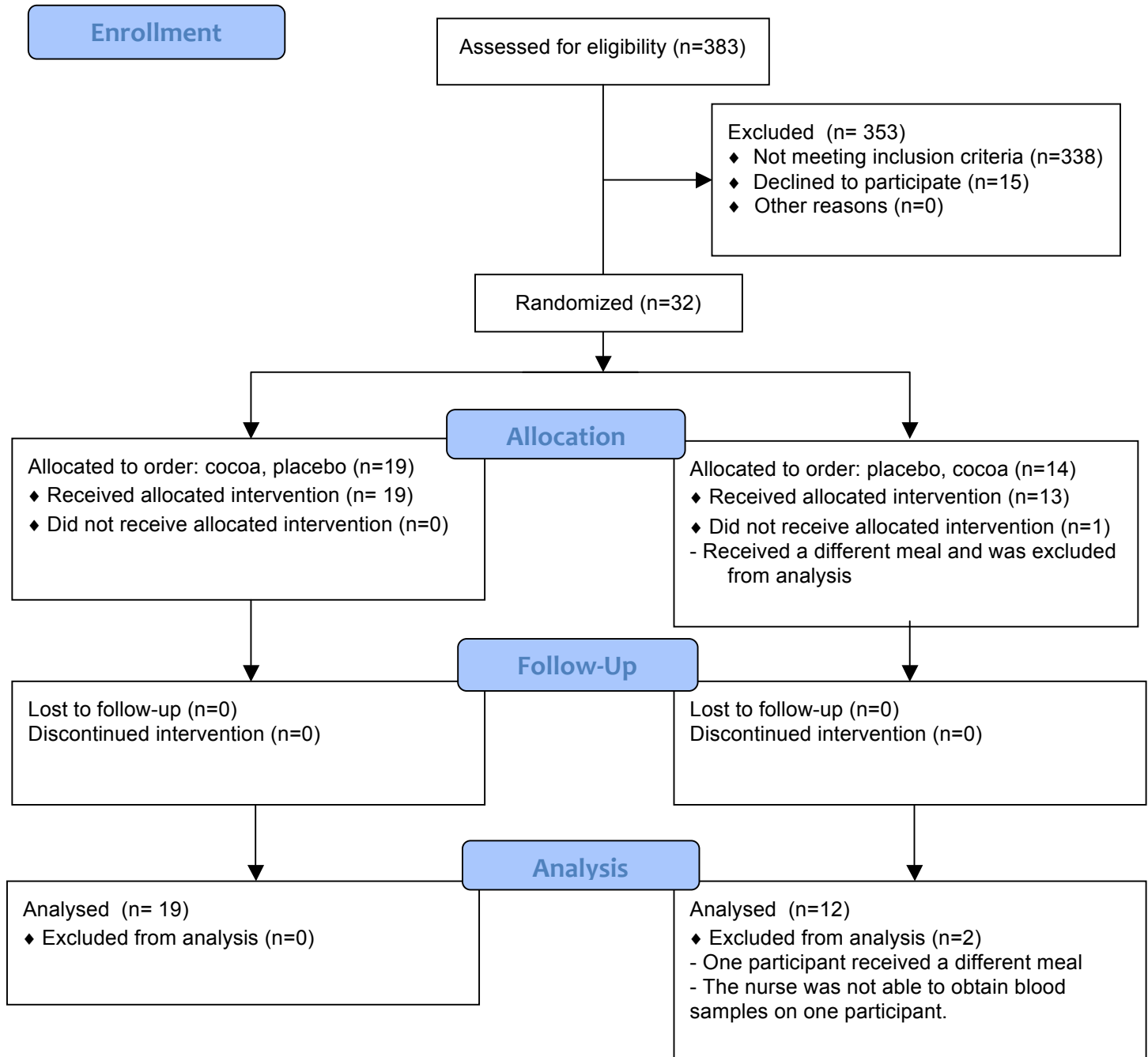


Figure S3.1. CONSORT flow diagram

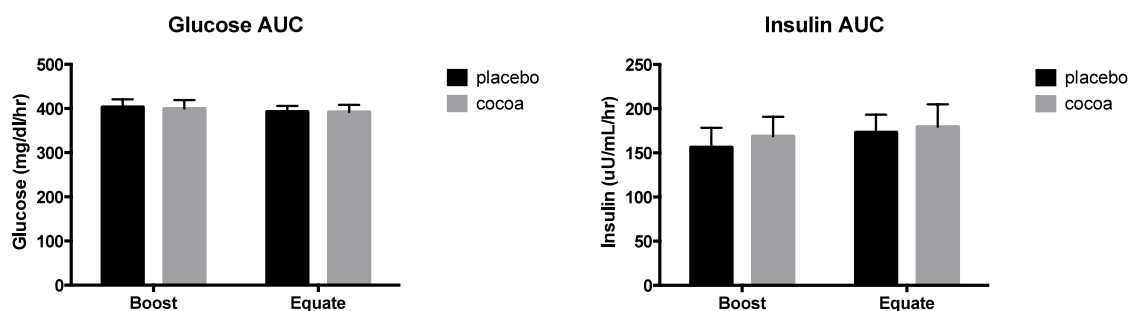


Figure S3.2. Comparison of glucose and insulin responses (AUC) to the two test beverages, Boost ($n = 16$) and Equate ($n = 14$). There were no significant differences ($p < 0.05$).

Table S3.1. Inclusion and exclusion criteria for subject eligibility and enrollment.

Inclusion Criteria	Exclusion Criteria
1. $25 \leq \text{BMI} < 40$	1. Diagnosed diabetes (type 1 or 2) or use of diabetes medications
2. Age 40-76 years	2. Antibiotic, prebiotic, or probiotic use in prior 3 months
3. Weight stable for previous 6 months (± 2 kg)	3. Taking anti-inflammatory medications (e.g., NSAIDs ^c , etc.) or antioxidant vitamins or supplements
4. Sedentary to recreationally active (< 2 d/wk, 20 min/d)	4. Total cholesterol > 300 mg/dL; total triglycerides > 450 mg/dL
5. Meets any 1 of the following 4 criteria:	5. Blood pressure $> 160/100$ mmHg
1) IFG ^a = 100-125 mg/dL	6. Exceeds any of the 3 following criteria ^d :
2) IGT ^b = 140-200 mg/dL	1) IFG > 125 mg/dL
3) HbA1c = 5.7-6.4%	2) IGT > 200 mg/dL
4) ADA risk assessment score ≥ 5	3) HbA1c $> 6.5\%$
	7. Diagnosed inflammatory disease (e.g., lupus, irritable bowel syndrome, periodontal disease, etc.)
	8. Diagnosed disease of the digestive tract (e.g., lactose intolerance, ulcers, cancer (stomach, liver, pancreatic, etc.), celiac's disease, etc.)
	9. Past or current ischemic heart disease, stroke, respiratory disease, endocrine or metabolic disease, neurological disease, or hematological-oncological disease.
	10. Smoking, tobacco use, or alcohol consumption (> 2 servings/d for males and 1 serving/d for females)
	11. Pregnant or planning to become pregnant

^aIFG: Impaired fasting glucose, measured by plasma glucose concentration after an overnight fast

^bIGT: impaired glucose tolerance as measured by plasma blood glucose concentration 2 h following standard Oral Glucose Tolerance Test (OGTT) with 75 g glucose beverage

^cNSAIDs: non-steroidal anti-inflammatory drugs

^dAny subject presenting with symptoms of diabetes during screening was immediately referred to a physician

Table S3.2. Nutrient and polyphenol composition of cocoa and placebo beverage dry mixes^a

Component	Cocoa	Placebo
Calories (kcal)	111	120
Total fat (g)	1.40	0.374
Saturated fat (g)	0.842	0.238
Trans fat (g)	0.0108	0.0136
Cholesterol (mg)	7.70	9.80
Sodium (mg)	299	330
Total carbohydrates (g)	19.0	18.088
Dietary fiber (g)	3.92	0.680
Sugars (g)	12.5	15.9
Protein (g)	10.6	10.9
Vitamin A (IU)	5.22	6.63
Vitamin C (mg)	1.62	2.04
Calcium (mg)	312	408
Iron (mg)	4.32	0.156
Magnesium (mg)	76.1	33.2
Potassium (mg)	581	543
Caffeine (mg)	23.3	0.00
Theobromine (mg)	206	0.136
Total Polyphenols (mg)	864	247
Catechin (mg)	12.5	0
Epicatechin (mg)	50.4	0
Total PACs ^b (mg)	531	0
Total PACs DP ^c 1-10 (mg)	308	<0.002
PACs DP 1 (mg)	68.4	<0.002
PACs DP 2 (mg)	40.7	<0.002
PACs DP 3 (mg)	27.0	<0.002
PACs DP 4 (mg)	32.0	<0.002
PACs DP 5 (mg)	32.8	<0.002
PACs DP 6 (mg)	39.6	<0.002
PACs DP 7 (mg)	47.9	<0.002
PACs DP 8 (mg)	16.2	<0.002
PACs DP 9 (mg)	20.9	<0.002
PACs DP 10 (mg)	7.20	<0.002

^aValues provided by the Hershey Co., Hershey, PA

^bPACs: proanthocyanidins

^cDP: degrees of polymerization

Table S3.3. Composition of meal replacement beverages for acute meal challenge

	Meal Beverage 1^a	Meal Beverage 2^b
Volume (fl oz.)	16	16
Calories (kcal)	480	500
Total fat (g)	8	12
Saturated fat (g)	2	2
Cholesterol (mg)	20	10
Sodium (mg)	300	200
Potassium (mg)	920	740
Total carbohydrate (g)	82	80
Dietary fiber (g)	0	0
Sugars (g)	50	46
Protein (g)	20	18

^aBoost, Very Vanilla Original, Nestle HealthCare Nutrition, Inc., Florham Park, NJ.

^bEquate Nutritional Shake, Vanilla, Wal-Mart Stores Inc., Bentonville, AR.

Table S3.4. Calculated fasting, AUC, C_{MAX} and excursion for glucose separated by phenotypes (NGT and IGT; NFG and IFG).

		Glucose (mg/dL)			
		Fasting	AUC	Excursion	C _{MAX}
All (n = 30)	Placebo	93.2 ± 1.9	398.6 ± 10.8	27.8 ± 3.4	121.0 ± 3.7
	Cocoa	91.0 ± 2.0	396.1 ± 12.8	28.3 ± 3.9	119.4 ± 4.7
	Δ	-2.1 ± 1.2	-2.6 ± 7.6	0.5 ± 3.3	-1.6 ± 3.5
	p value	0.0779	0.7385	0.8837	0.6452
NGT (n = 24)	Placebo	91.5 ± 2.1	384.3 ± 10.1	23.2 ± 3.2	114.6 ± 3.2
	Cocoa	88.6 ± 2.0	377.1 ± 11.4	23.4 ± 3.6	112.0 ± 3.9
	Δ	-2.9 ± 1.3	-7.2 ± 7.7	0.2 ± 3.8	-2.6 ± 3.7
	p value	0.0344*	0.3621	0.9504	0.4844
IGT (n = 6)	Placebo	99.9 ± 3.7	455.8 ± 26.5	46.5 ± 7.1	146.4 ± 6.4
	Cocoa	100.8 ± 3.7	471.7 ± 30.2	48.0 ± 9.9	148.8 ± 12.0
	Δ	0.9 ± 2.6	15.9 ± 21.8	1.5 ± 8.0	2.4 ± 9.8
	p value	0.7410	0.4987	0.8563	0.8158
Two-way interaction	p value	0.1979	0.2276	0.8809	0.5722
Main effects of phenotype	p value	0.0227*	0.0016**	0.0015**	<0.0001****
Main effects of treatment	p value	0.4961	0.6433	0.8377	0.9803
NFG (n = 25)	Placebo	90.2 ± 1.5	393.4 ± 12.2	28.4 ± 3.6	118.6 ± 3.8
	Cocoa	87.9 ± 1.7	384.9 ± 13.6	26.7 ± 3.9	114.6 ± 4.5
	Δ	-2.2 ± 1.3	-8.4 ± 7.8	-1.8 ± 3.3	-4.0 ± 3.3
	p value	0.1054	0.2884	0.6013	0.3293
IFG (n = 5)	Placebo	108.1 ± 3.9	424.8 ± 19.2	24.9 ± 10.2	133.0 ± 10.2
	Cocoa	106.6 ± 3.1	451.7 ± 25.6	36.6 ± 13.2	143.2 ± 13.4
	Δ	-1.6 ± 2.4	26.9 ± 20.3	11.8 ± 10.5	10.2 ± 12.7
	p value	0.5491	0.2564	0.3259	0.4668
Two-way interaction	p value	0.8396	0.0812	0.1319	0.1326
Main effects of phenotype	p value	<0.0001****	0.1035	0.7176	0.0331*
Main effects of treatment	p value	0.2388	0.3526	0.2610	0.5036

All values are reported as the mean ± the standard error of the mean (SEM). Delta (Δ) values are equal to cocoa minus placebo

Table S3.5. Calculated fasting, AUC, C_{MAX} and excursion for insulin separated by phenotypes (NGT and IGT; NFG and IFG).

