COMPARISON OF COTTONSEED OIL VS. OLIVE OIL DIET ENRICHMENT ON CARDIOMETABOLIC HEALTH MARKERS

By

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ABSTRACT

Cardiovascular disease (CVD) is the global leading cause of death; however, multiple cardiovascular and metabolic risk factors for CVD can be modulated through alterations in diet. Cottonseed oil (CSO), an oil rich in polyunsaturated fats, has been shown to elicit improvements in lipid profiles, appetite control, and metabolic responses in young healthy adults; yet, it has never been assessed in adults with hypercholesterolemia. Further, human CSO consumption has never been assessed for a period longer than seven days. The objective of this study was to compare effects of CSO vs. olive oil (OO; a well-characterized "healthy" oil rich in monounsaturated fats) diet enrichment on cardiometabolic health markers including blood lipids, angiopoietin-like proteins (ANGPTL), appetite control, metabolism, inflammatory cytokines, and markers of coagulation potential in adults with hypercholesterolemia. We conducted an 8-week partial outpatient feeding trial, where participants were randomized to either CSO or OO diet interventions. During the 8-week diet interventions, participants received prepared foods that provided approximately 60% of their daily energy needs. The provided intervention foods

delivered approximately 30% of participant energy needs as the assigned intervention oil (CSO or OO). In manuscript #1 (chapter #3), fasting lipid profiles including total cholesterol, low-density lipoprotein cholesterol, non-high-density lipoprotein cholesterol, and apolipoprotein B were improved with CSO compared to OO diets. Furthermore, in manuscript #1 (chapter #3) postprandial lipemia and glycemic control were worse after OO compared to CSO enrichment. In manuscript #2 (chapter #4) postprandial ANGPTL-3 and -4 worsened with OO vs. CSO diets. In manuscript #3 (chapter #5), there were no differences in substrate utilization at fasting or postprandially; however, OO led to a greater increase in diet induced thermogenesis compared to CSO. Conversely, manuscript #4 (chapter #6) shows greater improvements in appetite measures (fasting cholecystokinin, postprandial ghrelin, postprandial fullness, and suppression of energy intake) with CSO vs. OO diet enrichment. In manuscript #5 (chapter 7), there were no changes observed in fasting markers of inflammation or coagulation potential. Taken together, these results show daily CSO consumption to elicit greater improvement on markers of cardiometabolic health in adults with hypercholesterolemia compared to daily OO consumption.

INDEX WORDS: COTTONSEED OIL, OLIVE OIL, DIETARY FATTY ACIDS, BLOOD LIPIDS, CHOLESTEROL, TRIGLYCERIDES, ANGPTL, APPETITE, CCK, PYY, GHRELIN, METABOLISM, SUBSTRATE OXIDATION, HIGH-FAT, INFLAMMATION, IL-6, CRP, TNF-α, COAGULATION POTENTIAL, PAI-1, POLYUNSATURATED, MONOUNSATURATED

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DEDICATION

Special thanks to my husband and family for supporting and encouraging me on this journey. David, thank you for all of the wonderful meals, reminding me to take brakes, and helping me realize the importance of having fun and balance in life. Mom, thank you for the many phone calls, your willingness to listen, and your endless encouragement. Dad, thank you for reminding me that I can do hard things and for always believing in me. Phil, thank you for making me laugh at all of the silly inconveniences of life. The work detailed in this document is dedicated to my family who taught me persistence and curiosity.

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CHAPTER 1

INTRODUCTION

Cardiovascular disease (CVD) is the primary cause of death accounting for 32% of the global mortality in 2019 (1). A common underlying etiology for CVD is the inflammatory condition of atherosclerosis. As one of the early processes of atherosclerosis is the infiltration of the arterial wall by cholesterol, elevated blood lipids (hypercholesterolemia) have been proposed and shown to be an independent and primary risk factor for the development of CVD (2). In regard to nutrition factors influencing risk of CVD, dietary fatty acid composition has been shown to exert differential effects on CVD risk, especially with saturated fatty acids (SFA) having markedly unfavorable effects on blood lipid levels (3). Conversely, unsaturated fatty acids have repeatedly shown favorable effects in the modulation of blood lipids and CVD risk, with increasing evidence that polyunsaturated fatty acids (PUFA) may have more powerful effects on the reduction of blood lipids than monounsaturated fatty acids (MUFA) (4, 5). Potentially contributing to their greater responses in the reduction of blood lipids, PUFAs have been shown to be powerful regulators of the expression of genes related to lipid metabolism and cholesterol regulation (6, 7).

Cottonseed Oil (CSO) is a rich source of dietary PUFAs, especially the omega-6 (n-6) PUFA linoleic acid, and has shown the ability to improve cholesterol profiles (8, 9), modulate substrate utilization over time (10), and improve appetite responses (11) in young healthy men. Increased fat oxidation following a high fat meal and improved appetite regulation could both contribute to improvements in energy balance and weight maintenance, which ultimately

influences CVD risk. To date, CSO enrichment in the diet has not been studied for its effects on blood lipids, metabolism, or appetite responses in an older population displaying increased risk of CVD as measured by an unfavorable blood lipid profile (hypercholesterolemia). The impact of CSO enrichment in the diet has also never been studied in humans over a duration longer than one week.

The following literature review (Chapter 2) presents current research surrounding chronic disease, cholesterol metabolism, and dietary fatty acid composition. Further, this review focuses on the differential effects observed between types of fatty acids and their impact on 1) cholesterol metabolism, 2) energy metabolism and 3) appetite control. The purpose of this study was explored in Chapters 3 through 7. Broadly, the aims of these chapters are to compare the effects of 8-week dietary enrichment with CSO vs. olive oil (OO) on cholesterol profiles, glycemic control, angiopoietin-like proteins, metabolism, appetite, inflammation and coagulation potential, respectively. Our central hypothesis was that diets enriched with CSO would improve markers of cardiometabolic health and decrease CVD risk compared to OO in adults with hypercholesterolemia (elevated blood cholesterol profiles). For all manuscripts (chapters 3 through 7), dietary interventions were delivered using an 8-week partial outpatient feeding trial. In this trial all participants were provided study foods that accounted for ~60% of their daily energy needs and delivered ~30% of their daily energy needs as CSO or OO depending on their random group assignment.

Specific Aims and Hypotheses

- Aim 1: Assess the effects of diets rich in CSO or OO on blood lipids, and compare the change in responses from pre- to post- diet intervention between groups.

    - Hypothesis 1a: The CSO-enriched diet will result in improvements in fasting blood lipid profiles from pre- to post-diet intervention as indicated by decreases in total cholesterol (TC), low density lipoprotein (LDL), triglycerides (TG), lipoprotein (a), apolipoprotein B (apoB), LDL small, and increases in LDL particle size, high density lipoprotein (HDL), and HDL large compared to the OO diet with the OO diet.
    - Hypothesis 1b: The CSO-enriched diet will result in decreases in postprandial blood lipids (TG and free fatty acids (FFA)) from pre- to post-intervention vs. the OO-enriched diet.
- Aim 2: Assess the intervention effects of the CSO diets on markers of energy balance compared to OO diets.
    - Hypothesis 2a: The CSO-enriched diet will result in greater fasting and postprandial fat oxidation compared to the OO-enriched diet. No changes in DIT or RMR are expected in either group.
    - Hypothesis 2b: The CSO enriched diet will result in improved fasting and postprandial appetite regulation as indicated by visual analog scales and greater satiety hormone responses (cholecystokinin (CCK), peptide YY (PYY),) and hunger hormone (ghrelin) suppression from pre- to post- diet intervention compared to the OO enriched diet.
- Aim 3: Assess the effects of CSO rich diets on markers of inflammation, coagulation potential, glycemia, and regulation of lipid metabolism, compared to OO rich diets.

- Hypothesis 3a: The CSO enriched diet will result in favorable changes in fasting markers of inflammation (tumor necrosis factor-α, Interleukins (IL-1β, IL-6,), and c-reactive protein (CRP)) compared to the OO enriched diet.
- Hypothesis 3b: The CSO enriched diet will result in lower fasting coagulation potential as measured by Plasminogen activator inhibitor-1 (PAI-1), and tissue factor (TF) OO group.
- Hypothesis 3c: The CSO enriched diet will result in greater improvements in measures of glycemia as measured by a decrease in insulin and blood glucose both in the fasting and postprandial states compared to the OO diet.
- Hypothesis 3d: The CSO enriched diet will result in favorable changes to regulators of lipid metabolism as measured by a decrease in angiopoietin-like proteins (ANGPTLs)-3, -4, and -8 both fasting and postprandially vs. the OO diet.

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CHAPTER 2

REVIEW OF THE LITERATURE

Chronic disease, which is any condition that lasts one year or more and requires ongoing medical attention and/or limits activities of daily living (1), is the leading cause of death and disability in the world and have been since the turn of the century (2). The first World Health Organization (WHO) report in 2002 stated that almost 60% of all deaths and 43% of the global disease burden were attributable to chronic diseases (2, 3). From 1990 to 2017, chronic disease rates have increased 40% worldwide (4) showing the burden of chronic diseases is a pertinent and growing problem on the world forum.

The four most prominent chronic diseases are chronic obstructive pulmonary disease (COPD), type 2 diabetes, cancer, and heart diseases, such as cardiovascular disease (CVD) (2). The most common and preventable risk factors linked to those primary chronic diseases are high blood pressure, high blood cholesterol, and overweight, which are attributed to modifiable behavioral risk factors including unhealthy diet, physical inactivity, and tobacco use (2). There is a growing need for effective behavior modification and treatment of risk factors to control the disease burden and prevent the onset of chronic diseases.

CVD is the number one cause of death globally (5) as well as in the united states (6, 7) accounting for 32% of all deaths globally in 2019, 85% of which were heart attacks and strokes (8). This highlights the need for investigation into effective prevention, and implementation of preventative techniques. Similar to other chronic diseases, hypertension, obesity, type 2

diabetes, and hyperlipidemia all contribute to a greater CVD risk. Therefore, one approach to combating CVD has been to reduce or minimize these other risk factors, many of which are influenced by lifestyle choices. Before we can explore the relationship between hyperlipidemia and CVD risk, a detailed explanation of the components that make up our blood lipid metabolism is warranted.

Blood Lipid Metabolism and Cardiovascular Disease

Exogenous cholesterol and lipid transport

The Exogenous pathway of lipid metabolism begins in the intestine, with the hydrolyzation of triglycerides to free fatty acids (FFAs) and monoacylglycerol by intestinal lipases for the absorption of dietary lipids (9). The products of these enzymatic reactions are then emulsified with bile acids, cholesterol, plant sterol and fat-soluble vitamins, to form micelles, which then transport their contents to the enterocytes via diffusion (10). Sterol transporter, Niemann-Pick C1-like 1 protein (NPC1L1) also facilitates the transport of free cholesterol and plant sterols from the intestinal lumen into the enterocytes (10).

Once in the enterocyte, either ATP-binding cassette transporter G5/G8 (ABCG5/ABCG8) can transport cholesterol and sterols back into the intestinal lumen, or acyl-CoA cholesterol acyl transferase (ACAT) will esterify the sterols preventing their export back to the lumen (11). This process of efflux and esterification mediates cholesterol and plant sterol absorption (9, 11). FFAs either passively diffuse into the enterocytes or are absorbed via specific transporters including CD36 and fatty acid transport protein 4 (FATP4) (9, 11). While this is not a clear mechanism, animal studies suggest these transporters play a vital role in FFA absorption, yet humans appear to metabolize fats despite transporter mutations (12).

Fatty acid metabolites are initially stored in cytoplasmic lipid droplets of the enterocytes until their liberation for the purposes of oxidation or lipoprotein synthesis (13). Triglycerides are reformed from 1) monoacylglycerol and 2) FFAs, catalyzed by monoacylglycerol acyltransferase (MGAT) and diacylglycerol transferase (DGAT) for export in lipoproteins (13, 14). In the enterocyte, apolipoprotein (apo)-B48 is synthesized then affixed with triglycerides and cholesterol esters by the action of microsomal triglyceride transfer protein (MTP) resulting in the formation of chylomicrons (9, 15). Other major apo associated with chylomicrons include Apo C, Apo E, Apo A-I, Apo A-II, and Apo A-IV (9). Size and composition of chylomicrons is dependent of the amount and type of lipid absorbed by the enterocyte (15).

Newly formed chylomicrons are secreted to lymph for transport to systemic circulation by the thoracic duct (15). Circulating chylomicrons transport their nutrients to muscle and adipose tissue by the action of lipoprotein lipase (LPL) (16). LPL, synthesized in myocytes and adipocytes, is transported to the luminal surface of capillaries, mediated by lipase maturation factor 1 (LMF1)(16-18). Glycosylphosphatidylinisitol anchored high density lipoprotein binding protein 1 (GPIHBP1) anchors LPL to the capillary endothelium while LMF1 stabilizes it (16, 18). Apo C-II on chylomicrons activates LPL hydrolysis of the triglycerides within the chylomicrons resulting in FFAs (16, 19). Adjacent adipocytes and myocytes take up FFAs through FATPs and CD36 for oxidation or storage (16, 19). Some FFAs that are released from the chylomicrons by LPL bind to albumin and are transported to other tissues (9). The activity of LPL is regulated by angiopoietin like protein 3, 4 and 8, as well as Apo C-III, which target LPL for inactivation (20, 21). LPL expression is regulated by hormones (particularly insulin), nutritional status, and inflammation (16).

Characterized by their reduced size and enrichment with cholesterol, by way of reduction of triglyceride content, these chylomicron remnants also acquire Apo E (17). Phospholipids and Apos A and C on the surface of remnant particles are transferred to other high-density lipoproteins (HDL) (17, 19). The transfer of Apo C-II from chylomicrons to HDL impairs LPL hydrolysis of triglycerides in the chylomicron remnants (19). The liver then clears chylomicron remnants from circulation through binding Apo E to LDL receptors, LRP, syndecan-4 and other receptors, facilitating chylomicron remnant uptake by hepatocytes (17). Apo E mutations can decrease chylomicron clearance thus elevating plasma cholesterol and triglycerides levels (9, 22).

Similar to the process of chylomicron formation in enterocytes, triglycerides and cholesterol esters are transferred from the endoplasmic reticulum of hepatocytes to newly synthesized Apo B-100 in a process mediated by microsomal triglyceride transfer protein (MTP) for the formation of very low-density lipoproteins (VLDLs) (23, 24). The rate of VLDL synthesis is determined by the amount of available lipid, not the rate of Apo B-100 synthesis (23). Available lipid in the hepatocyte also determines whether Apo B-100 is degraded or exported from the liver via budding as VLDL (23, 24). Apo C is also known to be associated with VLDL (9).

Lipid and Cholesterol Distribution to Tissues

Once formed, VLDLs are then transported in the blood to peripheral tissues (23). The triglycerides they contain are hydrolyzed by the same LPL as chylomicrons, resulting in competition between the lipoproteins and the release of FFAs (16). VLDL remnants, or intermediate density lipoproteins (IDL), are a product of triglyceride hydrolysis from VLDL (17). IDL particles are richer in cholesterol than VLDLs and acquire Apo E from HDL (17). IDL

can be removed from circulation by the liver via binding of Apo E to LDL and LRP receptors (17). IDL are not cleared as readily as chylomicrons by the liver, leading to the continued hydrolysis of their triglycerides by hepatic lipase. (9, 17). The increase of cholesterol density and the transfer of Apos, except Apo B-100, to other lipoproteins results in low-density lipoproteins (LDL) (9, 17). Regarded as the product of VLDL metabolism, LDL particles mainly contain cholesterol esters and Apo B-100 (9, 17).

The term LDL cholesterol encompasses an array of particle sizes. Small dense LDL particles are considered to be more pro-atherogenic because their small size decreases their affinity for clearance from circulation by LDL receptors. This in turn contributes to their ability to enter the arterial wall, promoting the disease processes of atherosclerosis, and increases their susceptibility to oxidation and uptake by macrophages (9). LDL particle number is a measure of the number of LDL particles in a sample and has been shown to be a useful variable in predicting cardiovascular risk (25, 26), particularly in cases where levels of LDL cholesterol and apoB100 were discordant (27). Plasma LDL levels are determined by a balance between the rates of LDL production and clearance (28). Both processes are regulated by the number and activity of LDL receptors present on the liver (28, 29). High LDL receptor number and activity clears more VLDL and LDL by taking it up at the liver, resulting in decreased LDL concentration (28, 29). As much as 70% of LDL is cleared by hepatocyte LDL receptor mediated endocytosis, while the rest is taken up by extrahepatic tissues (28, 29).

The amount of cholesterol in hepatocytes regulates number of LDL receptors on their surface (28, 29). This is important to note as LDL mainly acts to deliver cholesterol esters to both hepatic and extrahepatic tissues (9). Sterol regulatory element binding proteins (SREBPs) are transcription factors that mediate the expression of LDL receptors in hepatocytes, as well as

other key genes involved in cholesterol and fatty acid metabolism (28, 29). Inactive SREBPs are transported from the endoplasmic reticulum to the golgi in response to decreases in cellular cholesterol levels (28, 29). In the golgi, proteases cleave the SREBPs into active transcription factors that move to the nucleus to stimulate the transcription of LDL receptors along with other genes (29). When cellular cholesterol levels are high, SREBPs remain inactive and LDL receptors are not synthesized (29).