		Insulin (uU/mL)			
		Fasting	AUC	Excursion	C _{MAX}
All (n = 30)	Placebo	8.2 ± 0.7	164.2 ± 14.8	71.6 ± 7.6	79.8 ± 8.0
	Cocoa	9.0 ± 0.8	173.8 ± 16.5	69.8 ± 7.9	78.9 ± 8.4
	Δ	0.8 ± 0.6	9.5 ± 10.0	-1.7 ± 5.5	-0.9 ± 5.7
	p value	0.1756	0.3465	0.7571	0.8785
NGT (n = 24)	Placebo	7.5 ± 0.7	156.4 ± 16.2	71.2 ± 9.2	78.7 ± 9.6
	Cocoa	9.0 ± 1.0	163.9 ± 17.6	68.2 ± 9.4	77.2 ± 9.9
	Δ	1.5 ± 0.6	7.4 ± 11.5	-3.0 ± 6.8	-1.5 ± 7.0
	p value	0.0332*	0.5250	0.6632	0.8276
IGT (n = 6)	Placebo	11.0 ± 1.9	195.5 ± 36.2	73.0 ± 10.4	84.0 ± 12.2
	Cocoa	9.4 ± 1.6	213.3 ± 41.8	76.3 ± 13.3	85.8 ± 14.6
	Δ	-1.6 ± 1.3	17.9 ± 20.3	3.3 ± 5.7	1.8 ± 6.2
	p value	0.2968	0.4183	0.5868	0.7855
Two-way interaction	p value	0.0460*	0.6831	0.6548	0.8205
Main effects of phenotype	p value	0.2911	0.2398	0.7893	0.7259
Main effects of treatment	p value	0.9373	0.3249	0.9804	0.9873
NFG (n = 25)	Placebo	8.0 ± 0.7	166.0 ± 15.6	74.1 ± 8.6	82.1 ± 9.0
	Cocoa	9.3 ± 1.0	173.6 ± 17.8	70.5 ± 9.2	79.8 ± 9.7
	Δ	1.3 ± 0.6	7.6 ± 11.1	-3.6 ± 6.0	-2.4 ± 6.2
	p value	0.0551	0.4961	0.5514	0.7081
IFG (n = 5)	Placebo	9.2 ± 3.0	155.4 ± 46.9	58.9 ± 14.7	68.1 ± 17.5
	Cocoa	7.8 ± 1.6	174.4 ± 47.7	66.7 ± 14.0	74.6 ± 15.5
	Δ	-1.4 ± 1.6	19.0 ± 27.1	7.9 ± 14.5	6.5 ± 15.3
	p value	0.4361	0.4883	0.6159	0.6936
Two-way interaction	p value	0.1067	0.6792	0.4474	0.5713
Main effects of phenotype	p value	0.9275	0.9046	0.6333	0.6469
Main effects of treatment	p value	0.9644	0.3347	0.7795	0.7904

All values are reported as the mean ± the standard error of the mean (SEM). Delta (Δ) values are equal to cocoa minus placebo.

Table S3.6. Calculated fasting, AUC, C_{MAX} and excursion for C Peptide separated by phenotypes (NGT and IGT; NFG and IFG).

		C Peptide (ng/dL)			
		Fasting	AUC	Excursion	C _{MAX}
All (n = 30)	Placebo	1.4 ± 0.1	19.0 ± 1.1	5.5 ± 0.3	6.8 ± 0.4
	Cocoa	1.5 ± 0.1	20.2 ± 1.5	5.8 ± 0.4	7.3 ± 0.5
	Δ	0.2 ± 0.1	1.2 ± 0.9	0.3 ± 0.4	0.4 ± 0.4
	p value	0.0084*	0.1743	0.4382	0.2482
NGT (n = 24)	Placebo	1.3 ± 0.1	19.0 ± 1.3	5.6 ± 0.4	7.0 ± 0.4
	Cocoa	1.5 ± 0.1	20.0 ± 1.8	5.7 ± 0.5	7.3 ± 0.6
	Δ	0.2 ± 0.1	1.0 ± 1.1	0.1 ± 0.4	0.3 ± 0.3
	p value	0.0044*	0.3797	0.8607	0.5270
IGT (n = 6)	Placebo	1.4 ± 0.2	19.1 ± 2.3	4.9 ± 0.4	6.3 ± 0.5
	Cocoa	1.5 ± 0.3	21.0 ± 3.2	5.9 ± 0.6	7.4 ± 0.9
	Δ	0.1 ± 0.2	0.1 ± 0.2	1.0 ± 0.6	1.1 ± 0.6
	p value	0.7533	0.2938	0.1417	0.1501
Two-way interaction	p value	0.3531	0.6937	0.3309	0.4066
Main effects of phenotype	p value	0.9841	0.8725	0.6979	0.7683
Main effects of treatment	p value	0.1015	0.2279	0.2570	0.1599
NFG (n = 25)	Placebo	1.4 ± 0.1	19.5 ± 1.2	5.7 ± 0.3	7.1 ± 0.4
	Cocoa	1.6 ± 0.1	20.6 ± 1.8	5.9 ± 0.5	7.4 ± 0.6
	Δ	0.2 ± 0.1	1.1 ± 1.1	0.2 ± 0.4	0.4 ± 0.4
	p value	0.0076**	0.3040	0.6885	0.4051
IFG (n = 5)	Placebo	1.2 ± 0.2	16.9 ± 2.8	4.4 ± 0.6	5.7 ± 0.8
	Cocoa	1.4 ± 0.3	18.1 ± 2.7	5.2 ± 0.6	6.5 ± 0.7
	Δ	0.1 ± 0.2	1.2 ± 1.1	0.7 ± 0.9	0.9 ± 0.9
	p value	0.5836	0.3272	0.4590	0.3780
Two-way interaction	p value	0.7341	0.9691	0.5818	0.6157
Main effects of phenotype	p value	0.6030	0.4601	0.2318	0.2813
Main effects of treatment	p value	0.0751	0.3504	0.3809	0.2381

All values are reported as the mean ± the standard error of the mean (SEM). Delta (Δ) values are equal to cocoa minus placebo.

Table S3.7. Calculated fasting, AUC, C_{MAX} and excursion for GLP-1 separated by phenotypes (NGT and IGT; NFG and IFG).

		GLP-1 (pM)			
		Fasting	AUC	Excursion	C _{MAX}
All (n = 30)	Placebo	26.6 ± 2.5	167.3 ± 13.7	25.0 ± 3.0	51.6 ± 4.1
	Cocoa	27.4 ± 2.9	166.4 ± 13.5	25.1 ± 2.4	52.5 ± 4.1
	Δ	0.8 ± 1.3	-1.0 ± 6.4	0.1 ± 1.9	0.9 ± 2.1
	p value	0.5652	0.8832	0.9508	0.6833
NGT (n = 24)	Placebo	29.2 ± 2.8	180.6 ± 15.1	26.3 ± 3.6	55.5 ± 4.6
	Cocoa	30.1 ± 3.4	176.6 ± 15.5	25.0 ± 2.8	55.1 ± 4.7
	Δ	0.9 ± 1.4	-4.0 ± 6.4	-1.3 ± 2.1	-0.4 ± 2.1
	p value	0.5370	0.5356	0.5358	0.8448
IGT (n = 6)	Placebo	16.3 ± 3.9	114.0 ± 22.7	19.7 ± 3.2	36.0 ± 7.0
	Cocoa	16.5 ± 3.2	125.3 ± 20.6	25.6 ± 4.9	42.1 ± 7.1
	Δ	0.2 ± 3.4	11.3 ± 20.4	5.9 ± 4.6	6.1 ± 6.9
	p value	0.9551	0.6024	0.2597	0.4221
Two-way interaction	p value	0.8348	0.3502	0.1432	0.2342
Main effects of phenotype	p value	0.0435*	0.0729	0.6451	0.1003
Main effects of treatment	p value	0.7428	0.6549	0.3479	0.2973
NFG (n = 25)	Placebo	29.5 ± 2.6	178.8 ± 14.4	24.8 ± 3.3	54.2 ± 4.5
	Cocoa	29.7 ± 3.3	172.7 ± 14.2	24.5 ± 2.6	54.2 ± 4.4
	Δ	0.2 ± 1.5	-6.1 ± 6.1	-0.2 ± 1.9	-0.0 ± 1.9
	p value	0.8895	0.3311	0.9094	0.9983
IFG (n = 5)	Placebo	12.4 ± 3.4	110.1 ± 30.2	26.1 ± 7.3	38.5 ± 9.7
	Cocoa	16.0 ± 4.5	134.9 ± 40.2	27.9 ± 7.0	43.8 ± 11.4
	Δ	3.5 ± 2.2	24.8 ± 21.7	1.8 ± 7.7	5.3 ± 5.8
	p value	0.1892	0.3168	0.8267	0.5988
Two-way interaction	p value	0.3553	0.0732	0.7067	0.3637
Main effects of phenotype	p value	0.0285*	0.1351	0.7412	0.2244
Main effects of treatment	p value	0.2984	0.2697	0.7670	0.3644

All values are reported as the mean ± the standard error of the mean (SEM). Delta (Δ) values are equal to cocoa minus placebo.

Table S3.8. Calculated fasting, AUC, C_{MAX} and excursion for GIP separated by phenotypes (NGT and IGT; NFG and IFG).

		GIP (pg/mL)			
		Fasting	AUC	Excursion	C _{MAX}
All (n = 30)	Placebo	37.3 ± 3.5	869.2 ± 71.8	289.5 ± 27.0	326.8 ± 28.4
	Cocoa	39.3 ± 3.6	825.9 ± 60.2	276.4 ± 25.4	315.6 ± 26.0
	Δ	2.0 ± 2.7	-43.3 ± 42.9	-13.2 ± 21.5	-11.2 ± 21.9
	p value	0.4744	0.3216	0.5455	0.6119
NGT (n = 24)	Placebo	35.8 ± 3.7	861.7 ± 81.5	288.9 ± 30.8	324.8 ± 32.4
	Cocoa	39.7 ± 4.0	801.1 ± 66.9	268.6 ± 29.4	308.3 ± 29.8
	Δ	3.9 ± 3.0	-60.6 ± 45.6	-20.4 ± 24.3	-16.5 ± 24.9
	p value	0.2155	0.1970	0.4102	0.5147
IGT (n = 6)	Placebo	43.3 ± 9.6	899.3 ± 164.4	291.9 ± 60.9	335.1 ± 63.2
	Cocoa	37.6 ± 8.4	925.2 ± 142.0	345.0 ± 55.4	345.0 ± 55.4
	Δ	-5.7 ± 5.4	25.9 ± 188.1	15.6 ± 49.1	9.9 ± 48.2
	p value	0.3418	0.8349	0.7641	0.8451
Two-way interaction	p value	0.1640	0.4297	0.5143	0.6373
Main effects of phenotype	p value	0.7493	0.6142	0.7331	0.7123
Main effects of treatment	p value	0.7911	0.7507	0.9303	0.9062
NFG (n = 25)	Placebo	36.4 ± 3.8	855.9 ± 74.8	287.4 ± 28.6	323.8 ± 30.0
	Cocoa	38.6 ± 3.9	765.7 ± 59.3	246.3 ± 24.5	284.9 ± 24.5
	Δ	2.2 ± 2.9	-90.1 ± 41.0	-41.0 ± 20.1	-38.8 ± 20.7
	p value	0.4570	0.0378*	0.0524	0.0723
IFG (n = 5)	Placebo	41.9 ± 9.9	935.8 ± 234.8	300.3 ± 84.9	342.2 ± 89.2
	Cocoa	42.7 ± 10.0	1126.8 ± 157.3	426.4 ± 56.7	469.1 ± 65.9
	Δ	0.8 ± 8.0	191.0 ± 114.9	126.1 ± 47.3	126.9 ± 47.6
	p value	0.9296	0.1717	0.0560	0.0559
Two-way interaction	p value	0.8451	0.0118*	0.0022**	0.0029**
Main effects of phenotype	p value	0.5899	0.1946	0.1345	0.1318
Main effects of treatment	p value	0.6924	0.3422	0.0975	0.0941

All values are reported as the mean ± the standard error of the mean (SEM). Delta (Δ) values are equal to cocoa minus placebo.

Table S3.9. Characteristics of subjects with NGT versus IGT

Parameter ^a	NGT ^b Subjects	IGT ^c Subjects	<i>p</i> value ^d
n	24	6	-
Female/male	15/9	4/2	-
Age (yr)	54.0 ± 2.0	58.5 ± 4.3	0.3283
Weight (kg)	95.2 ± 2.7	86.3 ± 5.6	0.1564
Body Mass Index (kg/m ²)	34.1 ± 0.8	30.4 ± 0.8	0.0361*
Serum triglycerides (mg/dL)	106 ± 12	129 ± 11	0.9921
Serum total cholesterol (mg/dL)	191 ± 9	218 ± 8	0.5020
ADA Risk Score ^e	5 ± 0	5 ± 0	0.5642
FBG ^f (mg/dL)	90.3 ± 1.7	97.8 ± 4.3	0.0725
2 h OGTT ^g (mg/dL)	102.1 ± 4.4	167.7 ± 8.3	<0.0001***
HbA1c ^h (%)	5.7 ± 0.1	5.9 ± 0.1	0.1511

^aValues are reported as mean ± SEM (except for n size and female/male)

^bNGT: subjects presenting with normal glucose tolerance (2 h plasma glucose concentration <140.0 mg/dL following 75 g glucose beverage) at screening

^cIGT: subjects presenting with impaired glucose tolerance (2 h plasma glucose concentration between 140.0-199.9 mg/dL following 75 g glucose beverage) at screening

^dt-test comparing IGT cohort to NGT cohort * = *p* < 0.05; ** = *p* < 0.001; *** = *p* < 0.0001

^eScore on American Diabetes Association Risk Assessment Questionnaire

^fFBG: fasting blood glucose concentration

^g2 h oral glucose tolerance test: blood glucose concentration 2 h after consuming 75 g glucose beverage

^hGlycosylated hemoglobin

Table S3.10. Characteristics of subjects with NFG versus IFG

Parameter ^a	NFG ^b Subjects	IFG ^c Subjects	<i>p</i> value ^d
n	25	5	
Female/male	14/10	4/1	
Age (yr)	54.3 ± 1.9	58.2 ± 5.7	0.4246
Weight (kg)	95.4 ± 2.7	87.2 ± 6.5	0.2537
Body Mass Index (kg/m ²)	33.9 ± 0.8	31.0 ± 1.6	0.1490
Serum triglycerides (mg/dL)	120 ± 12	136 ± 16	0.5590
Serum total cholesterol (mg/dL)	202 ± 9	228 ± 16	0.2232
ADA Risk Score ^e	5 ± 0	5 ± 0	0.3419
FBG ^f (mg/dL)	88.65 ± 1.1	107.7 ± 3.0	<0.0001***
2 h OGTT ^g (mg/dL)	113.3 ± 6.2	124.7 ± 22.2	0.5011
HbA1c ^h (%)	5.6 ± 0.1	6.1 ± 0.1	0.0071**

^aValues are reported as mean ± SEM (except for n size and female/male)

^bNFG: subjects presenting with normal fasting glucose (<100 mg/dL) at screening

^cIGT: subjects presenting with impaired fasting glucose (100-125mg/dL) at screening

^dt-test comparing IFG cohort to NFG cohort * = *p* < 0.05; ** = *p* < 0.001; *** = *p* < 0.0001

^eScore on American Diabetes Association Risk Assessment Questionnaire

^fFBG: fasting blood glucose concentration

^g2 h OGTT: blood glucose concentration 2 h after consuming 75 g glucose beverage

^hGlycosylated hemoglobin

Additional Data.

Visual Analog Scale. The second cohort of participants ($n = 14$ Equate beverage) completed Visual Analogue Scale (VAS) questionnaires about their perceptions of hunger, fullness, desire to eat, thirst, and nausea (**Figure E1**). The questionnaires were completed every thirty minutes during the meal challenge (9 times total). VAS measures were not split by NGT/IGT because there was only 1 participant in the IGT category of the 14 participants that completed the questionnaires.

The absolute data of the Visual Analog Scale is shown in **Figure E2**. Fourteen participants completed the questionnaires. In **Figure E2A**, a higher score indicated that there was a weaker desire to eat. In **Figure E2B**, a higher score indicated a higher perception of hunger. In **Figure E2C**, a higher score indicated a stronger feeling of fullness. In **Figure E2D**, a higher score indicated a higher perception of thirst. Lastly, in **Figure E2E**, a higher score indicated a stronger feeling of nausea.

There was a significant difference ($p = 0.0171$) in the maximum score in the question related to thirst (**Figure E3**), indicating that participants felt more thirsty when they consumed the placebo beverage compared to the cocoa beverage. In the absolute data (**Figure E2**), it appears as though the placebo treatment was only slightly higher than the cocoa treatment at the 4-hour time point. However, when calculating the average maximum score, we compared the maximum scores for each individual as opposed to the maximum average value for all participants as a group. Therefore, if we simply looked at the average score for the 4-hour time point (because it is the only time point where the placebo treatment appears to be higher than the cocoa treatment), it is, in fact, not significant ($p = 0.0578$). However, some participants felt most thirsty at other times

during the 4-hour test, and the maximum score indicated does not take into account the time at which they felt most thirsty.

In conclusion, there were no noteworthy changes in perceptions of desire to eat, hunger, fullness, thirst, or nausea, with the exception of a change in the maximum perception of thirst.

Hunger Questionnaire

Place an “x” on the line to indicate your response:

1. How strong is your desire to eat right now?

Very strong

Very weak

2. How hungry do you feel right now?

Not hungry at all

Extremely hungry

3. How full do you feel right now?

Not at all full

Extremely full

4. How thirsty do you feel right now?

Not at all thirsty

Extremely thirsty

5. How nauseated do you feel right now?

Not At all Nauseated

Very Nauseated

Figure E1. Copy of the Visual Analog Scale questionnaire.

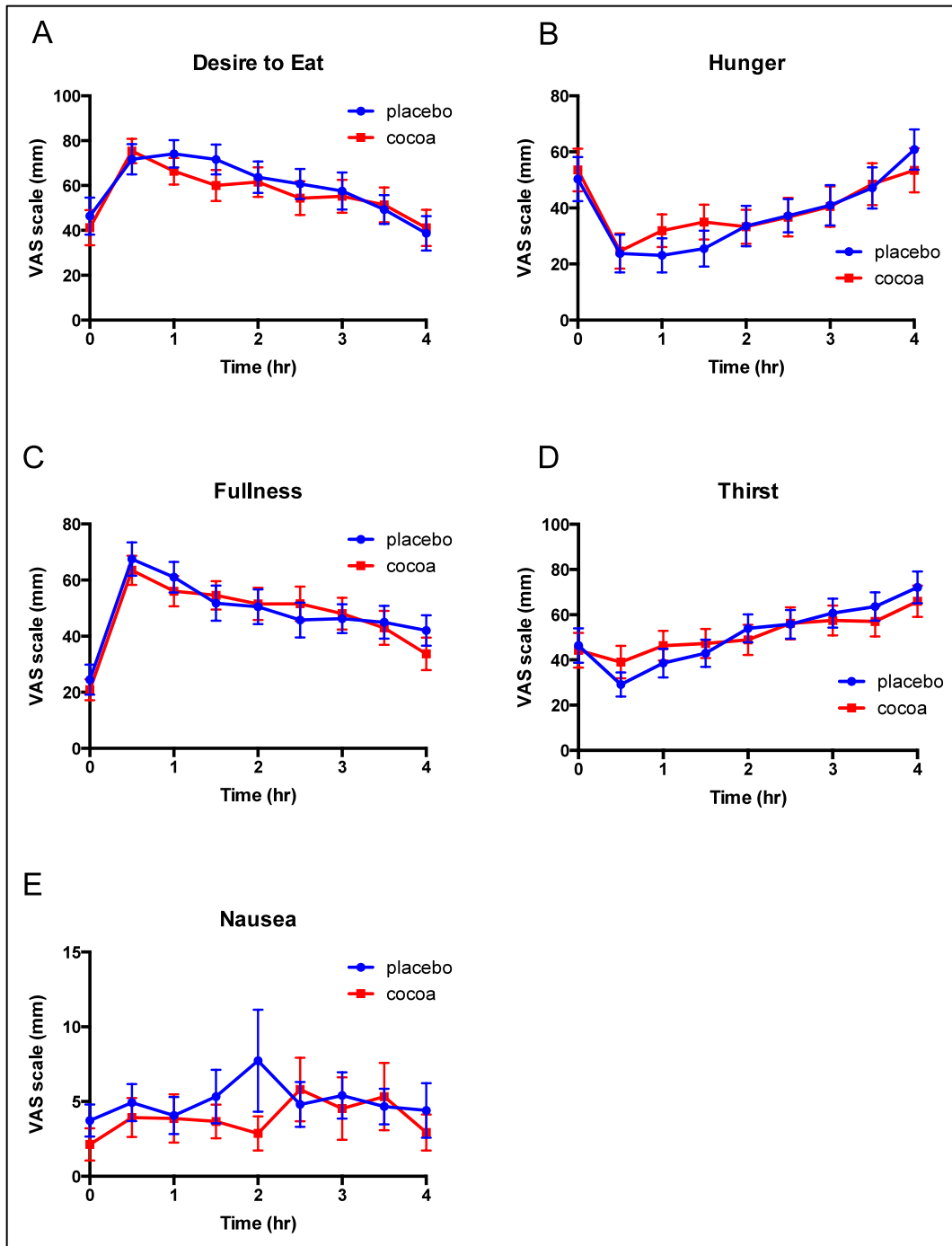


Figure E2. Visual analog scale (VAS) ratings of (A) desire to eat, (B) hunger, (C) fullness, (D) thirst, and (E) nausea reported in millimeters as the mean $\pm$ SEM. VAS measures were completed at baseline and every 30 min during the 4 h tests with cocoa (•) or placebo (•). No significant differences were detected between single time points throughout the tests by paired t-tests, $P < 0.05$, $N = 14$.

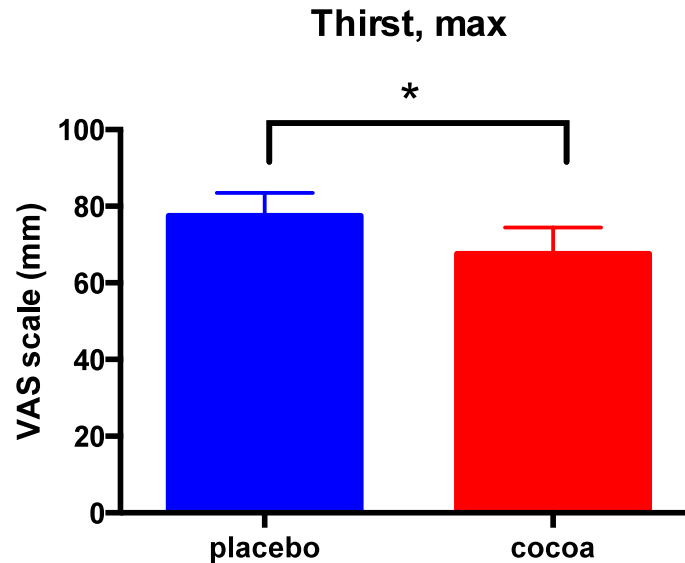


Figure E3. Maximum thirst perception recorded on VAS measures reported as the mean ($\pm$ SEM). A higher score indicates greater thirst. There was a significant difference between cocoa and placebo treatments as determined by a paired t-test, $p = 0.0171$.

Comparison of GLP-1 response between NGT and IGT groups. While there appeared to be no significant differences in GLP-1 due to the cocoa treatment, there were clear variations in the GLP-1 response between our two cohorts (NGT and IGT) that were worth characterizing.

Figure E4A depicts the raw GLP-1 data from the placebo beverage only. There is a notable difference in GLP-1 levels in response to the mixed meal. The fasting values, as indicated in **Figure E4B**, were significantly lower in the IGT cohort ($p = 0.0376$). GLP-1 concentration was also significantly different at 2 h ($p = 0.0495$). Differences in GLP-1 approached significance at 1 h, 3 h, and 4 h ($p = 0.0503$, $p = 0.1254$, and $p = 0.0724$, respectively). Fasting GIP, insulin, and C Peptide were not different between the two cohorts.

In conclusion, this study demonstrated important hormonal variations between adults with normal glucose tolerance and impaired glucose tolerance, differences worth characterizing further.