Lipoprotein (a) (Lp (a)) is an LDL molecule with an apolipoprotein (a) attached to the Apo B-100 (30). The functions of this lipoprotein are not well understood; yet, elevated Lp (a) is associated with increased risk of atherosclerosis, and LDL lowering therapies (statins) do not work on it (30). Circulating levels of Lp (a) in plasma can vary from undetectable to greater than 100 mg/dl (30). Lp (a) levels are reflective of production rates as Lp (a) production is primarily genetically regulated (30). People with higher molecular weight Apo (a) tend to have lower levels of Lp (a) and vice versa (31). The mechanism for Lp (a) clearance is unclear but does not seem to be LDL receptor mediated (30). Kidneys may play an important role in Lp (a) clearance because it is delayed in kidney disease (30, 31). Lp (a) reduction is a novel target for the reduction of atherosclerotic risk (30).

When cholesterol in the cell is oxidized, Liver X Receptors (LXR) are activated (32) (33). LXR is a nuclear hormone receptor that is a transcription factor, which stimulates the transcription of E3 ubiquitin ligase. This mediates the ubiquitination and degradation of the LDL receptor also known as inducible degrader of the LDL receptor (IDOL) (32). LDL receptors are also targeted for degradation by PCSK9, a secreted protein that binds to the LDL receptor and enhances its degradation in the lysosomes (34). PCSK9 loss of function mutations result in increased LDL receptor activity and decreased levels of circulating LDL (34).

Reverse Cholesterol Transport

Peripheral cells tend to accumulate cholesterol, as most do not have mechanisms to catabolize cholesterol on their own (9). Intestinal cells, sebocytes, and keratinocytes secrete cholesterol into the intestinal lumen or onto the skin to eliminate it (9). Other cells can synthesize steroid hormones, converting cholesterol to glucocorticoids, estrogen, and testosterone (9). These processes do not satisfy the need to eliminate excess cholesterol; thus, a reverse cholesterol transport is required.

High density lipoprotein (HDL) acquires lipid from peripheral tissues and other lipoproteins and brings the lipids to the liver. Apo A-I, synthesized mainly in the liver and intestines, is the main structural protein contained in HDL particles (35). Other proteins associated with HDL include Apo A-II, Apo C and Apo E (9). Cholesterol and phospholipids are effluxed from hepatocytes, enterocytes, and macrophages facilitated by ATP-binding cassette transporter A1 (ABCA1) to Apo A-I (pre-beta HDL), contributing to the maturation of the HDL (35-37). LXR activity has been shown to increase the expression of ABCA1 and ABCG1 which enhance the efflux of cholesterol from cells to HDL particles (33). Loss of function mutations in ABCA1 leads to a failure to lipidate Apo A-I, rapid catabolism of Apo A-I, and very low HDL levels (35, 36). Chylomicrons and VLDL can transfer cholesterol and phospholipids to HDL during their lipolysis by LPL facilitated by phospholipid transfer protein (PLTP) (9, 35). In circulation, triglyceride rich lipoproteins also transfer their apos to HDL (35).

Cholesterol status of the cell mediates cholesterol efflux through microRNAs. miR-33 targets ABCA1 and ABCG1 for degradation and is embedded in the SREBP2 gene (38). Increased cellular cholesterol decreases SREBP2 expression decreasing miR-33 and enhancing LXR expression (38). This combination of LXR and ABCA1/ABCG1 expression promotes the

efflux of cholesterol (33). When cellular cholesterol is low, miR-33 degrades ABCA1/ABCG1 and inhibits LXR activity, reducing the efflux of cholesterol. SREBP2 is also upregulated to promote LDL receptor activity, allowing the cell to acquire cholesterol (38).

The cholesterol in the core of mature HDL particles is esterified; yet, cholesterol that is effluxed from cells to HDL particles is free cholesterol, localized to the surface (39). Lecithin cholesterol acyltransferase (LCAT) is an HDL associated enzyme that catalyzes the transfer of a FFA to free cholesterol esterifying the cholesterol and permitting their translocation to the core of the HDL particle (39). Apo A-I from the HDL activates LCAT and facilitates the esterification process (39). LCAT activity is required for the formation of large HDL particles, ultimately allowing the HDL to be more effective at transporting cholesterol (39).

Cholesterol esters carried in the core of HDL particles may be transferred to Apo B containing particles in exchange for triglyceride, mediated by cholesteryl ester transfer protein (CETP) (9, 40). CETP activity deficiencies lead to large HDL particles, high HDL cholesterol levels, and decreased LDL cholesterol levels (40). The triglycerides that are transferred to HDL by CETP activity are catabolized by hepatic lipase, resulting in the formation of small HDL particles, the release of Apo A-I, and increased Apo A-I degradation (40). Hepatic lipase activity is increased in insulin resistant states which is associated with low HDL levels (35). Conversely, endothelial cell lipase is a phospholipase that hydrolyzes the phospholipids carried in HDL particles (35). Decreases in endothelial lipase activity have been shown to increase HDL levels in mice, yet is unclear in humans (35).

Apo A-I is metabolized independently of HDL cholesterol primarily in the kidneys and liver (9, 35, 41). Lipid poor Apo A-I is filtered by the kidneys and taken up by renal tubules depending on particle size (41). The degree of lipidation of Apo A-I determines the rate of its

catabolism (9, 41). At renal tubular cells, Apo A-I binds to cublin and megalin to facilitate the uptake and degradation of the filtered Apo A-I (41). The mechanism of liver catabolism of Apo A-I is not well understood, but HDL particles containing Apo E may be taken up via LDL and other Apo E receptors (9).

HDL particles primarily deliver their cholesterol to the liver, mediated by class B scavenger receptor B1 (SR-BI), promoting selective uptake of HDL cholesterol (42). HDL particles bind to SR-BI, transporting the cholesterol into the liver without the internalization of the HDL particle (42). This process forms a smaller HDL particle, depleted of cholesterol, that is released back into circulation (35). Despite SR-BI deficiency in mice raising HDL levels and atherosclerosis risk (42), human deficiencies have little impact on the risk for atherosclerosis because of the CETP mediated mechanism of transferring cholesterol from HDL to apo B containing lipoproteins and their subsequent return to the liver (9, 40).

Although reverse cholesterol transport is important for preventing the development of atherosclerosis, HDL cholesterol levels may not be indicative of the rate of reverse cholesterol transport (33). For example, the ability of HDL cholesterol to promote cholesterol efflux from macrophages has been observed to vary, which would indeed put an individual at increased risk for atherosclerosis development despite adequate levels of HDL (33, 43). There are also other components involved in this mechanism that are not the HDL particles themselves that have the potential to be dysfunctional (33). Thus, HDL cholesterol levels alone are not good indicators of the health of the reverse cholesterol transport mechanism (33, 43).

Cholesterol Elimination

After cholesterol is delivered to the liver, there are several pathways for cholesterol elimination. Two of which are either the conversion of cholesterol into bile acids for secretion in the bile, and the direct secretion of cholesterol into bile for elimination (43). The expression ABCG5 and ABCG8 genes is enhanced by LXR, which promotes the transport of cholesterol into the bile as a result of increases in cellular hepatic cholesterol (33, 43). Cholesterol is then eliminated by the secretion of bile into the intestines for elimination in the feces (44).

Endogenous Cholesterol Synthesis

Cholesterol can be absorbed from the diet, as explained above, or it can be synthesized endogenously. The formation of cholesterol involves many steps that can be generalized into 2 main processes. First is the condensation of isoprene units to from squalene. Second is the cyclization of squalene to produce lanosterol and eventually cholesterol (45). Cholesterol synthesis requires significant amounts of acetyl-CoA, ATP, oxygen, NADPH and NADH to be dedicated to the pathway, resulting in an energetically expensive process (46). This process begins with the formation of 3-hydroxy-3-methyglutaryl-coenzyme A (HMG-CoA) from 3 mol of acetyl-CoA (45, 47). HMG-CoA is then converted to mevalonate in the rate-limiting reaction catalyzed by 3-hydroxy-3-methylglutaryl-coenzyme A reductase (HMG-CoA reductase). This reaction is considered paramount as it serves as the target of statin therapy (48). Mevalonate is converted to 5-pyrophospho-mevalonate then to Isopetenyl Pyrophosphate (IPP), also known as an isoprene unit (49). Some IPP molecules are transformed to dimethyallyl pyrophosphate (DMAPP) by IPP isomerase. By the action of farnesyl pyrophosphate synthase (FPPS), DMAPP and geranyl diphosphate (GPP) are sequentially added to IPP to from farnesyl pyrophosphate (FPP) (50). Two FPP molecules are condensed to squalene by squalene synthase. (49). Next, squalene is converted to 2,3-epoxysqualene, by a more recently recognized rate-limiting enzyme,

squalene monooxygenase (SM) (46). SM is the first step in a series of reactions that result in the cyclization of the carbon chain and the intermediate known as lanosterol (49). The conversion of lanosterol to cholesterol is a complex multistep process that can proceed through either of two pathways: Bloch or the Kandutsch-Russell. Major differences in these pathways primarily involve the saturation or desaturation of the intermediates' sidechains, but both result in cholesterol (51), although much is left to be understood about either pathway. Cholesterol can be synthesized in most tissues of the body but is primarily formed in the liver, as well as absorbed from dietary sources and reabsorbed from bile.

Regulation of Cholesterol Synthesis

The cholesterol synthesis pathway is regulated by a negative feedback response to cellular cholesterol to create systematic homeostasis. As cellular cholesterol levels rise, the key enzymes including HMG-CoA reductase and SM are deactivated through mechanisms mediated by SREBP2 (45, 46). Cholesterol that is synthesized by the body or absorbed from the diet can contribute to the negative feedback regulatory mechanism of cholesterol synthesis. Many different factors, including body weight, hormones, circadian rhythm, genetics, gut microbiome, early nutrition, epigenetics and diet are being explored as potential mediators affecting the regulation of cholesterol biosynthesis (45). For the purpose of this document, we are going to focus on the effects of diet on cholesterol synthesis.

Nutrients, foods, or bioactive compounds have been explored for decades as regulatory factors for cholesterol homeostasis. One major theme that has emerged from this line of study is high saturated fatty acid (SFA) intake (45). SFA in humans has been shown to upregulate the synthesis of cholesterol especially when compared to polyunsaturated fatty acids (PUFAs) (52). SFA upregulation of cholesterol synthesis may be due to a decreased affinity of the enzyme that

converts free cholesterol to cholesteryl ester for SFAs, leading to a decrease in negative feedback in the cholesterol synthesis pathway in the liver (53, 54). At the same time, the increased free cholesterol in the liver is thought to be downregulating the expression of the LDL receptor leading to accumulation of LDL in the plasma (53, 54).

While SFA is associated with increases in cholesterol, PUFA replacement of SFA in the diet decreases plasma lathosterol concentration, indicating decreased cholesterol synthesis (55). Rodent studies suggest this reduction in cholesterol synthesis by both n-3 and n-6 PUFAs is a result of inhibition of HMG-CoA reductase and FPP synthase through the impairment of SREBP expression (56-58). Furthermore, linoleic acid (LA, 18:2n6) has been shown to counteract the hypercholesterolemic effect of SFA when intake of LA is greater than 4.5% of energy (59). Another component of the diet that is known to affect cholesterol synthesis is dietary fiber. Specifically, soluble dietary fibers increase gut viscosity by forming a layer in the small intestine, reducing the absorption of bile acids, and signaling for an increase in bile acid synthesis from cholesterol, reducing cholesterol in circulation (60).

Atherosclerosis

CVD is a term that is used to describe diseases that affect the heart or blood vessels (61). One of the most common types of CVD is coronary artery disease (CAD) (4). CAD involves a narrowing or blockage of the coronary arteries that can cause chest pain (angina), myocardial infarction, and death (62). Many factors influence the development of CVD, but a common underlying etiology is atherosclerosis warranting its discussion here.

Atherosclerosis is defined as a focal inflammatory fibro-proliferative response to multiple forms of endothelial injury (63). Endothelial injury and dysfunction are the result of the action of the various risk factors associated with the development of atherosclerosis such as smoking

cigarettes, chronically elevated blood pressure, diabetes mellitus, obesity status, and elevated cholesterol (64-69). The endothelial cells are a single cell layer creating the interior surface of the arterial wall.

Once endothelial dysfunction or injury occurs, there is an increase in the permeability of the arterial wall allowing LDL accumulation and oxidation by reactive oxygen species (ROS) resulting in pro-inflammatory particles referred to as oxidized LDL (70-72). These oxidized LDL particles stimulate the endothelia and vascular smooth muscle cells (VSMCs) (just below the endothelia) to produce adhesion molecules, chemoattractants, and growth factors, signaling for monocyte migration into the intima, and selective differentiation into macrophages and dendritic cells (73-76). The adaptive immune response modulates the inflammatory response and progression of the disease (72, 75).

Foam cells are produced in the arterial wall through the phagocytosis of oxidized LDL by immune cells and VSMCs resulting in the formation of xanthomas ("fatty streaks") and the onset of atherosclerosis (70, 73) (76, 77). Xanthomas progress to plaques through the aggregation and degranulation of platelets, collection and oxidation of LDL in the arterial wall, and the phagocytosis of oxidized LDL by macrophages resulting in more foam cells. (63, 70, 72). The foam cells release chemokines and growth factors that recruit more inflammatory cells and signal for VSMCs to proliferate and form the fibrous cap over the lesion as well as deposit calcium to form the atherosclerotic plaque (72, 76, 77). Apoptosis of the foam cells creates a lipid pool of cell debris and extracellular lipid called the necrotic core of the plaque (73). When the plaque is smaller and has a thicker fibrous cap it is termed a stable plaque due to its lower likelihood for rupture (63). Unstable plaques are more likely to rupture due to a larger necrotic core, high content of inflammatory cells and thinner fibrous cap (63).

Rupture of the plaque reveals the inner thrombogenic material. The resulting thrombus formation can occlude the lumen of the artery resulting in myocardial infarction or stroke (78). Repeated rupture and heling of a plaque is associated with greater incidence of fatal events (63, 79).

Hyperlipidemia and CVD

Lipid metabolism is an intricately regulated process of modulating cellular lipid and cholesterol content as well as the concentration of the many types of lipoproteins in circulation depending on the current needs of the body. Should these processes become dysregulated, elevated circulating lipoproteins, also known as hyperlipidemia, results (80). Characterized by elevated serum total cholesterol (TC), LDL, triglycerides, and lower serum HDL, hyperlipidemia has been well established as an independent risk factor for the development of atherosclerosis and CVD (80). Hyperlipidemia upregulates the differentiation of hematopoietic stem progenitor cells to inflammatory monocyte subtypes ($CD14^{++}CD16^{-}$ and $CD14^{++}CD16^{+}$) observed in humans and reproduced in rodent models (81). These types of monocytes preferentially adhere to vascular endothelium, infiltrate the arterial wall and promote the formation of atherosclerotic plaque macrophages (81). High levels of LDL specifically, tend to become oxidized, injure vascular endothelial cells promoting monocyte adhesion, very smooth muscle cell migration, and platelet activation, which all culminate in the initiation of the development of an atherosclerotic plaque (82). Knowing hyperlipidemia is an independent primary risk factor for the development of CVD, it is of note that other chronic diseases contribute to elevated blood lipids and vascular endothelial damage, further increasing the risk for CVD. In the following sections, the mechanisms of the relationship between age, chronic diseases, hyperlipidemia, and CVD risk will be explained.

Age as a Primary Risk Factor for CVD

It is known that blood lipids increase with age, specifically plasma TC, triglycerides, LDL, and apoB – 100, increasing the risk for the development of CVD (83, 84). The mechanisms responsible are difficult to study due to the instance of comorbidities but a brief discussion of proposed contributing factors is necessary.

Postprandial lipid oxidation is impaired in older adults compared to younger, and presents with increased postprandial fat deposition, contributing to impaired postprandial lipid clearance and weight gain (85, 86). Increased age and weight gain are associated with insulin resistance which has been shown to reduce LPL activity, leading to an accumulation of postprandial FFA (87, 88). Reduced LPL activity is also believed in older adults to produce a prolonged postprandial hypertriglyceridemic state after fat consumption (16, 84, 89, 90). The influx of triglyceride rich lipoproteins in the postprandial state results in direct competition for LPL, leading to the accumulation of lipoproteins (91-93). Dampened postprandial lipid clearance is also proposed to be resultant of impaired liver function (86). As humans in the developed world spend much of their waking hours in the postprandial state, the elevation and prolonged clearance of postprandial blood lipids imposes risk for not only the development of chronic hyperlipidemia but also CVD (94).

Chronic diseases as risk factors for CVD

While age and elevated blood lipids (hyperlipidemia) have been previously discussed as risk factors for CVD, other modifiable risk factors include hypertension, obesity, and type 2 diabetes mellitus (69). In this section, each of these risk factors will be described as to how they independently relate to the development of CVD, as well as how their synergistic effects compound CVD risk.

Hypertension

Hypertension, or chronically elevated blood pressure, is well known to be a major risk factor for CVD development both in the United States and worldwide (95). Sustained high blood pressure can result from age, diet, stress sedentary lifestyle, or a combination thereof (96). While there is more research to be done into the underlying mechanisms of hypertension, it is known to be related to many factors including genetics, sympathetic nervous system activation, the rennin-angiotensin-aldosterone system, endothelial dysfunction, impaired capillary blood flow and inflammatory mediators (96). A key component of hypertension in its development of atherosclerosis is the deficiency of Nitric oxide (NO). NO is regarded as a powerful regulatory molecule of vascular tone and its deficiency in hypertension is implicated in increased production of reactive oxygen species (ROS) (97). ROS, as well as reactive nitrogen species (RNS), contribute to oxidative stress that is thought to play a central role in the pathophysiology of hypertension, leading to vasoconstriction, vascular hypertrophy, vascular remodeling and increased systemic vascular resistance (98-100). This loss of vasomotor activity, characteristic of hypertension, promotes endothelial damage, increasing lipid permeability of the endothelial cells, promoting the formation of atherosclerotic lesions, and resulting in CVD (101).

Type 2 Diabetes

Type 2 diabetes is a disease characterized by insulin resistance and relative insulin deficiency (102). Diagnosed by HbA1c ≥6.5% and fasting plasma glucose ≥ 126 mg/dl, type 2 diabetes is a growing epidemic in the United States. With insulin resistance, pancreatic β-cells respond by secreting more insulin in an effort to offset the deficient action of insulin, resulting in elevated levels of circulating insulin in these individuals (103). Not only a hormone that signals for the cellular uptake of glucose, insulin is also a potent growth factor, that in these states of

elevated circulation, signals via the mitogen-activated protein kinase (MAPK) pathway for vascular smooth muscle cell growth, proliferation, and differentiation (104) which is likely to result in intimal thickening and the development of an atherosclerotic lesion (105). Another primary mechanism in which type 2 diabetes increases CVD risk is through hyperglycemia. This leads to the formation and accumulation of advanced glycation end products which promote the formation of reactive oxygen species. Immune cells are then activated promoting inflammation that is mediated by oxidative stress from the reactive oxygen species (106). Through these mechanisms of vascular damage and modification, endothelia dysfunction (also reflective of NO deficiency) is characteristic of insulin resistance, promoting atherosclerosis at the cellular level (107).

Independently, type 2 diabetes and hypertension are major risk factors for CVD, yet they are closely related to each other due to similarities in their pathophysiology. Both hypertension and type 2 diabetes can result in arterial remodeling, vascular fibrosis, vascular inflammation, endothelial dysfunction, and atherosclerosis. This milieu of vascular damage results in microvascular and/or macrovascular disease, which then is likely to progress to CVD (106). Diabetes is also known to promote hypertension through increases in inflammation, activation of the renin – angiotensin – aldosterone system, and oxidative stress that narrow vessels driving increases in blood pressure, and ultimately increasing the risk for the development of CVD (108).

Obesity

The last modifiable risk factor for CVD of this discussion is overweight and obesity. Characterized by excess body weight and adiposity, obesity is determined using a measure of body mass index (BMI). BMI is calculated by dividing weight in kilograms by height in meters

squared. Classifications of BMI are as follows: ≤18.4kg/m^2 is underweight; 18.5-24.9kg/m^2 is normal weight; 25-29.9kg/m^2 is overweight; 30-34.9kg/m^2 is obese class I; 35-39.9kg/m^2 is obese class II; ≥40 kg/m^2 is obese class III or extreme obesity. (109). BMI is used to classify obesity because it correlates strongly with body fat, morbidity, and mortality; and it is simple and inexpensive to implement in a clinical setting (110).

Obesity can independently increase CVD risk, but also increases the likelihood of the development of the other aforementioned risk factors, type 2 diabetes, hypertension and hyperlipidemia. Independently, obesity induces a state of low-grade chronic inflammation due to dysfunction of adipose tissue and dysregulation of adipokines. Dysfunctional adipose tissue may be the result of adipose tissue hypoxia from a reduced vascularization of the tissue and adipocyte hypertrophy (111). The dysfunctional adipose tissue releases pro-inflammatory adipokines, resulting in atherogenic systemic inflammation(112). Hyperlipidemia can result from obesity due to both excessive amounts of adipose tissue as well as hypothalamic leptin resistance, which is associated with vascular inflammation, oxidative stress, and insulin resistance (113). Hypertension results from hemodynamic and cardiovascular adaptations due to obesity. Such adaptations as increased cardiac output to compensate for increased metabolic demand of the enlargement of the adipose and lean tissues, and systemic vascular restriction resulting from hyperinsulinemia and low-grade inflammation, result in chronically elevated blood pressure and then hypertension (114). Insulin resistance is thought to develop in obesity not only from the low-grade chronic inflammation resultant of the hypoxic adipose tissue inhibiting insulin signaling activity, but also from hyperinsulinemia, oxidative stress, lipotoxicity from elevated free fatty acids, endoplasmic reticulum stress, and even hypoxia from obstructive sleep apnea (115). Obesity is arguably one of the most critical modifiable risk factors for CVD, because it not

only is an independent risk factor, but is also clearly implicated as a cause of the other primary risk factors described above.

Dietary Fats

Fat is an important macronutrient that has popularly been deemed "bad" by consumers since the 1980s. The diet-heart hypothesis proposed that high dietary fatty acid and cholesterol intake promoted coronary heart disease in "at risk" individuals (116). This recommendation to follow a low fat, high carbohydrate diet was originally suggested in an effort to reduce SFA intake and total energy intake (EI) (117). These recommendations were in response to large observational studies that were able to correlate excessive EI and SFA intake with higher instances of CVD (69). While lifestyle changes including low-fat diets and enriching the diet with fruits, vegetables, and whole grains have been successful in reducing CVD risk, the most clinically relevant effects were traditionally seen when drastic total lifestyle changes were implemented such as a combination of adopting a low- fat plant based diet, increasing daily physical activity, and attending group stress management sessions on a weekly basis (118). In contrast to the low-fat diet dogma, research on dietary lipids shows that fats are an important source of energy, especially in states of limited glucose availability (119). Most tissues have the ability to oxidize fats, which helps preserve glucose for tissues that are unable to oxidize fats including the brain and central nervous system (119). In addition to serving as a fuel source, dietary fats have other important functions including serving as structural components of cell membranes, facilitating inter and intra cellular signaling, and transporting and storing lipid soluble vitamins (120). Today, more research is being done with a focus on how different types of fats in the diet impact health and chronic disease risk. Before we can discuss these

physiological responses to dietary fats, it is important to first discuss the structure function and sources of these different types of fats.

Saturated fat

A fatty acid is defined in chemistry and biochemistry as a carboxylic acid with an often long, unbranched aliphatic tail, that is either saturated or unsaturated with hydrogen atoms (121). The length of the carbon chain can vary from being as short butyric acid which is 4 carbon atoms long to montanic acid which is 28 carbons long (121). Naturally synthesized fatty acids most commonly have an even number of carbons because their synthesis involves acetyl-CoA, which is a coenzyme that donates a 2 carbon atom group in the process of fatty acid synthesis (47, 121). Fatty acid saturation depends on the presence of double bonds in the carbon chain which are placed in enzyme mediated processes (121).

SFAs are single bonded chains of carbons that are completely saturated with hydrogens, giving them a straight structure. This chemical structure also lends the attribute of SFAs having a higher melting point than their unsaturated counterparts. Thus, fat sources that are rich in SFAs and are solid at room temperature are commonly referred to as solid fats (122). Common sources of SFA in the American diet include hamburgers, bakery products and snacks including potato chips, French fries, and buttered popcorn (123). Other sources of SFA include, palm oil, coconut oil, cocoa butter, and animal-derived fats such as lard, tallow, and butter (119). The American diet is rich in these fats as they are commonly used in cooking and baking. Current recommendations suggest transitioning to using less solid fats to reduce SFA intake to less than 10% of daily EI (122, 124).

Despite its implications in CVD development, SFAs do have some important functional roles in the body that cannot be ignored. Cell membranes require SFA incorporation into phospholipids to provide structure to these membranes. One part of a cell membrane that is commonly particularly rich in SFA containing phospholipids is called the lipid raft. Lipid rafts function as cell signaling platforms to which proteins involved in cell signaling are anchored. The SFAs that interact with these proteins in the lipid raft can also covalently modify them assisting in the regulation of signal transduction between cells (125).

Unsaturated fatty acids

Unsaturated fatty acids are similar to the previously described SFAs , except they have one or more alkenyl functional groups in their carbon chain (121). This alkene structure is substituting a single-bonded part of the carbon chain for a double bonded portion, creating a point of "desaturation"(121). The hydrogens on either side of the double bond can be in two different configurations: cis or *trans*. Cis refers to a configuration where the adjacent hydrogen atoms are on the same side of the double bond creating a bend in the chain (121, 126). Fatty acids are defined by the position of the first double bond. The omega system of naming assigns a number to the first double bond based on how many bonds it is from the end of the fatty acid chain and can be written in the format of n-x. In fatty acids with multiple cis conformation double bonds, the molecule takes on a bended and even hooked form. This pronounced bending of the structure of the fatty acid impairs the ability for these fatty acids to pack closely with others, resulting in lower melting points and increased fluidity of membranes as well as of the fats they are contained in (121, 126). The *trans* configuration is the opposite of the cis, meaning the hydrogens are on either side of this double bond, opposite from each other (121). This confirmation does not result in a bended structure, but rather a much straighter shape like that of

SFA. Most fatty acids in the trans configuration are not found in nature and are the result of hydrogenation (121).

Monounsaturated Fatty Acids

Monounsaturated fatty acids (MUFAs) contain only one point of desaturation. Oleic acid (OA, 18:1n9) is the most abundant MUFA in the human diet followed by palmitoleic acid (16:1n7) and vaccenic acid (18:1trans11) (119, 127). OA is an n-9 fatty acid that the body is able to synthesize on its own (119). Olive oil (OO), currently regarded as one of the most popular "heart healthy" oils by consumers, is about 75% OA making it a particularly rich source of MUFAs (128). Other sources of MUFAs include canola oil, sesame oil, nuts including almonds and pecans, and avocados (129, 130). There is no DRI for MUFAs as they are not an essential nutrient but the acceptable macronutrient distribution rang for total fat is 30-35% of energy (120, 131).

MUFAs are synthesized endogenously at cellular lipid droplets via adipose triglyceride lipase (ATGL) and have recently been shown to be involved in the stimulation of various transcription factors (132, 133) in addition to their general function as substrates for fat oxidation (134). Senarath et. al. showed some MUFAs to modestly down regulate expression of SREBP-1 and SCD1, regulating cellular lipid synthesis, while others increased expression of PPARγ and C/EBPα, and SREBP-1, promoting lipogenesis (132). Najt et. al. have shown MUFAs to be translocated to the nucleus via PLIN5 and interact with SIRT1 to enhance PGC-1α/PPARα signaling and promote oxidative metabolism (133). The insulin signaling pathway via IRS-1/PI3K has also been explored as being modulated by MUFAs and associated with preserved insulin sensitivity (135). At the cellular level, these data suggest that MUFAs play a role in the regulation of fatty acid oxidation and storage, yet these mechanisms need further study. Finally,

MUFAs are also well known as important structural lipids in cell membranes, especially in nervous tissue myelin (120).

Polyunsaturated Fatty Acids (PUFA)

PUFAs, are another form of unsaturated fatty acid that contains multiple points of desaturation (121). General sources of PUFAs include plant oils such as corn and soy bean, nuts, seeds and fish (136). Some PUFAs are essential fatty acids due to the body's inability to produce them. There are only two fatty acids that the human body cannot produce: linoleic acid (LA, 18:2 n2) and alpha-linolenic acid (ALA, 18:3 n3). Humans lack the ability to introduce double bonds in fatty acids beyond carbons 9 and 10, resulting in omega 3 (such as ALA) and 6 (such as LA) fatty acids being essential (121). These fatty acids are readily available in plants and plant oils. Good sources of ALA include green leafy vegetables, soy products, walnuts, and flax seeds (137). Adequate intake of ALA is 1.6 and 1.1 g/d for men and women, respectively (120). LA is more readily found in oils, such as canola, soybean, corn and cottonseed (138-140). Adequate intake of LA is 17g/d and 12 g/d for men and women respectively (120). Longer chain omega-3 fatty acids are also available in various plant and marine oils including eicosapentaenoic acid (EPA, 20:5n3), docosahexaenoic acid (DHA, 22:6n3), and docosapentaenoic acid (DPA, 22:5n6) and are known for their importance in neuronal, retinal, and immune function (141). These long chain PUFAs are not essential because the body can synthesize them from ALA and LA via elongases. However, it is important to note that less than 5% of the consumed ALA is converted to EPA and DHA highlighting the inefficiency of this biosynthesis and potential benefit of supplementation (142).

While other categories of fatty acids (MUFAs, SFAs) have important roles in the body, PUFAs may have even more important functions. They are integral constituents of cell

membranes so much so that on a macroscopic scale, insufficient LA and ALA intake results in a scaly dermatitis requiring supplementation (120). PUFAs are also precursors for powerful signaling molecules including eicosanoids, endocannabinoids and resolving lipids, which are involved in the modulation of many processes including the inflammatory response (143). Eicosanoids include prostaglandins, leukotrienes, and lipoxins. Prostaglandins have functions in sleep regulation, modulation of the immune response, and the promotion and resolution of the inflammatory process (144-146); leukotrienes function in the immune response, specifically related to bronchoconstriction (147); and lipoxins are key in resolution of inflammatory processes (143). Endocannabinoids are partial agonists of cannabinoids receptors, and have been shown to interfere with the transcription of nuclear factor κB (NF-κB) a key transcription factor for the expression of proinflammatory genes (148). Resolving lipids stimulate the resolution of inflammation through mechanisms including inhibition of dendritic cell function, promotion of IL-10 and hepatic growth factor, and reduction of cytokine production by inhibiting apoptosis through the phosphorylation of protein kinase B (149-151).

PUFAs are known regulators of transcription factors related to lipid storage, cholesterol synthesis, and fatty acid oxidation. More specifically, ALA and LA have been shown to repress the expression of lipogenic, glycolytic, and cholesterol genic enzymes' expression while upregulating genes of hepatic and skeletal muscle beta-oxidation. PUFAs can both directly and indirectly regulate transcription factors. In the direct regulation of transcription factors, PUFAs bind to ligand binding sites of the transcription factors inducing a conformational change in the receptor that repositions helix 12 (AF 2) promoting the dissociation of co-repressors (such as NcoR-Sin3-HDAC) and the recruitment of co-activators (including Src1, PGC-1). This mechanism has been documented for PPARs α, β, ϒ1, and ϒ2, LXRα, HNF-4s α and ϒ, RXRα

and ROR (152-156). Indirect mechanisms of gene regulation by PUFAs can occur via enzyme mediated pathways including cyclooxygenase, lipoxygenase, and protein kinase C, or changes in membrane lipid or lipid raft composition which would affect the function of receptors including g-protein receptors and tyrosine kinase-linked receptors. These indirect mechanisms effect the abundance of transcription factors in the nucleus and have been observed in the cases of SREBP, NFκB, c/EBPβ, and HIF-1α (157-160).

Peroxisome proliferator-activated receptors (PPARS) are steroid nuclear receptors that act by dimerizing with retinoid X receptors (RXR) and bind to peroxisome proliferator response elements (PPRE) on the target genes to upregulate their expression (161). PUFAs have been shown to regulate gene expression in various tissues and developmental stages differentially, but in general, both n-3 and n-6 PUFAs upregulate the mRNA of genes involved in peroxisomal beta-oxidation through the binding of PPAR-α. Binding of PUFAs to PPARβ has also been shown to increase the expression of uncoupling protein 2 (UCP2) which modulates thermogenesis and is also thought to upregulate fat oxidation (162, 163). PPARs are not the only pathway through which PUFAs affect transcription. In PPAR-α null mice, PUFA upregulation of beta-oxidation genes was impaired as expected, but PUFAs still suppressed lipogenic genes (164). This discovery that PPARs are not the only mechanism through which PUFAs exert regulatory effects on expression set the conditions for discovering SREBPs and their relation to PUFAs. It is important to note that different FAs can have different affinities for the various transcription factors. For example, in the PPAR family, FAs of 18-20c in length bind well to the α subgroup yet are poor activating ligands for the β subgroup (154).

SREBPs have been discussed previously as being responsible for cellular cholesterol homeostasis. When the concentration of sterols in the cell is low, SREBPs are activated and

promote transcription of cholesterol, triglyceride, and fatty acid synthesis genes. Inhibition of SREBPs, such as in the presence of PUFAs, downregulates the expression of enzymes required for these processes, through the inhibition of the proteolytic cleavage required for the activation of SREBPs (165, 166).

Hepatocyte nuclear factor-4α (HNF-4α) is a nuclear receptor involved in the transcription of a plethora of genes related to carbohydrate, lipid and protein metabolism, as well as the expression of other nuclear receptors (167). PUFAs have been shown to regulate the activity of HNF-4α by directly binding to its ligand binding domain inhibiting it's binding to promoter elements decreasing the expression of target genes(168, 169).