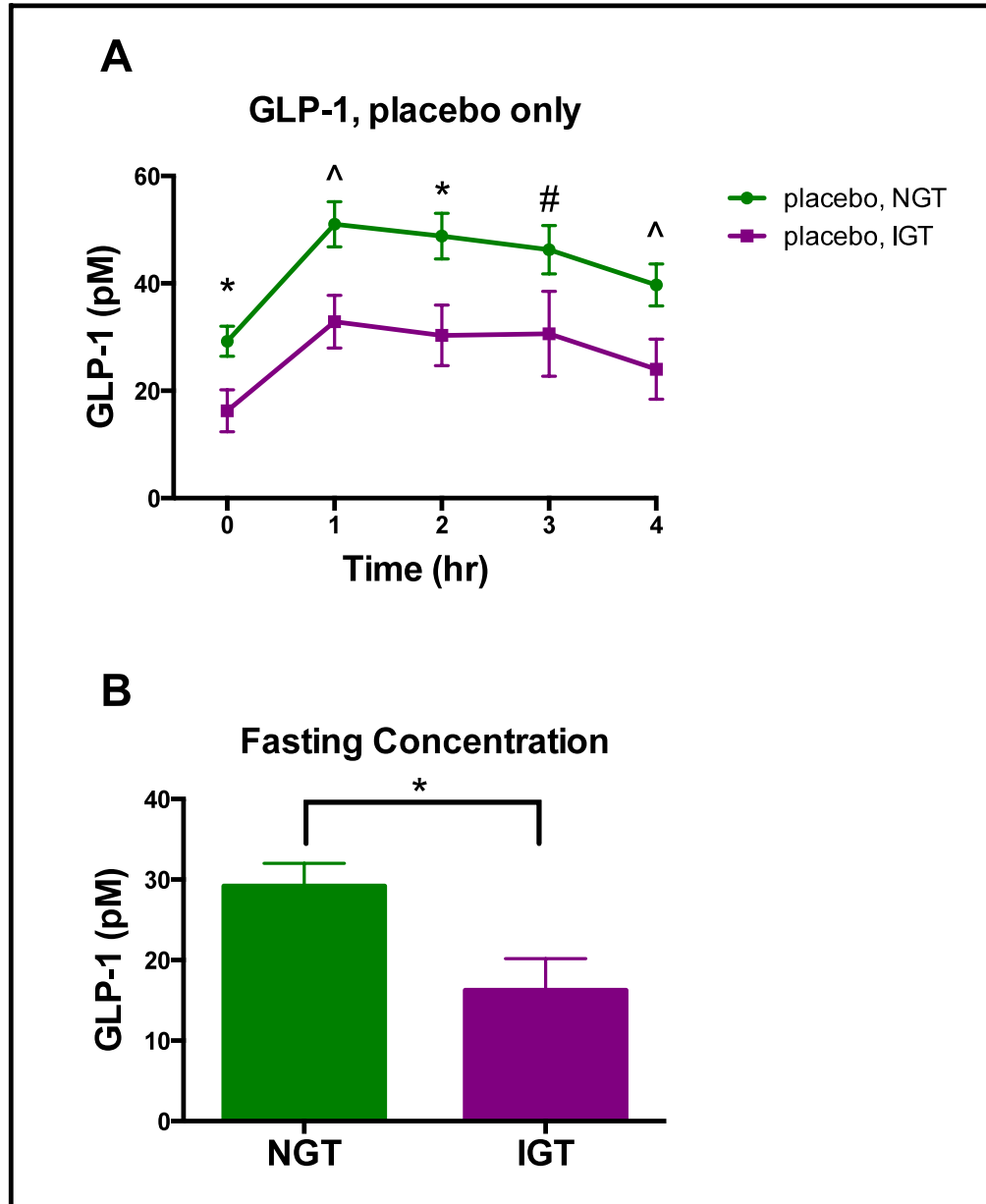


Figure E4. (A) Mean ($\pm$ SEM) GLP-1 concentrations after consumption of the meal with the placebo for subjects with NGT (●) compared to IGT (■). Unpaired t-tests detected *significant difference between groups $P < 0.05$; strongly approached significance ^ $P < 0.10$; approached significance # $P < 0.15$. (B) Mean ($\pm$ SEM) of fasting GLP-1 concentrations for subjects with NGT (●) compared to IGT (■). *An unpaired t-test detected a significant difference between groups ($p = 0.0376$).

Appendix E

Chapter 4: Chronic Study Detailed Materials And Methods

Subjects, recruitment, screening and enrollment. Approval from the Virginia Tech Institutional Review Board (IRB) was obtained for all aspects of the study involving human subjects (IRB# 13-755). The study was registered at ClinicalTrials.gov (NCT02203240). Volunteers were recruited from the New River Valley, VA via posted and mailed fliers, web postings, and newspaper advertisements. Interested individuals completed an online questionnaire to assess eligibility. Inclusion/exclusion criteria are shown in Table S4.1. Eligible and interested prospective participants provided written and oral informed consent and completed a health history questionnaire, Godin Leisure-time questionnaire,³⁰⁹ an infection/inflammation questionnaire, and the American Diabetes Association Risk-Assessment questionnaire²⁵⁷ at an initial informational session. Body weight was measured on a digital scale (Model 5002, Scale-Tronix, White Plains, NY) to the nearest ± 0.1 kg and height was measured on a stadiometer to the nearest ± 0.1 cm. Subjects also received instructions on how to complete a 4-day food diary (3 consecutive week days and one weekend day) to estimate habitual energy and macronutrient content. Once completed, the record was reviewed by a registered dietitian and analyzed using The Nutrition Data Systems for Research software (NDS-R 2013 University of Minnesota, Minneapolis, MN).

On a different day, participants who were still eligible and interested arrived at the lab between 6-9 A.M. after a 12-h overnight fast to complete screening procedures. Subjects provided a urine sample for a urinalysis conducted at a commercial lab (Solstas Lab Partners, Roanoke, VA) to test for white blood cell esterase and other markers of

infection or disease and a pregnancy test (Kroger, Cincinnati, OH) for premenopausal women. Participants were asked to sit quietly for 10 min prior to assessing their blood pressure using an automated blood pressure monitor (Omron Healthcare Inc., Bannockburn, IL). A fasting serum blood sample was taken from the antecubital vein to assess cholesterol and triglycerides by a commercial laboratory (Solstas Lab Partners, Roanoke, VA) using standard enzymatic techniques. The sample was allowed to clot for 15 min at room temperature (21°C) before being centrifuged (1148 x g, 15 min, 4°C). A finger stick blood sample was obtained to measure HbA1c (HbA1c Now⁺) (PTS Diagnostics, Indianapolis, IN). A fasting plasma sample was taken from the antecubital vein to measure glucose concentration. The whole blood sample was collected in a vacutainer with 12 mg K₃ EDTA (BD, Franklin Lakes, NJ) and immediately stored on ice until centrifugation (1148 x g, 15 min, 4°C). Blood glucose concentration was measured by the glucose oxidase method using a glucose auto-analyzer (Yellow Springs Instruments, Yellow Springs, OH, USA). Participants then consumed a 75 g glucose beverage (Sun Dex, Fisher, Pittsburgh, PA) within the first five min of the 2-hour oral glucose tolerance test (OGTT). An antecubital plasma blood sample was taken 2 h after the first sip of the beverage and analyzed for glucose as described above. Subjects were asked to remain seated and awake during the OGTT. Body composition was analyzed via dual-energy x-ray absorptiometry (Lunar iDXA enCORE v15) (General Electric Medical Systems Ultrasound & Primary Care Diagnostics, LLC, Madison, WI).

Twenty subjects were enrolled and 15 completed the study. Characteristics of subjects who completed the study are shown in Table 4.2.

Experimental design. This study was a randomized, double-blinded, placebo-controlled trial. Testing procedures were conducted in 3 visits before and after a 4-week intervention (Figure 4.2). The intervention included daily cocoa/placebo treatments and controlled feeding. Subjects were randomized to receive the cocoa or placebo treatment (with gender stratification). Treatment packets were coded and only the Hershey Co. and an investigator not involved with data collection and analysis knew the code.

Wash out diet. For one week prior to the first day of baseline testing, subjects followed a "wash out diet." Subjects ate their normal diet but avoided consuming foods and beverages that contained high amounts of polyphenols (i.e. apples, berries, wine, almonds, etc.) or other ingredients that may have affected the study outcomes (i.e. cocoa, chocolate, yogurt, prebiotics, probiotics, etc.) (see chapter 4 Supplementary Information for full list). Subjects continued the wash out diet through the three baseline testing visits.

Cocoa and Placebo Treatments. Cocoa and placebo beverage dry mixes were individually pre-packaged in coded sachets, from a single production lot and obtained directly from Hershey. The beverages were custom formulations produced by Hershey and designed to support human clinical trials. Random samples indicated that the composition of each mix was consistent between sachets. Upon receipt, beverage mixes were refrigerated (4°C) until use. The mixes are designed to be shelf-stable at room temperature for 2-3 yr. Full characterization and nutrition content of cocoa and placebo packets are described in Table 4.3 (The Hershey Co., Hershey, PA).

Participants consumed three treatment packets daily for four weeks. They were provided with a shaker bottle and were instructed to mix with hot or cold water and consume one beverage packet with each meal. Participants continued to take the treatments during post-testing, but only two packets a day so as not to interfere with the tests.

Controlled feeding. The controlled feeding portion of the study was conducted at the Metabolic Kitchen and Dining Lab at Virginia Tech. Subjects were provided with all of their food during the intervention and were instructed not to consume anything else or add any additional ingredients (condiments, salt, pepper, etc.) to their food. Subjects were required to come to the lab a minimum of 3 days per week in order to have a supervised breakfast and treatment, receive food, promote compliance, and track body weight. Subjects were weighed every time they came to the lab and were asked to report any intake of food or beverages not provided to them. Subjects remained on the diet during all post-testing procedures.

Subjects consumed an isocaloric, controlled diet (55% carbohydrate, 30% fat, 15% protein) during the 4-week intervention and post-testing. The energy and macronutrient content of the diets included the energy and macronutrients contained in the cocoa treatment (or placebo) products. The saturated fat content of the controlled diet was <10% of total energy intake. Fiber intake was matched to typical U.S. intake, at 7.5 g/1000 kcal.²⁹² Sodium was restricted to <3,000 mg/d for all diets except for the 3500 kcal diet which was restricted to 3,500 mg/d, which is similar to typical U.S. intake.²⁹³ Controlled diets were limited in polyphenol-rich foods (e.g., apples, tea, grapes, etc.). If

subjects were habitual coffee drinkers, they were provided with 12 oz. black coffee or 2 Tbs. coffee grounds per day.

Energy requirements for each participant were estimated based on height, weight, age, gender and an activity factor based on self-reported physical activity levels.²⁹⁴ Daily kcal amounts ranged from 1,500-3,500 kcal. A 7-day rotation menu was planned using Nutrition Data System for Research (NDSR) software version 2013 (University of Minnesota, Minneapolis, MN) by a registered dietitian. A sample menu with nutrient breakdown is included in the supplementary information. The individual macronutrients and saturated fat content of the diets were ± 5 g of the target values for each day. The controlled diet was designed to maintain body weight and to reduce the variability in the individual participant's diets and its potential impact on the study outcomes. Additional food items in the form of 250 kcal modules (with a macronutrient composition matched to the overall diet) were provided to adjust kcal level and counter any trends in weight loss or gain during the intervention. Average habitual daily energy and macronutrient intake for participants is displayed in Table 4.2.

The following procedures were performed before and after the 4-week intervention.

High Fat Challenge

During the high fat challenge, two skeletal muscle biopsies (details below) were conducted on alternate legs: one after an overnight fast, before the test meal, and the second 4 h after the meal in order to capture fasting and post-prandial measures (See Figure 4.3). After the first biopsy, an intravenous catheter was inserted into the antecubital vein to collect blood samples throughout the test. After a fasting blood

sample, subjects consumed a high fat meal (see Table 4.4). Subjects consumed the meal within ten minutes and were provided an optional bottle of water. Blood samples were collected 1, 2, 3, and 4 h after the first bite of the meal to track changes in circulating endotoxin in response to a high fat meal.