Liver X receptors (LXRs) are known to bind oxysterols and regulate fatty acid/cholesterol metabolism and transport. PUFAs have been shown to regulate LXRs in a few mechanisms including binding to the LXR, preventing the transcription of SREBP1c gene, decreasing lipogenesis (156, 170). PUFA upregulation of PPARS has also been shown to inhibit the lipogenic effects of LXRs by preventing their required dimerization with RXR (171). Furthermore, PUFAs may also inhibit the binding of the LXR-RXR heterodimer to their target response elements further impairing their ability to promote the expression of lipogenic genes (172).

PUFAs also regulate gene expression via more mechanisms than at the level of gene transcription. For example, PUFAs have been shown to destabilize the mRNA of the SCD-1 gene reducing the accumulation of mRNA resulting in a reduced expression of SCD-1 enzyme (173). PUFAs also have been shown to interact with response elements on DNA, directly controlling the expression of genes including FAS (174). Altogether, there are multiple mechanisms through which PUFAs exert regulatory effects on fatty acid and cholesterol

metabolism. Much of this mechanistic work has been done in vietro or in highly controlled animal models, leaving the question of how PUFAs incorporated into a mixed diet in free living humans affect cholesterol metabolism, fat oxidation and overall heath.

Effect of Dietary Fat Composition on Blood Lipids

While fat has been demonized in the public eye, it is well known in scientific literature for its power in modulating markers of cardiovascular health including blood lipids. We have previously discussed blood lipids and their metabolism in depth. Here we will explore the current understanding of how dietary fats affect blood lipids.

Saturated Fat and Blood Lipids

SFA is well known for its hyperlipidemic effect in humans (175). It repeatedly has been shown to result in elevated LDL cholesterol and TC when compared to both PUFA- (176-178), and MUFA-enriched (179-182) dietary RCT interventions. Acute SFA consumption also leads to longer elevations in postprandial triglycerides vs. MUFA or PUFA (183). This understanding of the hyperlipidemic effects of SFA is what informs the current recommendation to lower SFA intake and increase unsaturated fat intake.

While SFAs are important in cell signaling and structure, they are also implicated in influencing gene expression that contributes to the disease processes of CVD. Through stimulating the expression of PGC-1β, SFA has been shown to activate the SREBP transcription factor family stimulating lipogenic gene expression (184). SFAs have also been shown to upregulate the expression of LXR and bind to hepatocyte nuclear factor 4 (a transcription factor involved in liver lipid metabolism) (185). This pathway of gene activation is implicated in decreasing fat accumulation in the liver by mobilizing lipids in the form of VLDL and

triglycerides, producing a postprandial hyperlipidemic state that we have already discussed as being proatherogenic (184). SFAs have also been shown to induce NF-κB activation and expression of inducible cyclooxygenase (COX-2) in macrophages through the activation of toll-like receptor 4 (Tlr4) (186). COX-2 is thought to be a marker of inflammation as it catalyzes the conversion of arachidonic acid to prostaglandin endoperoxide, which is the rate limiting step in prostaglandin and thromboxane biosynthesis (187). Furthermore, the activation of Tlr4 results in the upregulation of multiple inflammatory cytokines along with COX-2, causing damage to β cells of the pancreas and inhibiting the phosphorylation of insulin receptor substrate 1, potentially influencing insulin insufficiency and resistance (188). These are some of the main mechanisms by which SFA can increase chronic disease risk in humans.

Monounsaturated Fat and Blood Lipids

MUFAs have been described as cardio protective for their prominence in population-based studies, often in relation to the Mediterranean diet (MED), which have commonly found lower instance of heart disease (189). In one study, a MUFA-rich diet was shown to significantly lower LDL, HDL, size of the major LDL fraction, LDL susceptibility to oxidization, and plasma concentrations of oxidized LDL when compared to SFA. These effects were observed for both a higher fat and lower fat diet (190). Beneficial changes of lipid profiles, similar to this trial, have been observed in other high MUFA trials (191-193). While these are not conclusive, these trials suggest that a MUFA-enriched diet is beneficial for improving blood lipids as well as preventing LDL oxidation when compared to SFA despite potentially decreasing the size of the LDL particles. In another study, both canola oil and high OA canola oil have resulted in lower TC, LDL, apo B, and non-HDL c compared to the western style control treatment, but saw no difference between each other for these measures (194). The high OA canola oil was able to

lower the TC:HDL c and apo B:apo AI ratios more than canola oil (194), showing a potential benefit to a high oleic version of canola oil. Both trials are evidence for the idea that even non-olive based MUFA oils have favorable effects on blood lipid compared to SFA.

One of the most prominent MUFAs, OA, has been shown in meta-analyses to have a mild TC and LDL cholesterol lowering effect with an inconsistent effect on HDL cholesterol when it is replacing SFA (127). Meta-analysis evidence suggests that the cholesterol lowering effect of MUFAs is not as powerful as would be needed to defend the idea that MUFA consumption alone would prevent the development of CVD or other disease states (195). A reduction in all measures of CVD and mortality risk has been found associated with MUFA consumption, but in sub group analysis these interactions were only significant of OO use (127).

Olive Oil

OO is especially popular as it is the main source of MUFA in the MED. A MED intervention, supplemented with OO or nuts, produced fewer incidence of major cardiovascular events when compared to a reduced fat diet in the PREDIMED study (196). This study was not able to differentiate the benefits of the MED from the MUFA supplementation. Canola oil, corn oil (regarded as PUFA rich), and OO enriched diets have shown a significant reduction of TC, LDL, and LDL apo B concentrations in adults with elevated LDL, yet OO had a significantly dampened effect (197). The OO treatment was shown to significantly preserve HDL levels, which is beneficial in preventing atherosclerosis by promoting reverse cholesterol transport (197). These results are mirrored by a meta-analysis of 27 other clinical trials comparing OO to PUFA interventions. (198). With this evidence, it is becoming apparent that PUFAs have a stronger blood lipid lowering ability than MUFAs and OO, potentially at the expense of HDL cholesterol, warranting a discussion of PUFAs specifically.

Polyunsaturated Fats and Blood Lipids

PUFAs are found in various vegetable oils as well as fish oils and fatty fish. The PUFAs in fish oil are primarily the longer chain n-3 fatty acids EPA and DHA, which are different than the predominant n-3 fatty acid found in vegetable oils, namely ALA. Many studies have shown beneficial effects of PUFA intake on blood lipids. In a study of aging adults with metabolic syndrome, a 6-month trial of the American Heart Association diet with the addition of PUFA or MUFA rich muffins showed a greater reduction of TG and improved vascular function with the PUFA diet compared to the MUFA diet (199). Corn oil (PUFA) has also been shown to favorably improve TC, LDL, TC:HDL, and apo B compared to OO (MUFA) supplementation in adults (200). Free living young adults have also mirrored these results (201). In another trial of mildly hypertensive men, both sunflower seed oil (n-6 PUFA) and linseed oil (n-3 PUFA) decreased TC and LDL compared to OO (MUFA), but linseed oil also decreased LDL:HDL ratio despite LCAT activity decreasing (202) . Therefore, linseed oil was more effective than sunflower oil at improving blood lipids, and OO had little effect. It is important to note that linseed oil has the highest PUFA to SFA ratio among these oils making it difficult to say if n-3 PUFA is the cause of these lipid lowering benefits or if it is the ratio of the components making up the respective oils that are exerting the beneficial effects. While n-3 PUFAs are a major component of this oil it is still difficult to determine if n-3 PUFAS in plant oils have a stronger lipid lowering effect than n-6 PUFAs. A different study in healthy men showed no difference in blood lipid responses between n-3 vs. n-6 PUFA (203); however, more research in this area is warranted before definitive conclusions can be drawn.

In this discussion of physiological responses following PUFA consumption, it is important to discuss the common stigma that n-6 PUFA intake increases inflammation. Recent

research has largely disproved this theory. The proposed mechanism behind this theory was the consumption of LA would promote the formation of arachidonic acid (AA, 20:4n6) which is a precursor for eicosanoid biosynthesis (204). Interestingly, in healthy young males, consumption of high AA diets directly did not have significant effects on blood lipids nor show other obvious adverse health effect (205). Also, dietary LA is not efficiently converted to AA nor directly influence plasma AA (206). Therefore, it is not surprising that a 2019 meta-analysis showed no association between LA intake and mortality risk. In fact, higher LA intake was associated with modestly lower risk of mortality from all causes of death, CVD, and even cancer (207). Further, recent work has analyzed inflammatory cytokine responses to CSO in healthy males and found a suppression in TNF-α after only 5 days (208). This highlights the potential of CSO to influence a primary underlying mechanism of CVD progression.

Cottonseed Oil (CSO) and blood lipids

CSO is a unique oil in that it has a high PUFA content as well as a relatively high SFA content. The fatty acid profile of CSO is 26% SFA, 20% MUFAs, and 55% PUFAs (209). More specifically, CSO is composed of primarily LA (53%), palmitic acid (24%) and OA (18%) (140). CSO is further proven to be unique in that even after the refining process, it has been found to contain trace amounts of dihydrosterulic acid (DHSA) (140, 210). DHSA is of note as it is a cyclopropyl intermediate in the synthesis of sterulic acid, which is a cyclopropyl fatty acid known to inhibit the hepatic lipogenic enzyme, stearoyl-CoA desaturase-1 (SCD1) potentially mitigating hepatic lipid accumulation in response to excessive dietary fat (140).

With new evidence constantly emerging about fatty acid and oil specific effects on blood lipids and cardiovascular health, CSO may be a unique dietary fat source for the modulation of cardiovascular health. Studies done in rodent models have shown a promising and unexpected

ability of CSO to lower cholesterol despite its relatively high SFA content. Radcliffe et. al. compared CSO to corn oil, both rich in LA, in a rat model. This study observed CSO lower total, LDL, and HDL cholesterol more than corn oil and postulated the potential for this unexpected lipid lowering effect to be due to the non-saponifiable portion of the oil (211).

CSO was later examined in mice by Patton et. al. who confirmed the presence of DHSA in the oil (140). DHSA is a cyclopropanyl intermediate in the synthesis of sterulic acid, a known inhibitor of Stearoyl-CoA desaturase-1 (SCD1) as mentioned before. More specifically, SCD1 catalyzes the desaturation of a range of SFAs to endogenously synthesize MUFAs, promoting the storage of lipid rather than the oxidation of it (212). Paton et. al. also showed the expression of SCD1 to increase in the livers of CSO fed mice without accompaniment of a reduction in SFAs, further indicating an inhibition of SCD1 (140).

In a human model, only two trials have studied the effect of CSO on blood lipids. The first was performed by Davis et. al. and was a simple 1-week partial outpatient feeding trial in young healthy men and women (210). Thirty-five grams of CSO was provided through carrot muffins, which were sent home to be consumed by the participants, as well as an in-lab lunch, which provided another sixty grams of CSO during the 5 days of the work week. After this one week of supplementation, significant reductions in total and LDL cholesterol were observed (210). The most recent CSO feeding trial was performed by Polley et. al. in young healthy men as a crossover outpatient feeding trial with OO as a comparator providing 44% of total energy as the respective oil (213). CSO was shown to significantly decrease TC, LDL cholesterol and triglycerides when compared to baseline as well as increase HDL cholesterol. OO had no significant results in these measures but showed a trend for decreasing triglycerides (213). In this study, CSO showed very favorable effects in altering blood lipid profiles and emphasizes the

potential of CSO as a dietary intervention to favorably modify blood lipids. To explore potential mechanisms behind the lipid lowering effect of CSO in humans, plasma palmitoleic acid (16:1n9) was measured as a marker of endogenous desaturase activity. 16:1n9 was significantly reduced post CSO intervention at fasting and postprandially indicating a reduction in endogenous desaturase activity in men, which mirrors the hypothesized mechanism presented by Paton et. al. (213). No significant differences in 16:1n9 were found with OO treatment (213). The observed lipid lowering effects, though understudied, have gained interest resulting in a scoping review of CSO trials, and used the Katan equation to predict the ideal dose of CSO required to see the most favorable lipid lowering (214). This displays the interest results of CSO are generating but it is important to note limitations of the literature to date. CSO has not been tested for its effects on blood lipids in longer interventions, at doses lower than 30% of energy needs, or in adults displaying poor or less healthy lipid profiles before this dissertation.

Energy Balance and the Influence of Dietary Fats

Obesity, which has previously been described as a major risk factor for CVD and comorbidity influencing other chronic diseases, is the result of energy imbalance. Energy balance is modulated by energy intake (EI) and energy expenditure (EE). Appetite regulation is known to affect EI, and fasting and postprandial metabolism is known to affect EE. Therefore, in this study, we have explored appetite and metabolic measurements as a means of indirectly assessing energy balance. Dysregulation of EI or EE can occur resulting in energy imbalance and either weight gain or weight loss (215). Early hypotheses suggested that the energy imbalance of obesity was due to a reduced metabolic rate or "slow metabolism" allowing the individual to consume more calories than they were expending thus gaining weight. The evidence for this hypothesis was that self-reported EI was not different than lean controls. This was later proven to

be false due to significant underreporting of EI in obese individuals, revealing the presence of over-consumption (216). It is now known that increased adiposity is associated with a greater overall EE likely driven by an increased basal metabolic rate in part due to a concurrent increase in fat free mass with weight gain (217). Obese individuals also expend more energy performing the same work or physical activity as lean individuals (218, 219). This evidence is combined with the repeated observation that obese subjects spend more time being sedentary than lean adults in multiple trials previously reviewed (217), suggesting that the imbalance of energy resulting in obesity is related to a sedentary lifestyle and over-consumption of energy.

Energy Metabolism

Modulation of EI and EE has classically been proposed to be in a homeostatic effort to maintain a set body weight, fat store, or amino acid pool (220). A more integrated hypothesis presented by Flatt suggests that the biological component that appetite and metabolism are homeostatically maintaining is glycogen stores (221). Glycogen stores are highly controlled, very from person to person, and are influenced by environmental conditions. Flatt found that glycogen stores tended to be higher in individuals on high carbohydrate diets, and lower in those on low carbohydrate diets (222). Flatt also demonstrated that when glycogen is maintained at increasing ranges, daily fat oxidation conceivably decreases, resulting in fat accumulation (223). The Flatt hypothesis suggests that macronutrient composition of the diet can modulate glycogen stores exerting influence on substrate oxidation subsequently affecting fat mass. In the context of weight change, a decrease in glycogen store such as is common in weight loss will promote orexigenic signals and decrease substrate oxidation in an effort to replenish glycogen reserves. This homeostatic response can result in increased EI in excess of needs, and lead to expanded fat stores. Due to the inability of fats to be converted to carbohydrates in the body, fat stores are

unable to compensate for the depletion of carbohydrate resulting in the biological drive to consume carbohydrates and Calories (224). Therefore, when studying metabolism, it is important to not only look at components of energy metabolism, but also at substrate oxidation.

Appetite and metabolism do change as body weight status changes, with some changes exceeding physiological expectations, especially with weight loss. This is evident in that as many as 80% of individuals relapse after weight loss, likely due to adaptations or modifications of metabolic, neuro-endocrine, autonomic and behavioral responses that oppose maintenance of the reduced weight state (225). Energy restrictions such as those associated with weight loss regimens have been shown to result in a reduced resting energy expenditure more than would have been predicted by the loss of weight (226). This attenuation of EE following acute and/or long-term underfeeding is termed adaptive thermogenesis (227). The reduce energy requirements of individuals as they lose weight contribute to the difficulty in progressively losing weight and results in the "plateau" phase or stalling of weight loss that is a common challenge to the efforts of many. Thermogenic adaptation in post-obese individuals has been shown to have a significant positive relationship with increased levels of hunger (227). Taking this phenomenon of reduced EE with an increase in feelings of hunger and placing it in the obesogenic environment of modern western society, we can conceptualize the cycle of weight remission of popular concern post-weight loss. Weight maintenance is extremely difficult post-obesity, highlighting the need for novel strategies of metabolic and satiety modulation to promote weight maintenance and avoid excessive weight gain. The following sections will explain metabolic and satiety mechanisms as well as the influences of dietary fats on theses homeostatic controls.

Effect of Dietary Fat Composition on Energy Metabolism

EE is the sum of energy converted to work and heat in the body (228). Indirect calorimetry (IC) is a method of calculating heat production (EE) from the measurement of the rates of oxygen consumption (VO_2) and carbon dioxide production (VCO_2) rather than directly measuring heat production (direct calorimetry) (228, 229). By using IC we are able to measure resting metabolic rate (RMR) as well as diet induced thermogenesis (DIT). DIT is the measure of the change in postprandial EE per kcal ingested to capture the thermic effect of a meal (228, 230). From a meal, energy is provided to the body in a chemical form from the macronutrients found in food: carbohydrates, protein, and fats. Because the chemical reactions for the combustion or breakdown of the different macronutrients consume and produce different ratios of O_2 to CO_2, it is possible to determine the percent of EE supported by each of these substrates. Substrate oxidation rates are calculated using the respiratory exchange ratio (RER) of VCO_2 and VO_2 (228, 229). The breakdown of one molecule of sugar can be represented by $C_6H_{10}O_6 + 6O_2 \rightarrow 6CO_2 + 6H_2O$. From this equation we can see that 6 molecules of CO_2 are produced and 6 molecules of O_2 are consumed. Therefor the RER for sugar (carbohydrates) is 1.0. The same analysis can be done for fats. The breakdown of one molecule of fat can be expressed by $C_{57}H_{104}0_6 + 80O_2 \rightarrow 57CO_2 + 52H_2O$. For fats, 57 CO_2 molecules are produced while 80 O_2 molecules are consumed resulting in an RER of 0.71. Protein complicates the calculation of substrate oxidation rates with RER due to the heterogeneity of structures from various amino acids leaving a variety of combustion equations that can result from the digestion of protein. Knowing that protein is 16% nitrogen by weight, urinary nitrogen can be measured to calculate protein oxidation (228). Using the knowledge that in healthy people protein oxidation rate does not typically fluctuate with dietary intake in the same manner as fat and carbohydrate oxidation

rates do, the Wier equation is used with IC to account for the protein oxidation rate allowing for the calculation of fat and carbohydrate oxidation rates from a non-protein RER (224, 229).

To understand how fatty acids affect measures of metabolism acutely and over time, both acute meal challenges and feeding trials are used. There is quite a bit of heterogeneity in the literature, pointing to a need to further explore the question of how dietary fat affects energy expenditure and substrate utilization. In most studies, the comparison is made between SFS vs. unsaturated fats, namely MUFAs. Far fewer studies show direct comparisons between PUFA vs. SFA, and even fewer compare all 3 categories of FA.

Eleven studies (6 acute, 5 longer-term) have examined responses of SFA treatments compared MUFA (231-241). Three acute meal challenges show a greater fat oxidation rate in response to a MUFA-rich vs. SFA-rich meal (238-240), while one showed no difference (241). There were no differences in fat oxidation rates with longer MUFA supplementation in three trials (231, 236, 237), yet one reported improved postprandial fat oxidation with a high MFUA diet (235). An increased EE on a MUFA diet compared to an SFA diet was shown in only two studies (233, 235), while three other studies showed no difference on EE (234, 236, 237). Significantly greater DIT in response to MFUAs compared to SFAs have been reported in three trials in both lean and obese individuals (233, 238, 240) while two studies reported no differences (232, 241). In general, when significant differences have been observed in comparison of MFUA or SFA interventions, MUFA has shown potential to be more beneficial than SFA on measures of metabolism but more research is needed to elucidate the exact relationship.

Fewer studies have compared PUFA-rich vs. SFA-rich meals or diets without also including a MUFA group. To date, no acute studies and only five intervention studies have been done

comparing PUFA vs. SFA without also including a MUFA treatment (242-246). Two of these studies reported higher RMR after PUFA-rich diets (242, 246); all other studies saw no difference. Four out of these 5 studies saw improved postprandial fat oxidation after a PUFA rich diet (242-245). Interestingly, in one of those studies Jones et. al. only found the higher fat oxidation response to a PUFA-enriched diet in obese vs. non-obese individuals which may point to a modulation of PUFA fat oxidation based on weight or adiposity (244). One study showed improved DIT with a high PUFA diet (246), yet PUFA-enriched diets have not been shown to affect EE significantly compared to SFA (244, 245). Based on these data, PUFAs have been shown to have a potentially beneficial effect on metabolism with the most evidence pointing towards improved postprandial fat oxidation with a high PUFA diet, but there is not enough information to make any clear determinations.

Seven studies have compared MUFAs, PUFAs, and SFAs in the same intervention, all of which were acute meal challenges. Two of these studies required participants to maintain energy balance for the duration of the study (247-253). DIT in response to a HF PUFA meal was greater than that of HF MUFA or SFA meals in normal weight women in one study, but this observation was not significant in obese women in a second study (247, 248). Another study examined postprandial responses to specific FAs and determined that medium chain FAs were oxidized more than longer chain FAs regardless of saturation. Among the longer chain FAs, they observed PUFAs to be oxidized more than MUFAs, and SFAs less than either (249). Conversely, Casas-Agusench et. al. observed no differences in postprandial fat oxidation between isocaloric PUFA, MUFA, or SFA rich meals, but reported a greater DIT in response to PUFA and MUFA meals compared to SFA with no significant differences between PUFA and MUFA (251). To confuse things even further, oxidation rates determined by stable isotope found fat oxidation of MUFAs

to be greater than PUFAs, which was greater than SFA in one study (250). In summary, 4 out of the 7 studies that included PUFA, MUFA, and SFA treatments in their protocols did not see significant differences of fat oxidation with any treatment. Two of the trials that did see significance are in agreement that SFAs are oxidized at a slower rate than MUFAs and PUFAs, but disagree on whether MUFAs or PUFAs are more favorably oxidized (249, 250). SFAs show less of a DIT response than PUFAs and MUFAs in 2 studies, but these two studies also do not agree on weather PUFAs induce a greater DIT than MUFAs or if there is no difference between MUFAs and PUFAs (247, 251). Further, two trials contradict this showing SFA to have similar DIT as long chain n-3 PUFAs and greater DIT than n-6 PUFAs or MUFAs (252, 253). Based on these studies SFAs may have a detrimental effect on fat oxidation and DIT compared to unsaturated fats but more investigation is needed. The present evidence is also unclear as to whether PUFAs or MUFAs more favorably affect metabolic outcomes requiring more direct investigation between the two.

As mentioned above, rarely are MUFA and PUFA compared against each other without also including a SFA treatment group. To date, only three studies have compared PUFAs and MUFAs directly to study their differential effects on measures of metabolism. The first was performed by Jones et. al. and involved measuring the responses of 15 normal weight men to meals rich in OO (MUFA-rich), sunflower oil (n6 PUFA-rich), or flaxseed oil (n3 PUFA-rich). OO demonstrated a higher DIT than flaxseed oil (rich in ALA) and a trend toward being greater than sunflower oil (rich in LA). There were no significant differences in fat oxidation observed (254). In a different study, Logan et. al. compared fish oil supplementation (n3 PUFA) with a placebo OO treatment for 12 weeks in older women. The fish oil treatment resulted in an increased RMR, EE during exercise, and fat oxidation rate in both resting and exercised states compared to the OO treatment

(255). Finally, the most recent comparison of MUFA- and PUFA-enriched diets on metabolism was performed in our lab by Polley et. al. in healthy young men using CSO and OO for PUFA and MUFA treatments, respectively. Responses to acute CSO and OO meal challenges revealed a greater RER and subsequent carbohydrate oxidation rate in response to the CSO meal, while OO elicited a greater fat oxidation rate and DIT acutely. However, after the 5-day interventions, carbohydrate oxidation rate and RER were decreased more with the CSO intervention than the OO intervention with a corresponding significantly greater increase in fat oxidation. This suggests that CSO may promote the oxidation of fats over carbohydrates which would be beneficial for weight maintenance over time, but not acutely (256). This observation could be explained by the inhibitory action of DHSA on SCD1 preventing the storage of dietary fatty acids and promoting their oxidation as explained by Paton et. al (140). Many questions still remain such as if this promotion of fat oxidation with CSO supplementation would still be evident in an older population including females despite the probability of already reduced rates of fat oxidation (257, 258). The following chapters of this dissertation address the question of age, and the effects of a longer interventions of high MUFA and PUFA diets on metabolism.

Appetite Regulation

Satiation (the process that leads to the termination of eating) and satiety (the feeling of fullness that persists after eating) are signaled by neuropeptide hormones and peripheral organ innervation, influencing both quantity and frequency of ingestion (259). It is hypothesized that despite complex biological controls of EI and EE, the stimuli of our current obesogenic environment, including extreme palatability of foods, poor sleep habits, distraction during meal times, and excessive cognitive demands, override appetite control permitting chronic excessive EI (220). In fact, palatability of meals undermines satiation leading to as much as 44% increase

in meal size compared to neutral and unpalatable meals (260). Increases in meal/ingestion frequency as influenced by daily snacking, has also been shown to increase EI with little effect on satiety resulting in increased EI (261). This evidence reinforces the hypothesis that excessive weight gain common in today's society is not driven by reduced metabolic rate but by increased EI.

Neural control of hunger and satiety occurs primarily in the arcuate nucleus (ARC) of the hypothalamus and the dorsal vagal complex (DVC) in the brain stem (262). In the ARC, there are two types of opposing neurons believed to control feeding behavior: the orexigenic neuropeptide Y/angouti-related peptide (NPY/AgRP) neurons, and the anorexigenic pro-opiomelanocortin/cocaine-and amphetamine-regulated transcript (POMC/CART) neurons (263). The location of the ARC (ventral to the third ventricle) is prime positioning for integrating endocrine and exogenous signals because it allows for neuroendocrine signaling via hormonal crossing of the blood brain barrier (264). Both NPY/AgRP and POMC/CART neurons are sensitive to hormones that signal energy balance (263). These two families of neurons are able to inhibit each other to control appetite stimulation as follows. In a low energy state (hunger) ghrelin stimulates NPY/AgRP neurons to inhibit melanocortin 4 receptor (MC4R) expressing neurons and activate non-MC4R expressing neurons in the paraventricular hypothalamic nucleus (PVH) via release of NPY, ϒ-aminobutyric acid (GABA) and AgRP (263, 264). This signal then stimulates neurons in the nucleus of the solitary tract (NTS), which resides in the DVC, along with vagal inputs that are hypothesized to excite GABA neurons in the intermediate and parvicellular reticular nuclei (IRt/PCRt) of the medulla oblongata to drive hunger responses (265). NPY/AgRP neurons are also know to extend to the lateral hypothalamus (LH) which is also nicknamed the "feeding center", the stimulation of which has shown strong stimulatory

effects on feeding behavior (263). The vagus nerve innervates the gut both afferently and efferently, allowing for neural communication of nutritional status and integrates at the DVC to affect hunger and satiety signaling (266). In high energy (satiated) conditions, POMC/CART neurons are stimulated to excite MC4R expressing neurons in the PVC by the release of α-melanocyte stimulating hormone (α-MSH), which activates an inhibitory pathway in the lateral parabrachial nucleus (LPB) to the IRt/PCRt which counteracts the hunger response circuit. Stimulatory signals (neuro-hormones such as leptin) of the POMC/CART neurons also inhibit NPY/AgRP neurons halting the stimulus of the hunger stimulating pathway. This is a brief overview of the complex neural pathways that integrate both homeostatic and hedonic signals to determine their influences on feeding behavior.

There are more than 30 hormones identified and implicated in appetite regulation. For the purposes of this document, we will briefly discuss five key hormones then review the current research of appetite responses to FAs of varying saturation.

Ghrelin is regarded as the only known peripheral orexigenic (hunger) hormone (267). Its release from p/D1 cells in the fundus of the stomach in humans is stimulated by norepinephrine via spinal efferent fibers in low energy stats. Ghrelin activates NPY/AgRP neurons after acylation via ghrelin-receptor (GHSR-1a) to stimulate hunger and promote feeding behaviors as discussed above (268). In terms of hedonic appetite stimulation, activation of the hypothalamus, to the medial prefrontal cortex, LH, to the dorsal motor nucleus of the vagus (also in the DVC), down through the vagus nerve to stimulate ghrelin release at the stomach (268). GLP-1 and somatostatin have been shown to inhibit ghrelin secretion (269, 270). Ghrelin is elevated in the fasting state, given its orexigenic responses, and decreases to baseline levels within the first hour after a meal (271).

The remaining hormones are anorexigenic or satiety hormones. Peptide YY (PYY) is released from L-cells in the lower gastrointestinal tract particularly the ileum and colon. It exists in two forms: PYY_{1-36} and PYY_{3-36}, with the 3-36 being the active form of the hormone. PYY_{1-36} binds and activates all five y receptors while PYY_{3-36} only binds the Y2 receptor. This is important because the Y receptors are the mechanism through which PYY acts on the NPY/AgRP neurons in an inhibitory manner to exert its satiating effects. As a satiety hormone, PYY concentrations are low during fasting and increase within 15 minutes postprandially (272). Release of PYY is thought to initially be a neural or endocrine stimulation related to the cephalic phase response as the initial secretion occurs before nutrients can reach the intestinal L-cells. Once nutrients reach the L-cells of the GI tract, PYY responses increase. Additionally, PPY is released in response to gastric acid, cholecystokinin (CCK), and bile acid infusion into the ileum or colon, yet gastrin exerts an inhibitory effect (273, 274). PYY also slows gastric emptying and gastrointestinal motility, inhibits the secretion of gastric acid, dampens gallbladder contractions, and reduces secretion of pancreatic exocrine enzymes (275).

Glucagon-like peptide (GLP-1) is co-released from L-cells of the lower intestines with PYY (276). GLP-1 levels are low in a fasted state and increase in response to nutrients entering the gastrointestinal tract, especially carbohydrates and fats (277, 278). The secretion of GLP-1 is known to be bi-phasic with a short early phase beginning 10-15 minutes after ingestion, then a more sustained later phase occurs 30 – 60 minutes following a meal (278). It is quickly degraded by the enzyme dipeptidyl peptidase IV (DPP-IV) resulting in a short half-life in circulation (279). It's major actions are its stimulation of insulin release from beta cells of the pancreas and inhibition of glucagon secretion from alpha cells (276). GLP-1 also exerts satiating signals on the brain through a still debated mechanism. It is known to have receptors in many areas of the

brain, including the NTS and the PVN and has demonstrated evidence of activating the CART/POMC neural pathway of satiety (280, 281). Because of its clear effects on the pancreas, GLP-1 receptors have been targeted in type 2 diabetes therapy.

Cholecystokinin (CCK) is released from intestinal I-cells in response to dietary lipid and protein via GPR40 G protein coupled receptors and calcium-sensing receptors (282). CCK exerts its control on nutrient delivery to the small intestine via stimulation of the CART/POMC neurons, signaling to inhibit food intake and slow gastric emptying promoting feelings of satiety. Somatically, CCK targets vagal afferent neurons and regulates the expression of receptors and peptide neurotransmitters by these neurons to further amplify signals of satiety (282). CCK levels raise from basal gradually over 10-30 minutes after initiation of a meal then remain elevated for as long as 3-5 hours before gradually returning to basal levels (283). The satiating effects of CCK are dependent on the action of leptin, indicating its regulation according to energy status. For example, in states of energy deprivation, leptin signaling will be low (signaling starvation), and attenuate the satiating effects of CCK (284). The inverse is also true. CCK is also known to influence the release of other hormones including PYY, GLP-1, PP, and ghrelin (274, 285-287).

The main action of insulin is the stimulation of glucose uptake by peripheral cells; however, insulin may have a role in appetite regulation. Insulin is released from β cells in the pancreas in response to ingestion of a meal, particularly in response to increased blood glucose levels, and returns to basal levels in the fasted state. When stimulated, insulin secretion rates follows a biphasic pattern with a greater relative rate of secretion occurring within ~5 minutes of the increase in blood glucose then a settling of insulin secretion rates after about 10 minutes (288). Insulin's role in appetite regulation is as a signal of adiposity. Insulin circulates in levels

according to fat mass (289). In states of increased fat mass there is increased insulin expression (along with leptin) increasing its action on the ARC (290). Insulin is known to stimulate CART/POMC neurons and inhibit NPY/AgRP neurons promoting anorexic behaviors. With reduced fat mass, insulin expression is reduced resulting in activation of NPY/AgRP neurons and inhibition of the CART/POMC neurons, promoting food intake behaviors (262). The hallmark deduction in insulin sensitivity with excessive weight gain may lead to the reduction of insulin's satiating effects, producing an additional conceivable mechanism for the excessive weight gain from over consumption promoting the maintenance of an obese state.

Effect of Dietary Fat Composition on Appetite Regulation

A total of 17 acute studies have assessed the effects of FA saturation on measures of appetite in humans. 12 of these studies compared acute MUFA, PUFA and SFA treatments, 4 compared MUFA to SFA, and only one compared MFUA to PFUA directly. All of these studies were crossover design, but the mode of delivery varied as well as source and quantity of FA in the acute treatments. Two studies used ileal (291) or duodenal (292) infusions and one used an oral fat feeding with intravenous heparin infusion (293) rather than traditional meal challenges. This results in a great amount of heterogeneity between the studies, complicating the interpretation.

Thirteen of 17 acute studies also collected subjective appetite ratings (294, 295), 10 of which used visual analog scale (VAS) questionnaires (232, 241, 251, 291, 292, 296-300). VAS questionnaires are a method to evaluate subjective or behavioral characteristics such as feelings of hunger or fullness. They correlate with, but do not reliably predict, EI such that they can be used as a proxy. They are able to predict meal initiation and amount eaten and are sensitive to experimental manipulations (301). It is noted that they are the most reliable for within subject analysis making them useful for assessing changes in feelings related to appetite as affected by

these FA interventions. Only four of these acute studies observed differences in subjective appetite with different FA saturations in a meal. SFA has been shown to increase fullness compared to MUFA and PUFA meals (298), and MUFAs have been shown to decrease fullness, and increase prospective consumption along with desire to eat compared to PUFA and SFA meals (297). In contrast, SFAs have also been shown to decrease fullness and satiation and increase hunger compared to MUFAs alone (241). When compared to MUFAs, PUFAs have been shown to increase fullness, while decreasing desire to eat, prospective consumption, and preoccupation with food, but only at 240 minutes postprandially (300). Only one third of the studies collecting data on subjective appetite have observed any differences with FA composition of the test meal, and these responses are not consistent. All four of these studies have different modes of delivery of test fats ranging from ileal infusion to liquid meal, to mixed meals. At this time, there is not enough information to determine the relationship of FA composition and subjective appetite responses from an acute exposure, highlighting the need for more research of homogeneous methods on this subject.