Skeletal Muscle Biopsy. Biopsies of the *vastus lateralis* were performed using a 5 mm modified Bergström needle (Cadence, Staunton, VA). Subjects were supine throughout the procedure. The skin was cleaned with a providone-iodine swab (PDI Inc., Orangeburg, NY) and a sterile field was created. The skin and muscle was numbed with a local anesthetic (1% lidocaine) (Hospira Inc., Lake Forest, IL). A 0.25 in incision was made with a #11 scalpel (Feather Safety Razor Co. LTD., Kita-Ku, Osaka, Japan). Suction was applied to the Bergström needle in order to remove small samples of the muscle. Multiple passes were taken until ~750 mg of tissue was collected. The site was cleaned with saline, closed with sterile bandage strips, and ice and compression were applied to minimize discomfort. Collected tissue was washed in 0.9% sterile saline to remove blood and tissue. Samples were weighed and either flash frozen in liquid nitrogen, added to buffer containing 250 mM sucrose, 1mM EDTA, 10 mM Tris-HCL, and 2 mM ATP for immediate analysis of substrate flexibility.

Substrate Flexibility. ^{14}C -CO₂ production and ^{14}C -labeled acid-soluble metabolites (ASM) were measured and summed to determine total palmitate (fatty acid) oxidation rates.²⁹⁸ ASM's are a measure of incomplete fatty acid oxidation that is correlated with dysregulated metabolism and insulin resistance.

Pyruvate oxidation was assessed by measuring ^{14}C -CO₂ production. Pyruvate oxidation was used to assess the activity of pyruvate dehydrogenase (PDH), the enzyme

that catalyzes the oxidation of pyruvate and the provision of glucose-derived acetyl CoA to the TCA cycle.

Gaseous ^{14}C - CO_2 produced from the substrate oxidation assays was measured in a sealed device by trapping ^{14}C - CO_2 in 0.4 mL in 1M of sodium hydroxide. Metabolic activity was stopped by the injection of 50 μL of 70% perchloric acid. To measure ASM's, acidified samples were collected, stored overnight (~12 h) at 5 degrees C, and centrifuged (18,000 x g, 15 min, 4°C) to remove albumin-bound $[1\text{-}^{14}\text{C}]$ -palmitate. One mL supernatant was collected and ASM's were quantified by counting in a scintillation vial ²⁹⁸. Metabolic flexibility was assessed by measuring ^{14}C - CO_2 generated from oxidation of $[1\text{-}^{14}\text{C}]$ -pyruvate with/without the presence of non-labeled palmitic acid. Changes in pyruvate oxidation in the presence of palmitic acid were examined to assess metabolic flexibility. Metabolic flexibility is expressed as the % decrease in pyruvate metabolism from $[1\text{-}^{14}\text{C}]$ -pyruvate only to $[1\text{-}^{14}\text{C}]$ -pyruvate + unlabeled palmitic acid. All samples were run in triplicate and data were normalized to total protein content and expressed in nmol/mg protein/h.

Endotoxin response to meal challenge. Endotoxin was measured in duplicate using Limulus Amebocyte Lysate (LAL) Pyrogen® 5000 assay kits (Lonza, Walkersville, MD). LAL assay plates, LAL reagent water, and pyrogen-free tubes (all from Lonza) were employed and serum was placed in a 15 min hot water bath (70°C) to minimize exogenous endotoxin contamination. Assays were performed using 100 μL serum diluted 40:1. Fluorescence was quantified using a BioTek Synergy 2 plate reader (Biotek, Winooski, VT), and data was processed using Gen 5 software (v1.08, BioTek).

Calculations. Psuedo-pharmacokinetic (PPK) curves and PPK parameters for

serum endotoxin concentrations will be constructed using PK plug-in functions for Microsoft Excel. PK parameters to be determined include: C_0 (fasting concentration), C_{MAX} (maximal concentration observed), excursion ($C_{MAX} - C_0$), and AUC (area under the PK curve).

Intravenous Glucose Tolerance Test (IVGTT). Insulin sensitivity was determined using Bergman's minimal model (MINMOD Millennium software, version 6.02, Minmod, INC., Pasadena, CA) via a frequently sampled IVGTT.²⁹⁵ This model characterizes the dynamic glucose/insulin system, given that glucose is removed from the blood stream via two predominant pathways: The first pathway is related to glucose-mediated glucose disposal. This is referring to the glucose transporter (GLUT) 1 – facilitated diffusion of glucose into organs and cells such as the brain, kidney, erythrocytes, and endothelial cells. This glucose uptake occurs in the absence of insulin. The second pathway is composed of insulin-mediated glucose disposal. This predominately utilizes GLUT4 in the skeletal muscles, the largest glucose sink in the body.

This model is applied to data from the IVGTT, where the glucose and insulin system is manipulated. A bolus of glucose is administered intravenously and the clearance of glucose from the blood is captured via frequent blood samples for 20 min. At this point, a dose of insulin large enough to influence glucose disposal but small enough to avoid hypoglycemia is administered in order to increase the sensitivity of the test and to calibrate the insulin response.²⁹⁵ Frequent blood sampling continues for 2-3 hours, until glucose and insulin concentrations return to baseline.

The minimal model can capture a variety of data related to the dynamics of glucose and insulin. First, glucose effectiveness (S_g) describes the first pathway, or the glucose-mediated disposal rate. β cells should respond to the bolus of glucose and secrete insulin; AIR_g, or the acute response of insulin to glucose, describes the responsiveness of the β cells and the concomitant increase in circulating insulin concentration. Insulin sensitivity (SI) describes the second pathway, which characterizes the effectiveness of insulin-mediated pathways. Lastly, the product of SI and AIR_g expresses the disposition index (DI). This describes the normality of the β cell response in relation to the degree of insulin resistance in the tissue. A tissue with normal insulin sensitivity would require a smaller amount of insulin to appropriately clear glucose compared to an insulin-resistant tissue. The disposition index has been shown to be excellent at predicting which pre-diabetic individuals will develop type 2 diabetes.³¹⁰

Procedures. Subjects arrived in the lab between 5-9 A.M. after a 12 h overnight fast. Dextrose (0.3 g/kg; 50% solution) (Hospira, Inc., Lake Forest, IL) and insulin (0.025 U/kg) (Lilly USA, LLC, Indianapolis, IN) dosages were calculated from body weight.²⁹⁶ An intravenous catheter was inserted into each antecubital vein (one arm was used for dextrose and insulin injections and the other for blood sampling). Baseline blood samples were taken prior to the dextrose injection (minute 0). Venous samples (10 ml) were collected at minutes 1, 2, 3, 4, 5, 6, 7, 8, 10, 12, 14, 16, and 18. Insulin was injected at minute 20. Venous sampling continued at minutes 22, 23, 24, 25, 27, 30, 40, 50, 60, 70, 80, 90, 100, 120, 150, and 180. Plasma samples were stored on ice and serum samples were allowed to clot at room temperature for > 15 minutes. Blood samples were centrifuged for (15 min, 4°C, 1140 x g). Glucose concentration was immediately

analyzed by the glucose oxidase method on an automated glucose oxidase analyzer (Yellow Springs Instruments, Yellow Springs, OH). Insulin was later measured from serum using the Immulite 1000 immunoassay analyzer (Siemens, Deerfield, IL).

Body Composition. Body composition was determined by dual-energy X-ray absorptiometry (Lunar iDXA enCORE v15) (General Electric Medical Systems Ultrasound & Primary Care Diagnostics, LLC, Madison, WI).

Gut Permeability. The four-sugar [40 g sucrose, 1 g mannitol, 1 g sucralose, (Spectrum Chemicals, New Brunswick, NJ) and 5 g lactulose (Qualitest Pharmaceuticals, Huntsville, AL)] probe test was used to assess total gut permeability.³⁰⁰ Sucrose is a suitable marker for gastric permeability because it is quickly degraded in the duodenum.³¹¹ Lactulose and mannitol are metabolized by colonic microflora, and thus are useful for determining small intestinal permeability.³¹² Lastly, sucralose is resistant to microbial degradation and will reside in the colon for the majority of its transit, making it a suitable probe for colonic permeability.³¹³

After an overnight fast and urine evacuation, participants were asked to consume the sugar-probe beverage in 5 minutes or less. Participants were given two breakfast sandwiches (see Table 4.4), which were the only items they were allowed to consume during the first 5 hours of the test in order to reduce confounding dietary factors (i.e. high sucrose content). This meal was selected for its ease of distribution and low sucrose content. Participants were also provided with one 16 oz. bottle of water, which they were instructed to finish by the first hour of the test to promote urination during the first

collection period. Participants were given two urine collection containers with 5 g of 10% thymol in methanol (w/v) (an antimicrobial preservative). Participants were instructed to collect all of their urine from the time the beverage was consumed (0 h) until 5 h in the first container, in order to capture gastric and small intestinal permeability. The second container was filled between 6 - 24 h to assess colonic permeability. Urine containers were returned after 24 h. Participants were asked to avoid consuming artificial sweeteners, caffeine, and alcohol during this test. The volume of urine in each container was measured and aliquots from each container were stored at -80°C degrees until further analysis.

Urine Chemistries. Urinary sugars were measured as described by Camilleri *et al.*³⁰¹ 50 μL urine was combined with 50 μL internal standard [20 mg/mL $^{13}\text{C}_6$ -glucose in water/acetonitrile (98:2)], diluted to 4 mL with water and vortexed with 4 mL dichloromethane. Following 30 min incubation and centrifugation (10 min, $3500 \times g$), 100 μL supernatant was diluted with 900 μL acetonitrile/water (85:15) and analyzed by UPLC-MS/MS. UPLC separation was performed on a Waters Acquity H-class (Milford, MA) equipped with an Acquity UPLC BEH Amide column (2.1 mm $\text{\AA}$ ~ 50 mm, 1.7 μm particle size). Isocratic elution was performed at 0.7 mL/min using acetonitrile:water (65:35) with 0.2% v/v triethylamine (TEA). Column and sample temperatures were 35 and 10°C , respectively. Detection by MS/MS was performed on a Waters Acquity Triple Quadrupole Detector (TQD). Negative-mode electrospray ionization [(-)-ESI] was performed with capillary voltage of -4 kV , and source and desolvation temperatures of 150 and 450°C , respectively. Desolvation and cone gasses were N_2 at flow rates of 900 and 1 L/h, respectively. For MS/MS, the collision gas was Ar. The cone voltages,

collision energy, and Multiple Reaction Monitoring (MRM) transitions for each compound are listed in Table 4.5. Peak widths were ~4 s, and AutoDwell was employed with required points-per-peak set at 12. The interscan delay time was 0.02 s. Data acquisition, processing, and quantification was performed using MassLynx v4.1 software (Waters, Milford, MA).

Calculations. Urine sugar concentrations were converted to total sugar excreted using urine volume. Excretion was calculated as a % of total sugar dose recovered in urine for 0-5 and 6-24 h samples. The lactulose/mannitol ratio (LMR) was calculated for both 0-5 and 6-24 h samples as the ratio of lactulose excretion to mannitol excretion,³⁰¹ as mannitol is a constant measure of epithelial surface area.³⁰⁰ Gastroduodenal permeability was defined as % sucrose excretion as well as sucrose/mannitol ratio (0-5 h).^{303,304} Small intestinal permeability was defined as the 0-5 h and 6-24 h LMRs, and colonic permeability will be defined as 6-24 h sucralose excretion and sucralose/mannitol ratio.^{304,305}

Appendix F

Informed Consent for Participants of Investigative Projects

Department of Human Nutrition, Foods and Exercise

Virginia Tech

TITLE: Effects of Dietary Cocoa on Inflammation and Insulin Sensitivity

INVESTIGATORS: Andrew Neilson, Ph.D.
Brenda Davy, Ph. D., R.D.
Kevin Davy, Ph.D.
Mathew Hulver, Ph.D.
Nabil Boutagy, Ph.D.
Kris Osterberg, Ph.D., R.D.
Elaina Marinik, Ph.D.
Cassie Mitchell, M.S.
Karen Strat, B.S.
Michael O'Rourke, R.N.

MEDICAL DIRECTOR: Jose M. Rivero, M.D.

PURPOSE:

Several factors, including age, excess body fat, a sedentary lifestyle, and family history, increase the risk of developing type 2 diabetes. Individuals at increased risk of type 2 diabetes tend to have leaky intestines, low-grade inflammation (a response of your immune system to defend your body against harmful substances) and reduced insulin sensitivity. Cocoa is rich in substances that, when eaten, promote improvements in leakiness of intestines, glucose levels, and insulin sensitivity in animals. However, it is not known whether these same benefits occur in humans. Therefore, the purpose of this study is to determine if cocoa improves leakiness of the intestines, inflammation, and insulin sensitivity in humans. Thirty-six (36) men and women at risk for developing type 2 diabetes will be included in this study. Support for this study is provided by the Hershey Company.