Nine of 17 acute meal challenge studies collected EI as a measure of appetite, 8 of these using some form of ad libitum buffet (232, 251, 291, 292, 295-297), 3 (294) using food records, and 2 studies using a combination of the two (297). In-lab measures of EI, such as the ad libitum buffet, should be interpreted with caution as the foods presented are limited to what the researchers are providing in both nutrient and energy density, and do not mimic free living conditions. It should also be noted that buffet style meals tend to promote overconsumption, in conjunction with offering the subject as much free food as they can eat creating a situation that is likely to influence the measure of EI to not accurately represent free-living conditions (302, 303). Despite potential differences from free-living conditions, this measure can provide useful

information as a response to a previously consumed meal challenge in a repeated measures study design, allowing an estimation of changes in EI related to appetite for a within subject analysis. Two of the acute studies found significant differences in EI after different FA meals, both showing that EI increased more following a MUFA- vs. SFA-rich meal (292, 297). This is not strong evidence as this is only two studies and the other 6 found no differences between types of FAs; however, this preliminary work shows that MUFAs may be the least satiating.

Nine of the acute studies measured hunger and satiety hormones although not all primary hormones of interest (ghrelin, CCK, PYY, GLP-1, and insulin) were measured in every study (291-293, 298-300, 304-306). Two studies found no effect of FA composition on insulin (299, 305) or ghrelin (299, 304). However, one observed an increased insulin response with MUFA meal compared to both PUFA and SFA meals (293). Another saw a greater reduction after a MUFA- vs. PUFA-rich meal in the first 60 minutes postprandially, but overall there was no difference in iAUC between the treatments (300). Similar inconsistent findings have been reported for PYY. Two studies found no differences based on FA composition (291, 306), while two studies reported opposing findings. Kozimor et. al. reported a lower PYY AUC after a MUFA liquid meal compared to PUFA and SFA meals (298), while Feltrin et. al. reported a greater PYY response with a MUFA meal (292) but only compares to a SFA meal. For CCK responses, 3 of 4 studies reported no significant effect of FA composition (291, 299, 306) while one reported a greater CCK response to a MUFA vs. SFA intraduodenal infusion (292). This should be interpreted with caution, however, as direct intraduodenal infusions may be amplifying the CCK response, as other modes of meal delivery are not able to detect differences. Finally, of the 5 studies measuring GLP-1, 3 showed MUFA meals to have a greater GLP-1 response compared to either SFA and PUFA or just SFA meals (293, 305, 306). The other 2 studies found

no differences based on FA composition. Taken together, significance appears to most commonly be observed in relation to the MUFA treatment in these acute intervention studies. This data suggests that MUFAs may elicit stronger satiety based on some hormone responses, but these results are inconsistent and not robust enough to be able to clearly determine. These are also contradictory of the EI and perceived appetite measures discussed above that suggest MUFA meals could elicit greater feelings of hunger and greater EI, although they do align with one study showing postprandially PYY to be the lowest following a MUFA-rich meal (298). The responses of PUFAs are even less clear and requires more research.

To date, six studies have assessed longer-term effects of dietary FA composition on appetite regulation. Five of those 6 studies measured subjective appetite using VAS with 3 showing no effect of FA composition in the diet (307-309). The 2 studies that did find significance showed PUFAs to have greater fullness (310) and decreased hunger (311) compared to SFA and MUFA. Three of those studies also measured EI, 2 using an ad libitum buffet (308, 311), and 1 using 24 hour recall (310), none of which had significant results. While not a robust analysis, perceived suppression of appetite has been observed with PUFA diet supplementation without measurable differences in EI. Relationships of MUFAs vs. SFAs remain to be elucidated.

At least one appetite hormone was measured in 5 of those 6 studies as well. Insulin was not affected by FA type (307, 309), and 2 studies found no effect on ghrelin as well (309, 312). Conversely, two other studies reported that a high PUFA diet decreased fasting (307) or postprandial ghrelin responses (311) compared to MUFA treatments. For PYY measures, 2 studies saw no differences (309, 311) while the third observed an increase in both fasting and postprandial PYY responses after a PUFA-rich diet compared to a SFA and MUFA mixed control (307). Finally, only 1 study measured CCK and found a greater postprandial response in

the PUFA treatment compared to the MUFA treatment (311). Based on this information, PUFA-rich diets may be beneficial for longer term appetite suppression based on both these hormone data and the aforementioned VAS data that also reported perceived increases in fullness and decreases in hunger after a PUFA rich diet. This highlights the need to continue studying PUFA supplementation or PUFA diet enrichment as a mode of appetite suppression to determine its clinical relevance and potential use in preventing obesity and weight gain, which we have discussed as being related to over consumption relative to EE.

When specifically examining the potential appetite differences between CSO vs. OO, only one study has been published. In a 5-day outpatient feeding trial, researchers observed a postprandial decrease in ghrelin and increase in CCK as well as lower feelings of hunger after a CSO-enriched (PUFA rich) diet intervention compared to an OO-enriched (MUFA rich) diet in normal weight men (311). There were no significant treatment effects found for the OO-enriched diet in either subjective feelings of appetite or appetite hormones (311). This initial study brings up the possibility of CSO's ability to potentially suppress hunger both subjectively and physiologically, which could have important clinical implications for appetite regulation and weight management. However, more research is needed on a longer-term diet as well as in different populations more at risk for weight gain and obesity.

Methodology Used in Study Design

Indirect Calorimetry (IC)

Indirect calorimetry (IC) functions on the principle of heat (energy) released by oxidative processes of metabolism, and can be calculated from the measurement of oxygen consumption and carbon dioxide production. IC is in contrast with direct calorimetry, which measures heat

losses of the subject directly but is unable to provide indications of substrate utilization. Resting metabolic rate is calculated in the IC method through the use of the Weir equation (313).

More specifically, the equation for total heat output in a given time is:

$$Total\ kcal = 3.9 * L\ O_2\ used + 1.1 * L\ CO_2\ produced$$

In this equation, protein is assumed to produce 12.5% of total energy expenditure at any one time. Substrate utilization is also calculated from the respiratory quotient as described by Frayn (314).

$$Fat\ oxidation\ \left(\frac{g}{min}\right) = \left(1.67 \times VO_2 \frac{L}{min}\right) - \left(1.67 \times VCO_2 \frac{L}{min}\right)$$

$$Carbohydrate\ oxidation \left(\frac{g}{min}\right) = \left(4.56 \times VCO_2 \frac{L}{min}\right) - \left(3.21 \times VO_2 \frac{L}{min}\right)$$

Blood Lipid Quantitative Measurements

Spectrophotometry is a method of quantitative analysis that measures the intensity of light after it passes through the sample to determine the concentration of the sample. This is based on the principle that every chemical compound absorbs, transmits, and reflects light within a certain wavelength. Knowing the path length and molar absorptivity of the molecule of interest, the absorbance of the sample measured can be used to calculate the concentration of the sample using Beer's Law (315). Spectrophotometry is used to measure TC, HDL, and non HLD-c.

Subclasses of lipoproteins are directly measured using ion mobility lipoprotein fractionation. In this process, a gas phase electrophoresis is used to separate unmodified and ionized lipoproteins based on size and electrical field. As the particles leave the separation chamber, they are each directly detected and counted. The lipoproteins migrate across the

laminar flow of the gas-phase as the voltage of the electric field of the separation chamber is ramped up incrementally allowing only one size and charge of lipoprotein to exit the chamber at a time. This method is used to measure small, medium and total LDL particle numbers, LDL peak size, LDL pattern, and large HDL particle number (Quest Diagnostics, Secaucus, NJ).

Enzymatic assays

Triglycerides and Non-Esterified Fatty Acids (NEFA) are measured by an enzymatic method (FUJIFILM Wako Diagnostics, Mountain View, CA). Briefly the triglyceride assay utilizes the enzymes glycerol kinase (GK), glycerol-3-phosphate oxidase (GPO), and catalase the decomposition of free glycerol in the sample to O_2 and H_2O. Then the sample is treated with LPL, GK, and GPO to oxidize the glycerol of the remaining triglycerides to hydrogen peroxide, which causes N-(3-sulfopropyl)-3-methoxy-5-methylanine (HMMPS) to undergo a condensation reaction, producing a blue color that is measured for absorbance to determine concentration of triglycerides. At the same time, a standard curve is prepared using serial dilutions of known quantiles of triglyceride and treated in the same manner as the unknown samples. The responses from the standard samples are graphed for the development of a standard curve equation (y=mx+b), allowing for the calculation of the concentrations of triglyceride present in the unknown samples. Standard curves are commonly used in many assay methods for the determination of the quantity of an analyte based on reaction responses. The NEFA assay utilizes the same principles as the triglyceride assay. The enzymes used in the NEFA assay oxidize the NEFAs in the sample to H_2O_2 for the purposes of initiating a condensation of an organic compound (in this case 3-methyl-N-ethyl-N-(β-hydroxyethyl)-alinine)(MEHA)) with 4-aminoantipyrine to produce a colored product who's optical density can be measured to determine concentration of the molecule of interest. Blood glucose is also measured using this

methodology (oxidation of glucose to produce H_2O_2 then condensation with dimethylaniline and 4-aminoantipyrine to produce a colored product) via an in-house glucose oxidase colorimetric enzymatic assay.

Radioimmunoassay (RIA)

In RIA, the target molecule (antigen) is measured by competitive binding to a specific antibody compared to a radiolabeled form of the antigen. A known amount of the antigen that is labeled with a radioactive isotope, such as I^{125}, will compete for limited binding sites on an introduced antibody with the unlabeled antigen that is present in the sample. In samples with greater concentrations of unlabeled antigen, less of the labeled antigen will be able to bind to the antibody. After incubation, a reagent is added to precipitate the bound reagents out of solution allowing for the separation of the bound and unbound antigens. When the unbound particles in solution are removed, a measurement of the radioactivity of the remaining bound sample can be used to calculate the concentration of the unlabeled antigen that was present in the sample.

In addition to the use of radiolabeled antigens, the RIA method requires additional control tubes. First, the total count tube is a measure of the I^{125} labeled antigen to confirm the quantity and quality of the labeled antigen. Second, non-specific binding tubes are a combination of labeled antigen and assay buffer that are processed as described above to estimate the "background noise" or the quantity of antigen that is remining in the tubes after precipitation not due to binding to the antibody. Third is the total binding tube (B0) which is a combination of the labeled antigen, assay buffer, and the antibody, measuring the total amount of labeled antigen that will bind without competition from the unlabeled antigen (sample). To calculate all standards and samples, the NSB is subtracted from the response and a percentage of tracer bound is calculated by dividing the difference by the B0. The quotients generated from the standard

points are plotted to generate the standard equation used to convert the quotients of unknowns to concentrations. In this study, RIAs were used to measure Insulin Ghrelin, PYY at fasting and at intermittent postprandial time points (EDM Millipore St. Louis, MO).

ELISAs

Enzyme Linked Immunosorbent Assays (ELISAs) use similar principals of binding the target antigen as RIAs. In ELISAs the target antigen is immobilized on to the surface of the microplate by binding to the capture antibody. A detection antibody then complexes with the captured antigen. This detection antibody is biotinylated before use in the assay, allowing streptavidin horseradish peroxidase to bind and serve as the reporter enzyme. To quantify the amount of substrate bound, a 1:1 mixture of H_2O_2 and tetramethybenzidine (TMB) are added which are condensed in the presence of the peroxidase enzyme, producing a colored product similar to the reaction described for enzymatic assays. The optical densities of the standards and samples are measured and the unknown concentrations of antigen are calculated based on a standard curve. The described mechanism is a "sandwich" ELISA which is similar to another method known as a competitive ELISA. Instead of a detection antibody, a competitive ELISA uses a biotinylated antigen that competes with the antigen in the sample for capture antibody. Further, the captured biotinylated antigen binds to the added streptavidin peroxidase. This way when the same mixture of H_2O_2 and TMB is added to produce the color generating reaction with intensities corresponding to the quantity of biotinylated antigen bound. The capture antibody has the same affinity for the biotinylated antigen and the unlabeled antigen, thus they bind proportional to their differences in concentration, allowing for the calculation of unknown antigen concentrations based on a standard curve prepared in the same manner. (Thermo Fisher Scientific, Waltham, MA). Inflammatory markers (IL-1beta, TNF-α, IL-6, CRP), markers of

coagulation potential (PAI-1, tissue factor) angiopoietin like proteins 3, 4, and 8 and CCK were quantified using ELISAs.

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CHAPTER 3

BLOOD LIPID RESPONSES TO DIETS ENRICHED WITH COTTONSEED OIL COMPARED WITH OLIVE OIL IN ADULTS WITH HIGH CHOLESTEROL IN A RANDOMIZED TRIAL [1]

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Abstract

Background: Increasing unsaturated fat intake is beneficial for cardiovascular health but the type of unsaturated fat to recommend remains equivocal.

Objective: To investigate the effects of an 8-week diet-intervention that was rich in either cottonseed oil (CSO; polyunsaturated fat-rich) or olive oil (OO; monounsaturated fat-rich) on blood lipids in hypercholesterolemic adults.

Methods: 43 men and women with hypercholesterolemia (53±10y, BMI 27.6±4.8kg/m2) completed this randomized parallel clinical trial consisting of an 8-week partial outpatient feeding intervention. Participants were given meals and snacks accounting for ~60% of their daily energy needs, with 30% of energy needs from either CSO (n=21) or OO (n=22). At pre- and post-diet intervention visits, participants consumed a high saturated fat (SFA) meal (35% of total energy needs; 70% of energy from fat). The primary outcomes of fasting cholesterol profiles and secondary outcomes of postprandial blood lipids and glycemic markers were assessed over a 5h period.

Results: There were greater reductions from baseline in fasting serum total cholesterol (TC) (-17.0±3.94 vs.-2.18±3.72mg/dL; p=0.008), LDL cholesterol (-19.7±3.94 vs.-5.72±4.23mg/dL; p=0.018), Non-HDL cholesterol (non-HDL-c) (-20.8±4.00 vs. -6.61±4.01mg/dL;p=0.014), and apolipoprotein B (-11.8±2.37 vs.-3.10±2.99mg/dL; p=0.05), in CSO vs. OO. There were also visit effects for increases in HDL cholesterol (CSO: 56.5±2.79 to 60.2±3.35mg/dL; OO: 59.7±2.63 to 64.1±2.24mg/dL; p<0.001), and decreases in TC/HDL ratio (CSO: 4.30±0.27 to 3.78±0.27; OO: 3.94±0.16 to 3.57±0.11; p<0.001) regardless of group assignment. In response to the high SFA meal, there were differences in postprandial plasma glucose (p=0.003) and triglyceride (p=0.004) responses, and a trend in non-esterified fatty acids (p=0.11), between

groups showing protection in the postprandial state from an occasional high SFA fat meal with CSO, but not OO, diet enrichment.

Conclusions: CSO, but not OO, diet enrichment caused substantial improvements in fasting and postprandial blood lipids, and postprandial glycemia, in hypercholesterolemic adults. This trial was registered at clinicaltrials.gov (NCT04397055).

Introduction

Cardiovascular disease (CVD) is the primary cause of death, representing estimated 17.9 million deaths globally in 2019 (1). A common underlying cause for CVD is the inflammatory condition of atherosclerosis. As one of the early processes of atherosclerosis is the infiltration of the arterial wall by cholesterol, elevated blood lipids (hypercholesterolemia) have been shown to be an independent and primary risk factor for the development of CVD (2). In 2015-2018, the National Health and Nutrition Examination Survey showed that 11.4% of adults had high total cholesterol (TC) and 17.2% had low high-density lipoprotein cholesterol (HDL-c) (3) demonstrating that unfavorable blood lipids are a relatively prevalent condition among U.S adults.

Nutrient intake and diet quality have a significant impact on the development of CVD risk. Dietary fatty acid composition is one component of the diet that has been shown to modulate fasting and postprandial blood lipids. It is well accepted that high saturated fatty acid (SFA) intake has unfavorable effects on blood lipid profiles including measures of cholesterol, triglycerides and related lipoproteins (4). While the strength of this effect may be dependent on the type and amount of SFA consumed (5), higher unsaturated fatty acid intake has been shown by meta-analysis to have favorable impacts on blood lipids (6). Additionally, increasing evidence suggest that polyunsaturated fatty acid (PUFA) intake has a stronger effect on lowering blood lipids compared to monounsaturated fatty acids (MUFA) (7, 8). This may be due in part to the regulatory roles that PUFAs elicit on the expression of genes related to lipid and cholesterol metabolism (9, 10). Importantly, we eat whole foods, rather than individual fatty acids, so studying the impact of specific PUFA- and MUFA-rich whole food sources is necessary.

Cottonseed Oil (CSO) is a rich dietary source of PUFAs, especially the omega 6 (n-6) PUFA, linoleic acid (LA, 18:2n-6) that has been in the food supply and consumed by Americans for over 100 years. Previous studies have shown that CSO-rich diets can improve cholesterol profiles in healthy young adults (11, 12). In addition to a high PUFA content, CSO contains a cyclopropyl fatty acid, dihydrosterulic acid (DHSA), a known inhibitor of the lipogenic enzyme stearoyl-CoA desaturase-1 (SCD1) (13). The combination of the DHSA-mediated inhibition of SCD1, and the regulation of lipogenic and cholesterogenic gene expression by the high PUFA content of CSO, make it a potentially ideal nutritional therapeutic to target cardiovascular health. To date, the cardiovascular benefits of CSO enrichment in the diet has only been shown in healthy adults over a single week. Olive oil (OO) is a rich source of MUFAs (14) that has gained popularity with consumers as a primary component of the Mediterranean diet (15, 16) due to a plethora of substantiated health claims (17). Olive oil is generally considered heart healthy by consumers (18), but it has been shown in meta-analysis to be less effective at lowering blood lipids compared to other plant oils (8). The purpose of this study was to examine the impact of a diet enriched with either CSO or OO for an 8-week period on our primary outcomes of fasting cholesterol profiles and secondary outcomes of postprandial blood lipids following a high SFA meal challenge in adults with hypercholesterolemia. We hypothesized that all measures of fasting and postprandial blood lipids would decrease (and HDL-c increase) following the CSO treatment compared to the OO treatment.

Methods

Study Design

This study was a single-blinded, randomized, parallel design clinical trial. Subjects were randomly assigned to one of two groups, either the CSO group or OO group with allocation ratio

of 1:1, using balanced blocks stratified by TC (desirable through "borderline undesirable" TC ≥ 180 mg/dL, or "undesirable" TC ≥ 240), low-density lipoprotein cholesterol (LDL-c) (desirable through "borderline undesirable" LDL-c ≥ 110 mg/dL, or "undesirable" LDL-c ≥ 160), and body mass index (BMI) ("healthy through overweight" 18.5-29.9 kg/m^2, or "Obese" ≥30 kg/m^2) . Participants were blinded to group assignment. Data collection occurred from May 2018 to June 2021 when the goal of 20 participants/group was obtained. This study included an 8-week diet intervention and 4 study visits: a screening visit (V0), pre-diet intervention visit (V1), mid-diet intervention visit (V2), and a post-diet intervention visit (V3) (**Figure 3.1**). In addition, there were weekly non-testing visits for the purpose of picking up study food and returning containers and paperwork from the previous week. The Institutional Review Board from the University of Georgia approved the study and written informed consent was obtained from all participants prior to beginning study procedures. This trial was registered at clinicaltrials.gov as NCT04397055 on April 30th, 2020.

Participants

Fifty-three sedentary adults between the ages of 30 and 75y with hypercholesterolemia or elevated blood lipids and BMI > 18.5 kg/m^2 were recruited for the study. To be included, an individual's blood lipids at the screening visit had to be "borderline undesirable" or "at risk" in two blood lipid measures or "undesirable" in one. "Borderline undesirable" or "at risk" was defined as TC ≥ 180 mg/dL, LDL-c ≥ 110 mg/dL, HDL-c < 50 mg/dL, or triglycerides (TG) ≥ 130 mg/dL, while "undesirable" were defined as TC ≥ 240, LDL-c ≥ 160, HDL-c < 40, or TG ≥ 200 (19-22).

To rule out individuals with familial hypercholesterolemia, participants with LDL-c levels greater than the 95th percentile or HDL-c levels lower than the 20th percentile were excluded.

Other exclusion criteria included women on hormone replacement therapy for < 2 years, regular exercise (>3h/week), weight gain or loss >5% of body weight in the past 3mo, plans to begin a weight loss/exercise regimen during the study, allergies to study foods, a medically prescribed diet, history of medical or surgical events that could affect digestion or swallowing, gastrointestinal surgeries conditions or disorders, chronic or metabolic diseases (including atherosclerosis, previous myocardial infarction or stroke, cancer, diabetes, moderate to severe asthma, chronic lung disease, and kidney disease), or fasting blood glucose levels > 126 mg/dL or blood pressure >180/120 mmHg were also excluded. Individuals with medication use affecting digestion and absorption, metabolism (e.g. thyroid medications), lipid-lowering medications, medications for diabetes, steroid/hormone therapies, or ADD/ADHD were excluded. Finally, fish oil or calciumfloroboron supplement use within three months of their participation, excessive alcohol use (> 3 drinks/d (42g ethanol/d) for men; > 2 drinks/d (28g ethanol/d) for women) or tobacco or nicotine use were also excluded.

Screening visit (V0)

Participants reported to the Human Nutrition Lab (HNL) following an overnight fast of 8 – 12 hours and abstaining from exercise and alcohol for at least 24 hours. Anthropometrics including height, weight, waist and hip circumference, and blood pressure were measured, and a fasting blood draw was performed to assess blood lipids and glucose. The validated Alcohol Use Disorders Identification Test (AUDIT) (23) was used to assess alcohol consumption habits and confirm habitual consumption of < 3 drinks/d in men or 2 drinks/d in women. Resting metabolic rate (RMR; kilocalories/day) was measured using indirect calorimetry (TrueOne 2400, Parvo Medics, Sandy, Utah, USA) under standard conditions (24) as described previously (25, 26). Participant's RMR was multiplied by an average activity factor of 1.65 to estimate daily energy

needs (27). Individuals that qualified for the study were then randomized into one of the two diet groups (CSO or OO) by a researcher who was not involved in data collection or analysis using a random-number generator. Participants were contacted and instructed to complete a two-day food diary including one week day and one weekend day that was turned in at visit 1 (28).

Pre-Diet Intervention Visit (V1)

The night before the V1, participants consumed a lead-in dinner and a snack (provided by research personnel) that contained 50% or total energy from carbohydrate, 15% of energy from protein, and 35% of energy from fat. The same fasting procedures from the screening visit were repeated for V1. Upon arrival at the HNL at 0700 hours, height, weight, hip-waist circumference, blood pressure, and body composition were measured. Body composition was assessed using the Bod Pod (Cosmed USA, Inc.). Two questionnaires, 1) the Perceived Stress Scale (PSS) (29) and 2) the International Physical Activity Questionnaire (IPAQ) (30) were administered. Physical activity was assessed by calculating total metabolic equivalent task (MET) min/wk from the IPAQ responses. An intravenous (IV) catheter was then placed in the antecubital vein and fasting blood samples were collected 5 minutes later. The line was kept patent with a saline solution.

After fasting measures were taken, a high fat liquid meal challenge rich in SFAs was administered. The nutrient content from this meal was designed to provide 35% of the participant's total estimated daily energy needs (determined from RMR at screening) as described previously (25). The meal was made from an original milk chocolate ready-to-drink shake (Ensure, Abbott Laboratories, Inc., Columbus, OH), unsalted butter, red palm oil, coconut oil, soy lecithin granules, and powdered chocolate drink mix. Briefly, this meal had 5.0% energy from protein, 25.0% from carbohydrate, and 69.5% from fat. SFA, MUFA and PUFA contributed 46.9%, 15.7%, and 6.9% of energy from fat, respectively. Participants had 10

minutes to drink the meal. To ensure the entire meal was consumed, four ounces of water were used to rinse out the container, which was then also consumed. Following the meal challenge, blood was drawn at 30, 60, 90, 120, 150, 180, 240, and 300 minutes (5h) postprandially. Participants were also given 4oz of water every hour.

8-week Dietary Intervention

The day following V1, participants began the 8-week OO or CSO enriched diet intervention. The OO used was California Olive Ranch® (Chico, CA) Destination series Everyday Extra Virgin Olive Oil. The CSO was Chef's Pride® (Pacific Grove, CA). Fatty acid analysis of a sample of each test oil (**Table 3.1**) shows expected differences in fatty acid composition between the two oils, however; other components that may differ between the two oils, such as phenolic content were not measured, nor was an analysis of fatty acid compositions done when different batches or lot numbers were used.

By design, this partial outpatient feeding trial provided participants with most, but not all, of their required daily energy needs thus allowing them to maintain some of their usual diet. Before leaving the HNL after V1, participants were provided a one-week supply of daily meals and snacks that corresponded to their assigned diet group. Researchers, who were dietetic interns, also counseled participants on their estimated energy needs including how much energy (kilocalories) they had each day to fill with foods of their own choosing to maintain energy balance. The provided diets were identical between groups with the only difference being the type of oil used in the preparation of the foods (CSO vs. OO). Foods were prepared in the research kitchen with all ingredients weighed to the 0.01g. A 7-day rotating menu of meals were used providing two meals (breakfast and a lunch or dinner entrée) and snacks daily. Therefore, participants typically ate only one meal of their own choosing each day. Examples of meals

provided include soups, sautéed vegetable medleys, pasta with sauce, turkey meatloaf and mashed potatoes, breakfast muffins, and brownies or cookies as desserts. These meals were selected based on the successful incorporation of oils into them. Five days per week, a breakfast shake was provided as one of the two meals. Participants were instructed to prepare this liquid meal by mixing provided individually portioned instant meal shake mix, with milk of choice, and a designated amount of the assigned oil provided. The rest of the meals were pre-portioned by weighing ingredients to the 0.01g and were then individually packaged in microwave safe containers by research personnel and frozen. Participants were instructed on safe reheating practices of the meals.

For the provided diets, participants were instructed to consume all of the provided foods and follow their normal dietary patterns for any meals not provided. Along with being randomly assigned a diet treatment (CSO or OO), participants were non-randomly assigned to a kilocalorie tier. The tier assignment was dependent on the participant's estimated energy requirement determined from their RMR at screening (**Table 3.2**). The provided foods accounted for about 60% of estimated energy needs of the participants, and allowed each tier to provide about 30% of estimated daily energy needs from the assigned oil (CSO or OO).

Weekly Responsibilities

Participants completed a daily Meal Compliance Checklist where they checked off meals consumed each day. Meal compliance checklists were submitted to research personnel once per week for analysis. Participants consuming less than 75% of provided foods were deemed non-compliant, and were to be removed from the final data analysis. In addition to compliance records, participants were asked to keep food diaries once per week, alternating between week

days and weekend days. Daily nutrient intakes from the food diaries were assessed using The Food Processor SQL software (ESHA Research; version 10.12.0).

Weekly visits to the HNL

Participants returned to the HNL once per week to pick up weekly supplies of meals. During these visits, participants returned meal compliance checklists, and food diaries. Participants were also asked to fill out the IPAQ questionnaire in 2-week intervals during the intervention.

Mid-Diet Intervention Visit (V2)

Four weeks after V1, participants reported to the HNL following an 8-12 hour fast and 24 hours without exercise and alcohol for V2. The same lead-in meal provided at V1 was consumed for dinner the night before V2. Anthropometrics, PSS, IPAQ, and a fasting blood draw were repeated as explained in V1. No SFA-rich meal challenge was performed at V2.

Post-Diet Intervention Visit (V3)

Four weeks after v2, participants returned for V3. The same lead-in procedures from V1 and V2 were repeated. Participants then completed the exact same study procedures and measurements that took place at V1, including anthropometrics, questionnaires, SFA meal challenge, and blood draws.

Blood Lipid Analysis

All blood samples were drawn into K2 EDTA-coated vacutainers (Becton, Dickinson and Company, Franklin Lakes, NJ), and immediately placed on ice. For the lipid panel at all 3 testing visits (V1-V3), a portion of the fasting blood sample was drawn into a serum separator clot activator vacutainer (Greiner Bio-One North America Inc., Monroe, NC) and kept at room

temperature for 30 minutes before centrifugation for 15min at 3,000rpm at 4°C. The serum was transferred into a transport tube and kept at 4°C until the advanced blood lipid panel was performed (Quest Diagnositics). The lipid panel was the primary outcome and included TC, HDL-c, TG, LDL-c, LDL particle number (LDL-P), LDL small, LDL medium, HDL large, and apoB through spectrophotometry and ion mobility lipoprotein fractionation.

The remainder of the fasting blood sample and all postprandial samples were centrifuged as described above. The plasma was aliquoted and stored at -80°C until further analysis. Sample analysis of secondary outcomes included TGs and non-esterified fatty acids (NEFAs), glucose and insulin. Postprandial responses of plasma TGs, NEFAs, and glucose were measured by enzyme-based calorimetric assays (Wako Chemicals USA, Inc., Richmond, VA). Glucose was measured using a colorimetric glucose oxidase/peroxidase method (glucose oxidase: G2133, peroxidase: P8250; Sigma Aldrich, St. Louis, MO, USA). Insulin was measured by radioimmunoassay (MilliporeSigma, Darmstadt, Germany).

Statistical Analyses

The SAS version 9.2 statistical package (SAS Institute Inc, Cary, NC, USA) was used for all data analyses. All results are reported as mean ± SEM unless otherwise noted. Statistical significance was set at $p<0.05$ for all measures. A sample size of 38 (19/group) was estimated to detect a change in TC of 3.72 mg/dL between groups with a pooled SD of 25.26 mg/dL (Cohen's D of 0.14) in healthy males using G*power 3.19.7 assuming at least 80% power and an α of 0.05 based on the previous CSO study by Polley et al (11). Sample size calculations were run in the same manner to detect at least a 10% change between groups based on results for LDL-c, TG and postprandial TG AUC from Polley et. al. (11) resulting in estimated sample sizes of 30, 36, and 32 (Cohen's Ds of 0.17, 0.15, and 0.16), respectively. To ensure appropriate power was reached,

we used the TC sample size estimation for this trial since it was the most conservative estimate. The decision to utilize per protocol analyses was made a priori. An unpaired T-test was used to compare differences in compliance and change from baseline fasting biochemical markers between groups. A two-factor (treatment, visit (time)) repeated measures analysis of variance (ANOVA) using PROC MIXED was used to determine between and within group differences for fasting biochemical data, anthropometrics, perceived stress, total MET minutes, and self-reported intake. An intervention average was calculated for total MET minuets and self-reported intake. Intervention averages were compared to baseline averages. For all time course (meal challenge) data, a three-factor (treatment, visit, time point) repeated measures ANOVA using PROC MIXD was used to determine differences between and within groups. Subjects were modeled as random effects while treatment, visit, and time point were fixed effects in both ANOVA models with no covariates. Additionally, area under the curve was calculated for all postprandial measures and analyzed using the same two-factor repeated measures ANOVA as described for fasting biochemical data. When significance was found, post hoc analyses were done using Tukey's test. Continuous variables were examined for normality using the Shapiro–Wilk test, and an appropriate transformation was applied to non-normal data before analysis. No such transformations were required in the current analysis.

Results

Participants

Fifty-three participants were randomly assigned to the intervention (n = 26 CSO; n = 27 OO); however, 10 participants did not start or complete the intervention and were not included in the final analysis (**Figure 3.2**). Therefore, forty-three participants completed the intervention (12 women and 9 men in the CSO group; 15 women and 7 men in the OO group) and were included

in this per protocol analysis of primary and secondary outcomes. One of the 42 participants did not complete the meal challenge, thus only their fasting data is included. Participant characteristics at baseline are presented in **Table 3.3**. For body weight and BMI, there was a significant main effect of visit ($p<0.001$ for both) but not for treatment ($p=0.98$; $p=0.77$) and no treatment by visit interaction ($p=0.99$; $p=0.98$), respectively. Post hoc analyses revealed that this visit effect was driven by increases in body weight and BMI from pre- to mid- ($p=0.03$; $p=0.04$) and post-intervention ($p<0.001$; $p< 0.001$), regardless of group assignment. There were no other main or interaction effects in any other measure of anthropometrics, perceived stress, or reported physical activity.

On average, participants in the CSO and OO groups reported consuming 91 ± 2% and 92 ± 1% of provided foods, respectively, and compliance was not different between groups. No participant reported poor compliance (defined as <75% of study products consumed throughout intervention). A baseline value for self-reported intake was calculated as an average of the two food records collected before the start of the intervention. An intervention average was calculated as an average of all food diaries collected during the intervention. There were no significant differences between groups at baseline for any of the analyzed nutrients (**Table 3.4**). There were significant visit effects for percent energy from protein ($p<0.001$), carbohydrate ($p<0.001$), fat ($p<0.001$), and alcohol ($p=0.007$) as well as grams of grams of fat ($p<0.001$), alcohol ($p=0.24$), and fiber ($p<0.001$), with decreases in all measures except fat across both groups.

For fatty acid composition, there was a treatment by visit interaction ($p<0.001$) for an increase in MUFA intake in the OO group compared to CSO. Conversely, there was a treatment by visit interaction ($p<0.001$) for increases in total PUFA and omega 6 in the CSO vs. OO group.

Finally, there was a main effect of treatment (p=0.013) for omega 3 fatty acids showing greater intake in OO vs. CSO regardless of visit, and a main effect of visit (p<0.001) showing a decrease in omega 3 intake from baseline to intervention, regardless of group. There were no significant main or interaction effects for self-reported intake of protein, carbohydrate, saturated fat, trans fat or sugar.

Fasting biochemical markers

Fasting blood lipids are presented in **Figures 3.3 and 3.4.** There were no differences between groups for any outcome at baseline. For the intervention, there was no effect of treatment (p=0.42) but, there was a significant effect of visit (p=0.002), and a treatment by visit interaction (p=0.027) for TC. The interaction effect was driven by a decrease in TC from pre- to post-intervention in the CSO group (p<0.001) with no change in the OO group (p=0.97). Similarly, for LDL-c, there was no treatment effect (p=0.31), but there was a visit effect (p<0.001), and a treatment by visit interaction (p=0.04) which was driven by reductions in CSO from baseline to mid- (p=0.009) and post-intervention (p<0.001) with no change in OO. Likewise, for Non-HDL-c there no treatment effect (p=0.76), but there was a visit effect (p<0.001), and a treatment by visit interaction (p=0.04). The interaction was driven by reductions in CSO at both the mid- (p=0.04) and post-intervention visits (p<0.001) with no change in OO.