You will not be eligible to participate in this study if any of the following apply:

- You are a smoker
- You have unstable heart disease (chest pain or heart failure) or diabetes
- You have untreated high blood pressure or high cholesterol
- You have health problems that would make it unsafe for you to participate
- You gained or lost more than 5 pounds in the last 6 months
- You take medications or supplements that would influence study variables
- You have taken an antibiotic in the past 3 months
- You exercise two or more times a week at level that causes you to breathe hard and sweat
- You have certain food allergies (e.g., dairy foods, gluten allergy)
- You are allergic to lidocaine
- You are pregnant, nursing or plan on becoming pregnant

METHODS:

You are being asked to participate in a 6-week study. There will be two testing periods during this study: before and after a 4-week intervention during which you will consume either a cocoa or placebo supplement along with a controlled diet. Prior to the first testing period, there will be a one-week "wash-out" period with no cocoa or placebo (details to follow). The testing procedures involve blood tests, a muscle biopsy of your upper leg, and a urine test. The tests are described in detail later. If you agree to be involved in this study, you will first have to fill out a health history questionnaire. Your results may be discussed with the study's medical director to determine if you can be a participant. You may be able to participate if you are between 40 and 75 years of age, your body mass index (a measure of obesity) must be between 25-40 kg/m² (indicating overweight or obese), and at least one of the following:

1. You have a blood glucose level between 100 and 125 mg/dl after an overnight fast.
2. You have a blood glucose level between 140 and 200 mg/dl after consuming a glucose drink.
3. You have an HbA1c level between 5.7 and 6.4%.
4. You are considered at risk for developing type 2 diabetes by the American Diabetes Association risk assessment questionnaire.

If you don't have at least one of the above OR if you test above any of these ranges, you will not be able to participate. We will conduct these measurements during "session 2."

Wash out period. You will follow a one-week wash-out diet before the first testing period. This period is necessary to "wash out" any effects of flavanoid compounds that you typically eat that may affect outcome variables measured in our study. You will not be provided food during this time, but you will be instructed to avoid certain foods that contain flavonoids. Flavanoids are found in a variety products, such as tea, apples, berries, chocolate products, and almonds. We will provide a comprehensive list in advance.

Intervention diet. Between the two testing periods, you will be provided all of your food for four weeks. This food will have the same number of calories you usually eat. The diet will have 55% of the calories from carbohydrates, 30% from fat, and 15% from protein. During this 4-week intervention, you will be randomized (a process similar to flipping a coin) to either a cocoa supplement powder or a placebo (sugar powder) group. The testing procedures (high fat meal challenge, blood samples, muscle biopsies, and 24 hour urine collection) will be performed at two time points during the study: once in the beginning before starting the 4-week intervention and once immediately following the intervention. You will need to come to the laboratory each day during the 4-week study period to have your body weight measured as well as to pick up your food and return any uneaten foods from the previous day.

There will be about 40 visits if you decide to participate in the study. This will require approximately 55 hours of time commitment. The real number and order of visits will depend on the compatibility of the study's staff schedules and your schedule. In addition, the order may differ from the order of appearance in this document. All subject responsibilities are described below. Sessions 1 and 2 are screening procedures. Following session 2, we will be able to determine if you qualify for the study. Sessions 3, 4, and 5 comprise the "testing period" which will be repeated following the 4-week intervention with cocoa or placebo treatment.

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STUDY PROCEDURES:

Session 1: (1 hour)

- **Medical History:** You will be asked to complete a medical history questionnaire. This questionnaire is used to screen for health problems or reasons you should not participate in this study. If you have a history of coronary heart disease without current chest pain or heart failure, we will need written permission from your physician for you to participate.
- **Physical Activity Questionnaire:** You will be asked to complete a physical activity questionnaire about the type, intensity, and duration of physical activity you perform each week.
- **Infection/Inflammation Questionnaire:** You will be asked to complete a questionnaire about any recent illnesses or infections that you may have had in the past month.
- **American Diabetes Association Diabetes Risk Assessment Questionnaire:** You will be asked to complete a brief questionnaire about your health history and your family's health history to determine if you are at risk to developing diabetes.
- **Diet Records:** To get an idea of what and how much food you eat, you will be asked to record all of the food you eat for 4 days (3 weekdays and one weekend day). You will be taught how to properly fill out the diet records during this session; they must be completed before the 1-week washout diet.

Session 2: (2.5 hours)

- **Overnight Fast:** You will be asked to avoid eating or drinking anything except water and to avoid consuming any caffeine-containing beverages for 12 hours prior to this visit so that the test results will not be influenced by the food you eat or by the normal digestion process. You may drink only water during this time.
- **Urine test:** You will be asked to urinate in a small cup that we provide to you. We will measure the amount of sodium and other electrolytes, glucose, proteins and pH. Also, we test if there are blood cells present to determine whether it is safe for you to participate in the study.
- **Pregnancy Test:** If you are female you will be required to have a pregnancy test. This will require you to collect 2-3 teaspoons of your urine. If you are pregnant or the test indicates that you are pregnant you will not be able to participate in this study. If you are a postmenopausal female who has not menstruated for at least 1 year then you do not have to complete this test.
- **Blood draw:** A small needle will be inserted into one of your arm's to draw blood (approximately 3 tablespoons). We will measure glucose, cholesterol, triglycerides and other factors to determine your eligibility. You will have your blood drawn twice during Session 2.
- **Oral Glucose Tolerance Test (OGTT):** For this procedure you will be asked to drink a very sweet sugar solution (75 grams of glucose). Blood samples will be taken both before and 2 hours after you drink this solution. The purpose of this procedure is to determine whether you have a normal glucose control after drinking this sweet tasting drink (oral glucose tolerance). This procedure will take approximately 2 hours of your time. If your glucose response indicates

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you may have diabetes you will not be able to continue participation in this study and you will be referred to your personal physician.

- **Hemoglobin A1c test:** This is a measure of your average blood glucose level over the period of several months. For this test, we will make a small prick in your finger to produce a drop of blood. This blood will be applied to a testing kit to determine whether or not you are in the prediabetic range. If your results indicate your hemoglobin A1c is too high you will not be able to continue participation in this study and you will be referred to your personal physician.
- **Blood pressure:** You will be asked to rest quietly for 15 minutes. We will then measure your resting blood pressure using a stethoscope and standard blood pressure cuff or an automatic blood pressure monitor.
- **Body weight and height:** Your body weight will be measured without shoes on a hospital scale and your body height will be measured, as well.

Session 3 (4.5 hours)

- **Overnight Fast:** You will be asked to avoid eating or drinking anything except water, and to not consume any caffeine-containing beverages for 12 hours prior to this visit so that the test results will not be influenced by the food you eat or by the normal digestion process. You may drink only water during this time.
- **Infection/Inflammation Questionnaire:** You will be asked to complete a questionnaire about any recent illnesses or infections that you may have had in the past month.
- **High Fat Meal Challenge:** You will be asked to eat a test meal consisting of two sausage, egg, and cheese breakfast sandwiches. Two muscle biopsies will be performed during this test. The first biopsy will be done before you eat the breakfast sandwich and the second biopsy will be done 4 hours after the meal (details to follow). Your blood will be drawn during this test, as well.
- **Catheter and Blood Draw:** A small needle will be inserted in your arm to draw blood (approximately 1 tablespoons/draw). We will measure various hormones that influence your metabolism (how your body burns calories and produces body heat). Blood will be collected before the meal challenge and at 1, 2, 3, and 4 hours after the meal. The catheter will remain in your arm throughout the entire test.
- **Muscle Biopsy:** Throughout the entire study, you will be asked to undergo this procedure 4 times: 2 muscle biopsies will be performed during each high fat challenge, and the high fat challenge is done twice (before and after the 4-week diet). You should not take aspirin, ibuprofen or other non-steroidal, anti-inflammatory medications (such as Advil, Motrin, Celebrex or Vioxx), or other medications or substances that may affect bleeding or bruising, for 72 hours prior and after this procedure. This procedure is used to sample a small amount of muscle (about 350-450 mg/or about the size of 2 or 3 erasers on a pencil) from underneath the skin from the thigh. The actual biopsy site will be on the top of either the right or left leg half way between the knee and the hip.

Neither a physician nor nurse may be present during the procedure. This procedure will be performed by a study investigator (Kevin P. Davy, Ph.D.) or co-investigator (Matthew Hulver,

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will not be influenced by the food you eat or by the normal digestion process. You may drink only water during this time.

- **Intestinal Permeability:** You will be asked to use the restroom before consuming about two cups of a sugar drink. You will then drink an additional two cups of water to make collection of urine easier. You will be asked to collect and save your urine. Urine will be collected in two containers. The first will be used for collecting your urine between the point at which you consume the sugar drink and 5 hours after consuming the drink, and another between 6 and 24 hours after consuming the sugar drink. You will be provided with urine collection containers and asked to return them to the lab at the end of each collection period. You should only consume the breakfast sandwiches that we provide during the first 5 hours after drinking the sugar drink.

Take-Home Tests

- **Diet Records:** To get an idea of what and how much food you eat, you will be asked to record all of the food you eat for 4 days (3 weekdays and one weekend day prior to baseline testing).
- **Urine Collection:** You will be asked to collect a 24-hour urine sample to bring to the laboratory on two occasions (each time you come in for session three).

SUMMARY OF SUBJECT RESPONSIBILITIES

- Provide an accurate history of any health problems or medications you use before the study begins.
- Inform the investigators of any discomfort or unusual feelings before, during or after any of the study sessions.
- Be on time and attend all of the scheduled experiments (every day for approximately 40 days).
- Follow all participant instructions for each session.
- Record any food you eat that has not been provided by the investigators.
- Return any uneaten food that has been provided by the investigators.
- Follow physical activity instructions provided by the investigators.
- Carefully read the instructions on consuming any food provided to you.
- Inform the study investigators if you are pregnant or intend to become pregnant during the study.

RISKS OF PARTICIPATION

- **Catheter and Blood Draw:** Some pain or discomfort may be experienced when the catheter is inserted in the vein, but this should persist for only a short time. During the blood draws, you may have pain and/or bruising at the place on your arm where the blood is taken. In about 1 in 10, or 10% of the cases, a small amount of bleeding under the skin will cause bruising. The risk of a blood clot forming in the vein is about 1 in 200, while the risk of infection or significant blood

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Ph.D.) who have been specifically trained to perform the biopsy. You will be lying down and your skin will be cleansed with iodine-type solution (Providine or Betadine). If you are allergic to iodine, we will use chlorhexadine which does not contain iodine. A sterile drape will be placed over the area and your skin and muscle tissue will be numbed by injecting numbing medication (lidocaine/bupivacaine) into the area with a small needle. If you allergic to lidocaine or bupivacaine, you cannot participate in this study. Then, a small incision (about 1/4 of an inch) will be made in the skin and a needle (a little thinner than a pencil) will be inserted to remove a small amount of muscle. Some suction may be applied to the other end of the needle to help remove the muscle.

After the biopsy is completed, pressure will be applied and the skin will be closed with sterile tape. To ensure cleanliness, the skin will be cleaned with saline and will be covered with gauze and a clear adhesive dressing. The site will then be wrapped with an ACE bandage. You will be asked to keep the ACE bandage on for at least 10-15 minutes. You may take Tylenol for any discomfort you may experience following the biopsy. We will use the biopsy samples to measure factors that contribute to inflammation. The biopsy will take place at Dr. Jose Rivero's medical office in Christiansburg or the Human Integrative Physiology Laboratory (228 War Memorial Hall). Directions will be provided to you. You will be provided with instructions on how to care for the biopsy sites as well as what to look for if a problem were to occur.

Session 4: (4 hours)

- **Overnight Fast:** You will be asked to avoid eating or drinking anything except water, and to not consume any caffeine-containing beverages for 12 hours prior to this visit so that the test results will not be influenced by the food you eat or by the normal digestion process. You may drink only water during this time.
- **Intravenous Glucose Tolerance Test (IVGTT).** Two small plastic tubes (intravenous catheters) will be placed in two arm veins (different arms), and about 3 tablespoons of blood will be taken to measure hormones or proteins that influence your metabolism and cardiovascular system. We will then inject a small amount of glucose (0.3 mg/kg body weight) and insulin (0.03 unit/kg body weight) into your veins (insulin is a hormone which helps your body's cells metabolize glucose). We will draw a small amount of blood (less than one half teaspoon) about 28 times over a 3-hour period. A registered nurse will be present to perform this test with the assistance of the research staff.
- **Blood Pressure:** You will be asked to relax and sit quietly for 15 minutes. We will then measure your resting blood pressure using a stethoscope and standard blood pressure cuff or an automatic blood pressure monitor.
- **Body Composition:** This test is to measure your body fat. You will lie on a hospital-type bed and a small amount of x-ray will be passed through your body to determine the amount of bone, muscle and fat in your body. This unit is called a DEXA scan. This test takes approximately 15 minutes and there is no pain associated with the procedure. Your weight and height will also be measured at this time.

Session 5: (1 hour)

- **Overnight Fast:** You will be asked to avoid eating or drinking anything except water and to not consume any caffeine-containing beverages for 12 hours prior to this visit so that the test results

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will not be influenced by the food you eat or by the normal digestion process. You may drink only water during this time.

- **Intestinal Permeability:** You will be asked to use the restroom before consuming about two cups of a sugar drink. You will then drink an additional two cups of water to make collection of urine easier. You will be asked to collect and save your urine. Urine will be collected in two containers. The first will be used for collecting your urine between the point at which you consume the sugar drink and 5 hours after consuming the drink, and another between 6 and 24 hours after consuming the sugar drink. You will be provided with urine collection containers and asked to return them to the lab at the end of each collection period. You should only consume the breakfast sandwiches that we provide during the first 5 hours after drinking the sugar drink.

Take-Home Tests

- **Diet Records:** To get an idea of what and how much food you eat, you will be asked to record all of the food you eat for 4 days (3 weekdays and one weekend day prior to baseline testing).
- **Urine Collection:** You will be asked to collect a 24-hour urine sample to bring to the laboratory on two occasions (each time you come in for session three).

SUMMARY OF SUBJECT RESPONSIBILITIES

- Provide an accurate history of any health problems or medications you use before the study begins.
- Inform the investigators of any discomfort or unusual feelings before, during or after any of the study sessions.
- Be on time and attend all of the scheduled experiments (every day for approximately 40 days).
- Follow all participant instructions for each session.
- Record any food you eat that has not been provided by the investigators.
- Return any uneaten food that has been provided by the investigators.
- Follow physical activity instructions provided by the investigators.
- Carefully read the instructions on consuming any food provided to you.
- Inform the study investigators if you are pregnant or intend to become pregnant during the study.

RISKS OF PARTICIPATION

- **Catheter and Blood Draw:** Some pain or discomfort may be experienced when the catheter is inserted in the vein, but this should persist for only a short time. During the blood draws, you may have pain and/or bruising at the place on your arm where the blood is taken. In about 1 in 10, or 10% of the cases, a small amount of bleeding under the skin will cause bruising. The risk of a blood clot forming in the vein is about 1 in 200, while the risk of infection or significant blood

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loss is 1 in 1000. There is a small risk of the vein becoming inflamed and/or painful in the hours or days after the catheter is removed. If you feel faint during/after a blood draw, you should notify the study doctor or study staff immediately and lie down right away to avoid falling down. Having staff who are experienced in catheter placement and blood draws will minimize these risks.

- Oral Glucose Tolerance: Because this procedure requires the placement of a catheter in a vein in each arm, the risks here are identical to those stated above. In addition, there is a small risk of low blood sugar occurring during or after the test. If this happens, orange juice (with table sugar) or some other sugar containing food will be given to you.
- Intravenous Glucose Tolerance Test: Because this procedure requires the placement of a catheter in an arm vein, the risks here are identical to that stated above. In addition, there is a small risk of low blood sugar occurring during or after the test. We will be monitoring your blood sugar frequently and can usually anticipate this before your blood sugar drops too low. If this happens, orange juice (with table sugar) or some other simple carbohydrate containing food will be given to you. We will monitor your glucose until it returns to normal. A registered nurse will perform the test with the assistance of the investigators.
- HIV/AIDS: In the event a researcher or other staff person is improperly exposed to your blood, your blood will be tested for the presence of HIV, the Hepatitis B Virus, and the Hepatitis C Virus. There will not be any cost to you for this test. The research team will follow proper procedures for testing and reporting as outlined by Virginia State Law, which includes sending the sample to a certified laboratory. Please note that, should your blood require testing, you will be informed of your test results and provided with the opportunity to receive appropriate and timely counseling. In addition, your results will be sent to the local health department.
- Muscle Biopsies: If you are allergic to lidocaine, you will not be allowed to participate in this study. There may be slight discomfort and burning when the local anesthetic is injected prior to the biopsy, but you are not expected to experience discomfort during the biopsy procedure. Bruising in the area of the muscle biopsy for 1-2 weeks will likely occur, but local pressure and ice are applied to the site immediately after the procedure to limit this potential effect and its accompanying tenderness. There is a slight risk of infection at the biopsy site. There is a small risk that you will become lightheaded, dizzy, or anxious before or during the procedures. There is also a small risk of fainting. If you feel dizzy, lightheaded or feel like you might faint before, you should sit down or lie down immediately to avoid falling. These reactions are usually temporary and resolve within a short time after sitting or lying down. If these feeling do not go away soon after sitting or lying down, you should call 911 or have someone take you to the nearest emergency room.!! We did have one individual faint and her their head after leaving the laboratory. This required a trip to the emergency room and stitches. However, this occurred only once in the over 350 biopsies performed in our research studies. All of these reactions are temporary and resolve within a short time after completing or stopping the procedure. Having a trained individual perform the procedure minimizes these risks. You will be asked to return to the physiology laboratory within 5 days after the biopsy to have the site checked to ensure proper healing.

You will likely receive a scar from each of the biopsies performed but these are expected to be very small. These scars usually turn a purple color in the weeks to months following the biopsy and then fade considerably over time. The study staff will show you several pictures of examples of the scarring (greater than 1 year old) that can occur following similar biopsy

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procedures. It is important that you understand that these are just examples of the scarring that can occur. The actual scar you receive may be smaller or larger or differ in coloring. Individuals with darker skin (e.g., African Americans, Hispanics and Asians) tend to scar more than those with lighter skin. You should consider this before you agree to participate.

- **DEXA Scan:** The amount of radiation that you will receive in the DEXA exam is less than the amount permitted by the Food and Drug Administration (FDA) per year. The amount you will receive is equal to 1/20 of a chest x-ray. The more radiation you receive over the course of your lifetime, the more likely your risk increases in developing cancerous tumors. The radiation in this study is not expected to greatly increase these risks; however the exact increase in such risk is not known.
- **Sugar Drink:** Potential risks related to ingestion of the different types of sugar found in the sugar/water drink may be associated with gastrointestinal symptoms such as gas, bloating, and diarrhea.
- It is not possible to identify all potential risks in an experiential study. However, the study doctors and study staff will take all possible safeguards to minimize any known and potential risks to your well-being. We believe the overall risks of participation are minimal. All of the procedures are well established and used routinely in the study investigators laboratory.
- Side effects are possible in any research study despite high standards of care, and could occur through no fault of your own or the study doctors or study staff.

BENEFITS OF PARTICIPATION

As a result of your participation you will obtain health information related to your body composition, blood pressure, blood glucose, and cholesterol. However, you should not consider this a wellness or medical exam. You should discuss any concerns about your health information with your personal physician. Your participation will contribute to improving the understanding of how cocoa supplementation impacts health.

COMPENSATION

You will be compensated \$50 dollars each time you complete session 3, 4, and 5 (each performed twice) and another \$100 dollars for completing the entire study. The total compensation you can receive for participation in this study is \$400.

CONFIDENTIALITY

The data from this study will be kept strictly confidential. No data will be released to anyone but those working on the project without your written permission. Data will be identified by a code, without anything to identify you by name. In the event that any of your tests indicate a problem, your results may be shared with the medical director, Dr. Rivero, and your personal physician.

FREEDOM TO WITHDRAW

You are free to withdraw from the study at any time for any reason. Simply inform the experimenters of your intention to cease participation. In addition, circumstances could arise which would lead to your exclusion from the study. For example, lack of compliance to instructions, failure to attend testing sessions, and illness could be reasons for the researchers to stop your participation in the study. Other reasons include an inability by the researchers to obtain an adequate muscle sample or other measurements that are necessary for the study. All of the sessions and measurements are required components.

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INJURY DURING PARTICIPATION IN THIS STUDY

Neither the researchers nor the University have money set aside to pay for medical treatment that would be necessary if you are injured as a result of your participation in this study. Any expenses that you incur, including emergencies and long term expenses would be your own responsibility. You should consider this limitation before you consider participating in this study.

APPROVAL OF RESEARCH

This research has been approved, as required, by the Institutional Review Board for Research Involving Human Subjects at Virginia Tech. You will receive a copy of this form to take with you.

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SUBJECT PERMISSION

I have read the informed consent and fully understand the procedures and conditions of the project. I have had all of my questions answered, and I hereby give my voluntary consent to be a participant in this research study. I agree to abide by the rules of the project. I understand that I may withdraw from the study at any time.

If you have questions, you may contact:

- Principal Investigator: Kevin Davy, Professor, Department of Human Nutrition, Foods, and Exercise. (540) 231-3487; After hours: 540-230-0486
- Chairman, Institutional Review Board for Research Involving Human Subjects: David Moore, Associate Vice President for Research (540) 231-4991

Name of Subject (please print) _____

Signature of Subject _____ Date _____

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CONSORT 2010 Flow Diagram

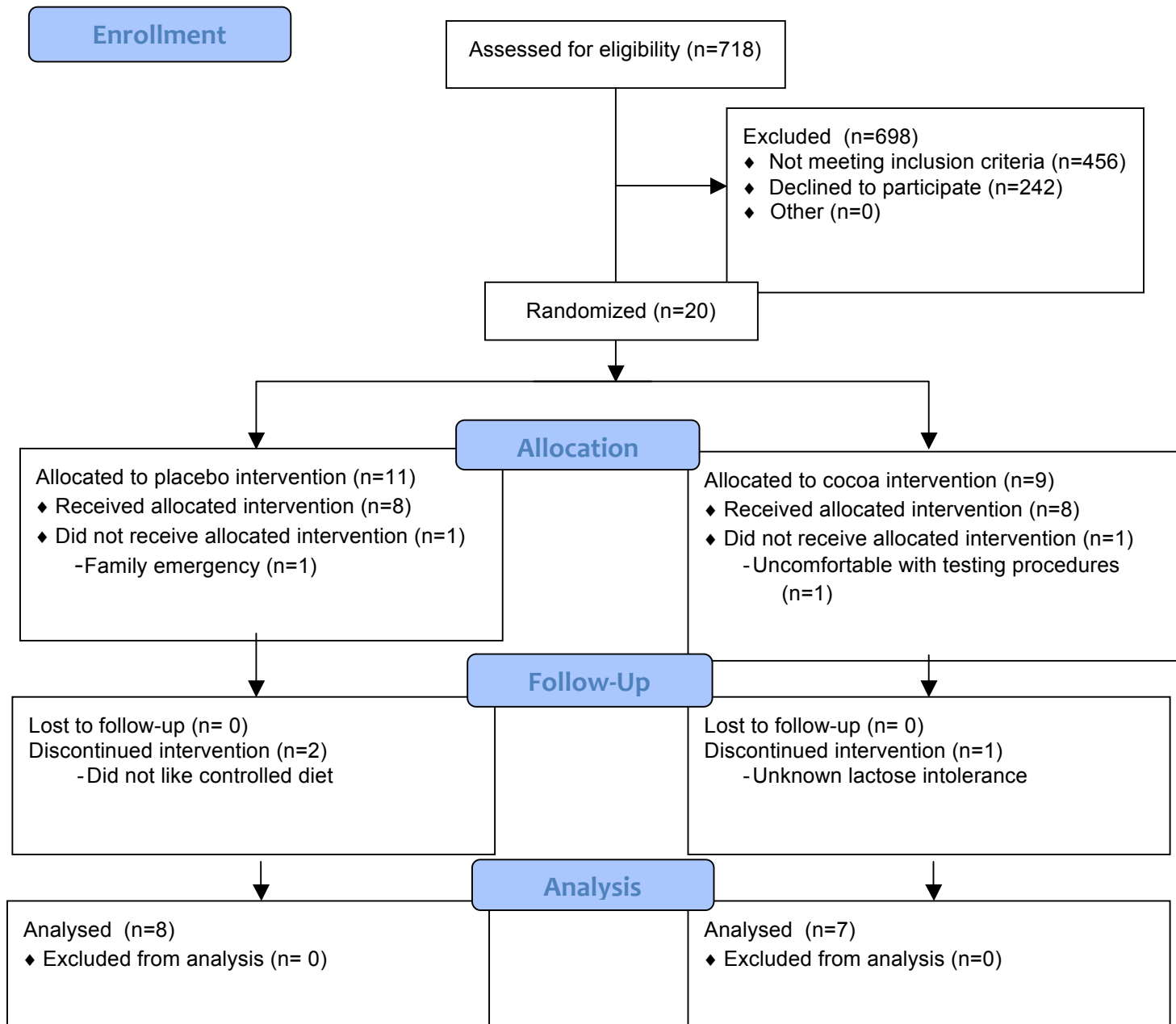


Figure S4.2. CONSORT Flow Diagram.

Table S4.1. Inclusion and exclusion criteria for subject eligibility and enrollment.

Inclusion Criteria	Exclusion Criteria
6. $25 \leq \text{BMI} < 40$	12. Diagnosed diabetes (type 1 or 2) or use of diabetes medications
7. Age 40-76 years	13. Antibiotic, prebiotic, or probiotic use in prior 3 months
8. Weight stable for previous 6 months (± 2 kg)	14. Taking anti-inflammatory medications (e.g., NSAIDs ^c , etc.) or antioxidant vitamins or supplements
9. Sedentary to recreationally active (< 2 d/wk, 20 min/d)	15. Total cholesterol > 300 mg/dl; total triglycerides > 450 mg/dl
10. Meets any 1 of the following 4 criteria: 1) IFG ^a = 100-125 mg/dL 2) IGT ^b = 140-199 mg/dL 3) HbA1c = 5.7-6.4% 4) ADA risk assessment score ≥ 5	16. Blood pressure $> 160/100$ mmHg 17. Exceeds any of the 3 following criteria ^d : i) IFG > 125 mg/dL ii) IGT > 200 mg/dL iii) HbA1c $> 6.5\%$ 18. Diagnosed inflammatory disease (e.g., lupus, irritable bowel syndrome, periodontal disease, etc.) 19. Diagnosed disease of the digestive tract (e.g., lactose intolerance, ulcers, cancer (stomach, liver, pancreatic, etc.), celiac's disease, etc.) 20. Past or current ischemic heart disease, stroke, respiratory disease, endocrine or metabolic disease, neurological disease, or hematological-oncological disease. 21. Smoking, tobacco use, or alcohol consumption (> 2 servings/d for males and 1 serving/d for females) 22. Strict vegan or vegetarian 23. Strong aversion or allergy to major food groups used in the controlled diet 24. Pregnant or planning to become pregnant 25. Allergic to lidocaine

^aIFG: Impaired fasting glucose, measured by plasma glucose concentration after an overnight fast

^bIGT: impaired glucose tolerance as measured by plasma blood glucose concentration 2 h following standard Oral Glucose Tolerance Test (OGTT) with 75 g glucose beverage

^cNSAIDs: non-steroidal anti-inflammatory drugs

^dAny subject exceeding these threshold levels were referred to their personal physician

Table S4.2. List of items subjects were instructed to avoid during the wash out diet.

Completely avoid:	Consume in moderation:
Yogurt	Onions
Cocoa/chocolate and chocolate products	Garlic
Almonds	Artichokes
Apples	Leeks
Apple juice	Peaches
Berries	Pears
Cranberry juice	Apricots
Grapes	Cherries
Grape juice	
Metamucil	
Prebiotics	
Probiotics	
Wine	
Tea	

^bIGT: impaired glucose tolerance as measured by plasma blood glucose concentration 2 h following standard Oral Glucose Tolerance Test (OGTT) with 75 g glucose beverage

^cNSAIDs: non-steroidal anti-inflammatory drugs

^dAny subject presenting with symptoms of diabetes during screening was immediately referred to a physician

Table S4.3 Sample menu for 1 day (2500 kcal diet)

	Amount	Weight (g)	kcal	Protein	CHO	Fat	Sat Fat	Fiber	Na (mg)
TARGETS			2500 kcal	94 g	344 g	83 g	22 g	19 g	<3000
Cocoa/Placebo	3	54/51	167/180	16/16	29/27	2/1	1/0	6/1	448/495
NDS, Cereal, cooked, Quaker instant oatmeal		48	175.1	4.3	36	2.3	0.4	3.2	298
NDS, margarine, regular, stick, salted, soybean oil		38	271.7	0	0	30.685	5.23	0	285
NDS, juice, orange juice, RTD, not fortified		480	249.2	2.28	58.28	0.58	0.06	1.6	10.06
NDS, white bread, regular, commercial		172	455.8	15.74	84.384	5.488	1.2	4.644	844.52
NDSR: (Beef, roast, round, top, no visible fat eaten, no fat or salt added, 56 g after cooking, edible portion)		100	198.2	36.07	0	5	1.71	0	44.6
NDSR: Swiss Cheese, Natural, low-sodium		28	106	7.5	1.5	7.78	4.98	0	4
NDS, Mayonnaise, Brand Name, Hellman's, Best Foods real mayonnaise		33	236.1	0.297	1.04	26.2	3.92	0	187
NDS, granola bar, Nature Valley Oats and Honey		21	91.266	2.105	14.459	3.051	0.318	1.28	80.033
NDS, juice, lemonade, RTD, regular		480	202	0.352	52.43	0.202	0.03	0	20
NDSR (Peaches, canned, water-packed)		108	25.92	0.475	6.599	0.065	0.006	1.404	3.24
Kroger Stir Fry Veggies with Rice		340	279	8.763	59.401	0.898	0.203	4.794	14
NDSR Stir Fry Sauce, Commercial		30	48	0.54	11.32	1.72	0.04	0.346	328
NDSR ACTUAL AMOUNTS			2509	96.017	349.27	84.715	18.747	20.368	2563

Table S4.4. Composition of test meals for high fat challenge and sugar probe tests.

	High fat challenge meal ^a	Sugar probe meal ^b
Serving size (g)	256	232
Calories (kcal)	820	500
Total fat (g)	58	18
Saturated fat (g)	24	8
Cholesterol (mg)	260	270
Sodium (mg)	1,660	1,380
Potassium (mg)	600	Not provided
Total carbohydrate (g)	52	54
Dietary fiber (g)	4	2
Sugars (g)	8	4
Protein (g)	24	26

^aTwo Jimmy Dean Sausage, Egg, and Cheese Biscuit sandwiches (Tyson Foods, Inc., Springdale, AR)

^bTwo Jimmy Dean Ham, Egg, and Cheese on a Muffin sandwiches (Tyson Foods, Inc., Springdale, AR)

Table S4.5. UPLC retention times and MS/MS transitions for detection of sugar probes.

Compound	Retention time (min)	[M-H] ⁻ (m/z)	Daughter ion (m/z)	Cone voltage (V)	Collision energy (eV)
Sucralose	0.24	395.2	359.0	42	10
Mannitol	0.38	181.2	88.9	28	14
Sucrose	0.43	341.3	179.0	38	12
Lactulose	0.46	341.3	160.9	12	8
¹³ C ₆ -glucose	0.39	185.2	91.9	18	8

Urinary Sugar Analysis Methodology

Extraction and Quantification of Sugar Probes. Total urine volume will be measured, and aliquots will be frozen at -80°C. Urinary sugars will be measured as described by Camilleri et al.⁴³ 50 µL urine will be combined with 50 µL internal standard [20 mg/mL ¹³C₆-glucose in water/acetonitrile (98:2)], diluted to 4 mL with water and vortexed with 4 mL dichloromethane. Following 30 min incubation and centrifugation (10 min, 3500 x g), 100 µL supernatant will be diluted with 900 µL acetonitrile/water (85:15) and analyzed by UPLC-MS/MS. UPLC separation will be performed on a Waters Acquity H-class (Milford, MA) equipped with an Acquity UPLC BEH Amide column (2.1 mm Å~ 50 mm, 1.7 µm particle size). Isocratic elution will be performed at 0.7

mL/min using acetonitrile:water (65:35) with 0.2% v/v triethylamine (TEA). Column and sample temperatures will be 35 and 10°C, respectively. Detection by MS/MS will be performed on a Waters Acquity Triple Quadrupole Detector (TQD). Negative-mode electrospray ionization [(-)-ESI] will be performed with capillary voltage of -4 kV, and source and desolvation temperatures of 150 and 450°C, respectively. Desolvation and cone gasses will be N₂ at flow rates of 900 and 1 L/h, respectively. For MS/MS, the collision gas will be Ar. The cone voltages, collision energy, and Multiple Reaction Monitoring (MRM) transitions for each compound are listed in Table 5. Peak widths will be ~4 s, and AutoDwell will be employed with required points-per-peak set at 12. The interscan delay time will be 0.02 s. Data acquisition, processing, and quantification will be performed using Waters MassLynx v4.1 software.

Skeletal Muscle Substrate Flexibility Full Analysis

Glucose oxidation values were not normally distributed for the cocoa, post, fed states as assessed by Shapiro-Wilk's test of normality ($p = 0.007$) (log transforming the data set did not improve normality). However, these data were distributed normally for all other groups ($p > 0.05$). The assumption of homogeneity of variances was violated, as assessed by Levene's test for equality of variances ($p < 0.05$) indicating that there was unequal variance between the placebo and cocoa groups (log transforming the data set did not reduce the difference in variance). Because the log transformation did not influence the assumptions and ANOVA's are considered to be fairly robust to deviations from normality and heterogeneity of variance, the analysis was pursued with the untransformed data set. There was no statistically significant three-way interaction between treatment, time, and meal $F(1,13) = 2.572$, $p = 0.133$. There was a statistically

significant two-way time*treatment interaction $F(1,13) = 6.340$, $p = 0.026$, meal*treatment interaction $F(1,13) = 5.894$, $p = 0.030$, and time*meal interaction $F(1,13) = 6.053$, $p = 0.029$.

For total fatty acid oxidation, there were 3 outliers in the data, assessed as a value greater than 1.5 box-lengths from the edge of the box, and 1 extreme value, assessed as 3 box-lengths from the edge of the box. Two outliers were in the cocoa, pre, fed group, and one was in the cocoa post, fed group. The extreme value was in the coca, post, fasted group. They were not influenced by a log transformation and they were within physiologically relevant ranges so they were left in the analysis. Total fatty acid oxidation values were not all normally distributed, as assessed by Shapiro-Wilk's test of normality. The cocoa, post, fasted group was not normally distributed ($p = 0.017$) (this was not influenced by a log transformation). All other groups were distributed normally ($p > 0.05$). The assumption of homogeneity of variances was violated, as assessed by Levene's test for equality of variances ($p < 0.05$) (this was not reversed after a log transformation). Because ANOVA's are considered to be fairly robust to deviations from normality and heterogeneity of variance, the analysis was pursued. There was a statistically significant three-way interaction between treatment, time, and meal $F(1,13) = 9.819$, $p = 0.008$. The placebo group had a statistically significant simple two-way interaction between time and meal ($F(1,7) = 8.125$, $p = 0.025$) but the cocoa group did not ($F(1,6) = 2.473$, $p = 0.167$). There was a statistically significant simple simple main effect indicating that the placebo group pre-fasting to pre-fed was significantly different ($F(1,7) = 24.610$, $p = 0.002$). The following data are mean $\pm$ standard deviation. For the placebo group pre-intervention, simple simple pairwise comparisons were run between

the two meals. A Bonferroni adjustment was applied. Total fatty acid oxidation was 7.676 ± 0.638 nmol/mg protein/h during pre-testing and 18.814 ± 2.453 nmol/mg protein/h at post-testing, a statistically significant difference of 11.138 (95% CI, -16.446 to -5.829) nmol/mg protein/h, $p = 0.002$. There were no differences between post-intervention fasting compared to fed for the placebo group ($F(1,7) = 3.295$, $p = 0.112$) but the cocoa group approached significance ($F(1,6) = 5.713$, $p = 0.054$).

For oxidative efficiency, there were 2 outliers and 1 extreme value in this data set. One outlier was in the placebo, post, fasted group, and one was in the cocoa, post, fasted group. The extreme value was in the placebo, pre, fasted group. Log transformation resulted in only one outlier in the cocoa, post-intervention, fasted group. They were left in the analysis since they were within physiologically relevant ranges. Oxidative efficiency values were not all normally distributed, as assessed by Shapiro-Wilk's test of normality. The following were not normally distributed: placebo, pre, fasted group ($p = 0.001$), cocoa, post, fasted group ($p = 0.006$) and placebo, post, fasted ($p = 0.015$) (not influenced by log transformation). All other groups were distributed normally ($p > 0.05$). The assumption of homogeneity of variances was violated, as assessed by Levene's test for equality of variances ($p < 0.05$). Log transformation resulted in no violation of Levene's test ($P > 0.5$). There was no statistically significant three-way interaction between treatment, meal, and time $F(1,13) = 1.027$, $p = 0.073$. There were no significant two-way interactions, either. Log transformation did not alter these results.