For Apo B there was no effect of treatment (p=0.52), but there was a main effect of visit (p<0.001), which was for reductions at both the mid- (p=0.04) and post-intervention (p<0.001) visits. There was also a trend for a treatment by visit interaction (p=0.09) driven by reduction in CSO from pre- to post-intervention (p<0.001) with no change in OO. There were visit effects for HLD-c (p<0.001), TC:HDL-c ratio (p<0.001), and LDL medium (p=0.04) but not treatment effects (p=0.33, p=0.46, p=0.21) or interactions (p=0.92, p=0.38, p=0.04). The visit effects were

for increases (mid: p<0.001; post: p<0.001) for HDL-c and decreases in TC:HDL-c ratio (mid: p<0.001; post: p<0.001) and LDL medium (post: p=0.049) regardless of intervention group. Finally, there were no significant main or interaction effects in fasting TG, NEFA, LDL-P, LDL small, LDL medium, HDL large, insulin, or glucose.

Change in fasting biochemical markers between groups

The change from pre- to post-intervention for fasting biochemical markers are presented in **Table 3.5** as both absolute changes and percent changes. For the absolute changes, the decreases in TC (p=0.008), LDL-c (p=0.018), Non-HDL-c (p=0.014), and ApoB (p=0.05) were greater in the CSO group compared to the OO group from pre- to post-intervention. There were no differences between groups for the change in HDL-c, TG, NEFA, TC:HDL-c ratio, LDL small, LDL medium, HDL large, LDL-P, fasting insulin, or glucose. To examine the magnitude, we also calculated the mean difference in change between groups. This is also presented in **Table 3.5** with 95% confidence intervals and mirrors the significance mentioned above.

Postprandial biochemical markers

The meal responses for TG, NEFA, insulin, and glucose are presented in **Figure 3.5**. For postprandial TGs there was no effect of treatment (p=0.45) but significant effects of visit (p=0.02) time point (p<0.001) and a treatment by visit interaction (p=0.004). The interaction was driven by higher postprandial TG at post intervention compared with baseline in OO (p=0.002) (**Figure 3.5 A, B**) with no difference in CSO. This effect, however, was not observed when examining AUC data (CSO pre: 213±27.3 and post: 213±24.9; OO pre: 179±15.9 and post: 194±14.6 mg/dL · 5h; p=0.22. For NEFAs (**Figure 3.5 C, D**) there was no treatment effect (p=0.98), but a significant effect of time point (p<0.001), visit (p= 0.003) and a trend for

treatment by visit interaction (p=0.11). The interaction trend was driven by a reduction in postprandial NEFA from pre- to post-intervention in CSO (p=0.008) and no change in OO (p=0.68). For AUC, there was a visit effect (p=0.05) for NEFA (CSO pre: 0.45±0.03 and post: 0.41±0.03; OO pre: 0.44±0.03 and post: 0.42±0.04 mEq/L · 5h) showing a decrease from pre- to post-intervention regardless of treatment. There were no main or interaction effects for insulin (**Figure 3.5 E, F**). Finally, there were also no main effect of treatment or visit for glucose (p=0.35 and p=0.76, respectively); however, there was a treatment by visit interaction (p=0.003) (**Figure 3.5 G, H**) driven by a trend for increase in OO from pre- to post- visit (p=0.09) and a non-significant decrease in CSO from pre- to post-intervention (p=0.20). There was also a significant treatment by visit interaction for glucose AUC (CSO pre: 99.7±2.50 and post: 97.5±2.68; OO pre: 99.7±2.52 and post: 103±2.67 mg/dL · 5h; p=0.028), again showing an increase in OO compared to CSO after the intervention.

Discussion

For the first time, we have shown that a diet enriched with CSO (high in PUFA) for 8 weeks resulted in significant improvements in fasting TC, LDL-c, HDL-c, TC:HDL-c ratio, non-HDL-c, and ApoB in adults with hypercholesterolemia. CSO-diet enrichment also suppressed postprandial NEFA and glucose following the intervention. The only changes in the OO diet (high in MUFA) were improvements in fasting HDL-c and TC:HDL-c ratio and a worsening of the postprandial TG and glucose response to a SFA-rich meal. Together, this broadly provides additional evidence on the comparative effects of dietary oils rich in PUFA versus MUFA for blood lipid control, and specific evidence that CSO has a greater effect than OO for blood lipid improvements in an at-risk population.

The suppression of fasting TC and LDL-c in the CSO group amounted to 7.4% and 12.2% reductions, respectively. These findings are clinically meaningful because every 1.0% reduction in LDL-c has been estimated to reduce the risk of coronary artery disease (CAD) by 1.2-2.0% (31, 32) resulting in an estimated 14.6-21.4% reduction in CAD risk in the CSO group. Additionally, of the CSO participants in our study, the reductions in TC and LDL-c led to a reduction in at least one diagnostic category (e.g. "High" to "Borderline High") in 57% of participants. The CSO diet also lowered LDL-c in a similar magnitude to bile acid sequestrants, fibrates, and nicotinic acid. While not as effective as statins, our magnitude of change in LDL-c is similar to extremely low dose of pravastatin without the adverse side effects commonly reported with these pharmacologic lipid lowering therapies, especially statins (33, 34). Finally, ApoB is a major structural protein found on atherogenic lipoproteins (35), and is gaining recognition as a potentially more useful marker for predicting CVD risk than LDL-c (36). By conclusion of meta-analysis, reductions of 10 mg/dL in apoB reduce the risk of coronary heart disease (CHD) and overall CVD risk by 9% and 6%, respectively (37). Based on this, the magnitude of reduction in apoB in our CSO group would correspond to a 10% and 7% reduction in CHD and CVD risk, respectively. Therefore, the reductions in TC, LDL-c, and apoB demonstrate the clinical significance of CSO's superior lipid lowering effect, and subsequent lowering of chronic disease risk, compared to OO. These effects were observed despite reductions in intakes of other nutrients known to improve lipid profiles during the intervention such as fiber and slight weight gain, which would be expected to have detrimental effects on blood lipid profiles.

It is well established that the replacement of SFA with unsaturated fats reduces blood lipids and CVD risk (6, 38-41). This was well demonstrated in the DIVAS study which observed

similar improvements in fasting blood lipids when replacing SFA for unsaturated fats but no differences between MUFAs and PUFAs for these outcomes (41). The direct comparison on the effect of MUFAs versus PUFAs, rather than comparing MUFA or PUFA to a SFA control, on blood lipids and CVD risk is less well understood. This is especially true for CSO versus OO. High MUFA sources, including OO, often lack the robust lipid lowering effects regularly observed by high PUFA sources (8, 42, 43). Conversely, trials have shown stronger lipid lowering effects from high PUFA intake, although they usually have little to no effect on HDL-c (44). While data comparing MUFA and PUFA diets have not been directly analyzed in a meta-analysis, individual trials exist comparing the two unsaturated fats. PUFA-rich oils, including corn, soybean, and sunflower, have been shown to reduce TC, LDL-c, non-HLD-c, and VLDL-c to a greater degree than MUFA-rich oils such as OO, rice bran oil, and modified high oleic oils (45-49). From this small body of literature, it appears that consumption of PUFA-rich oils tend to generate greater reductions in blood lipids than MUFAs.

Not only does PUFA seem to be superior to MUFA for its lipid lowering effects, but CSO appears to have benefits beyond other high PUFA food sources. In animal models, there are larger improvements in lipid metabolism following CSO consumption, all compared to high PUFA treatments including corn, soybean, and safflower oils (13, 50-55). Additionally, CSO improves glucose tolerance and hepatic lipid accumulation compared to safflower oil in mice (13). This evidence is limited as there are no human studies comparing CSO to other high PUFA foods/diets; however, two previous studies in humans, both one week in duration in healthy, young adults, showed similar lipid lowering effects to what we have observed presently with CSO consumption (11, 12). These combined data from multiple studies shows that CSO, rich in n-6 PUFA, has a consistent effect of improving blood lipids and appears to be more effective

than other high PUFA food sources. However, as mentioned above, since this data originates in animal models, clinical trials are needed.

Certain properties of CSO may explain its superior effects on lipid metabolism. CSO is a unique oil in that is high in PUFA, but SFA as well (23% SFA, 20% MUFA, and 57% PUFA) (see **Table 3.1**). The high SFA content of CSO makes it's lipid lowering properties somewhat surprising (52). There are multiple potential mechanisms to explain the lowering of blood lipids by CSO. The first is transcriptional regulation of lipid and cholesterol metabolism by the high PUFA content of the oil (9, 10). The second was presented when Paton et al (13) confirmed the presence of trace amounts of DHSA in CSO and confirmed that CSO had a more pronounced effect on lipid metabolism than a safflower oil-enriched diet (high n-6 oil devoid of DHSA). DHSA is of note because it is a cyclopropyl intermediate in the synthesis of sterulic acid, which is a known inhibitor of the hepatic lipogenic enzyme, SCD1, potentially mitigating hepatic lipid accumulation in response to excessive dietary fat (13) which could support improvements in cholesterol metabolism. More specifically, SCD1 catalyzes the desaturation of a range of SFAs to endogenously synthesize MUFAs, promoting the storage of lipid rather than the oxidation of it (56). Paton et al (13) was able to prove this mechanism in a mouse model with the use of desaturation indices, liver lipid quantification, and expression of SCD1 mRNA. Polley et al (11) confirmed the possibility of this mechanism in humans using a similar desaturation index while also having observed a cholesterol lowering effect following CSO consumption. It is also plausible that the two pronged mechanism (SCD-1 inhibition by trace dihydrosterulic acid (DHSA), and blood lipid metabolism regulation by PUFAs) is necessary to produce the same observed changes in blood lipids observed in the present trial. This mechanism remains to be confirmed in humans. The effects of CSO may also be a result of other components of its non-

saponifiable portion including tocopherols and beta-sitosterol (12, 57) as both may modify blood lipid control by modifying expression of regulatory proteins in the metabolism of cholesterol. Lastly, it is important to recognize CSO as a whole may be required to exert the observed changes in blood lipid metabolism rather than a singled out nutrient.

Since adults spend most waking hours in the postprandial state, it is now recognized as a critical period in which disease development can be exacerbated (58, 59). In the current trial, we utilized a high-fat, SFA-rich meal challenge at pre- and post-intervention, rather than a high-fat meal rich in the oil of the assigned intervention group (CSO or OO). This unique study design allows us to determine the chronic effects of daily CSO or OO consumption, and its potential protective effect against an occasional meal high in SFAs. Only the CSO group experienced postprandial improvements in NEFA and glucose following the intervention. Insulin signaling regulates both NEFA and glucose by the delayed activation of lipoprotein lipase (LPL) and the immediate translocation of GLUT4, respectively (60, 61). The improvements of NEFA and glucose in CSO suggest protection from lipotoxicity of the high SFA meal challenge and improved insulin sensitivity, further improving the participants' abilities to handle an occasional high SFA meal. While we did not expect outcomes to improve with OO, we were somewhat surprised with the worsening of postprandial TG and glucose following OO treatment. A small 1kg of weight gain was observed in the trial, regardless of group. Theoretically, this could lead to slight detriments in terms of lipid control; however, the differences between groups for the postprandial outcomes suggests that CSO offered protection from changes that could be due to weight gain while OO did not. This appears to contradict other studies where OO is used as part of Mediterranean diet studies (62, 63), so more work on the isolated effects of OO consumption is warranted.

This study is not without limitations. We chose a relatively high dose of each oil to match the doses used in previous short-term CSO studies. While high-fat diets, such as the ketogenic diet, are quite popular, the dose used here affects the generalizability to individuals following a lower-fat diet. Another limitation was the decision to compare PUFA vs. MUFA rather than PUFA vs. a true control group that had no intervention. This design allowed us to first detect within group differences of the intervention diets, and then to be able to compare the magnitude of change directly between the diets. Since OO is a popular "healthy oil" choice among consumers (15, 16), and CSO is less familiar, this design sheds light on the physiologic responses to diets enriched in each oil. The lack of change in most outcomes for the OO group, and lack of change in known dietary components as seen in the self-reported intake analyses, strengthens our findings that it was CSO, and not some other confounding variable of participating in a dietary intervention study, that improved outcomes. However, only the fatty acid composition of each oil was measured in this trial, so other compounds that can vary in oils such as phenolic content potentially limit our ability to determine what is driving our observed effects on blood lipids. Another limitation may be that we only controlled dinner the night before each testing visit rather than a multi-day, lead-in diet. This was intentional to isolate the effects of the intervention, and to have baseline measures closely reflect their usual intake rather than a manipulated baseline level. Additionally, we only used a 2-day baseline food diary rather than a longer one in order to reduce participant burden, but we acknowledge this is a relatively short period of time and may cause our intake analysis to be less sensitive than a longer food record. This trial was also single-blinded rather than double-blinded limiting the strength of the design. Finally, the measurements of dietary intake, physical activity, stress, and compliance were all

self-reported, which contains some degree of under- or over-reporting, and we did not have direct measures or biomarkers to assess compliance.

In conclusion, we have shown that a CSO-enriched diet reduced fasting blood lipids, including TC, LDL-c, ApoB, postprandial NEFA and glucose, and improve HDL-c, in adults with hypercholesterolemia. The results of this study are clinically meaningful because the magnitude of reduction in LDL-c by CSO (12.2%) could correspond to a 14.6-21.4% reduction in CAD risk. Additionally, the reduction in postprandial NEFA and glucose response provides further protection in the fed state. This study shows that these sources of MUFAs and PUFAs have different responses with respect to blood lipid metabolism and glycemic control. Furthermore, this study provides evidence that CSO in particular may be a beneficial oil to incorporate into the diet for adults with hypercholesterolemia. In addition to its cholesterol lowering effects, cottonseed oil is a practical cooking oil with neutral flavor and high smoke point meaning it is easily incorporated into common foods. It is also easily accessible to consumers, is already commonly found in food items such as salad dressings, condiments, and packaged goods (crackers, chips), and is currently used by many restaurants due to the aforementioned high smoke point which is good for frying. Cotton is primarily known as a textiles crop, thus its ability to contribute to food supply highlights the utility of this U.S. crop. Future studies should investigate the effects of enriching the diet with different high PUFA oils to see if CSO is superior to other PUFA-rich oils. Future studies should also examine CSO diet enrichment at lower doses over different durations, and in various populations. Finally, additional studies should also investigate other markers of CVD risk, including inflammation, to fully characterize CSO's effect on CVD risk.

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Table 3.1. Fatty Acid Distribution of Oils

Fatty acids	Name	CSO (%)	OO (%)
Total SFA		22.73	17.46
14:0	tetradecanoic acid (myristic)	0.78	0
16:0	hexadecanoic acid (palmitic)	18.83	14.30
18:0	octadecanoic acid (stearic)	2.68	2.37
20:0	eicosanoic acid	0.27	0.45
21:0	Methyl heneicosanoate	0	0.08
22:0	docosanoic acid (behenic)	0.18	0
Total MUFA		19.94	70.85
16:1	*cis*-9-hexadecenoic acid (palmitoleic *n-7)*	0.57	1.30
18:1	*cis*-9-octadecenoic acid (oleic *n-9)*	19.27	68.95
20:1	*cis*-11-eicosenoic acid (gadoleic *n-9*)	0.10	0.32
Total PUFA		57.33	11.69
18:2	*cis*-9,12-octadecadienoic acid (linoleic *n-6)*	56.97	10.67
18:3	*cis*-9,12,15,-octadecatrienoic acid (ALA *n-3)*	0.36	0.74
20:2	cis-11,14,-eicosadienoic (n-6)	0	0.09
20:4	Arachidonic acid (n-6)	0	0.10
20:5	Eicosapentaenoic acid (n-3)	0	0.10

CSO = cottonseed oil; OO = olive oil; SFA = saturated fatty acids; MUFA = monounsaturated fatty acids; PUFA = polyunsaturated fatty acids;

Table 3.2. Nutrient breakdown of provided intervention foods for each kilocalorie tier.

	Cottonseed Oil				Olive Oil			
Intervention Tiers (kcal)	<1,600	1,600-2,299	2,300-3,000	>3,000	<1,600	1,600-2,299	2,300-3,000	>3,000
Energy (kcal)	1,090	1,402	1,678	2,107	1,090	1,402	1,678	2,107
Energy from protein (%)	7.2	6.7	6.5	6.8	7.2	6.7	6.5	6.8
Protein (g)	19.1	23.0	26.8	35.2	19.1	23.0	26.8	35.2
Energy from carbohydrates (%)	36.9	36.3	36.3	39.0	36.9	36.3	36.3	39.0
Carbohydrates (g)	98.0	124.0	148.5	200.6	98.0	124.0	148.5	200.6
Fiber (g)	3.2	4.1	5.1	7.9	3.2	4.1	5.1	7.9
Sugar (g)	51.3	64.8	76.5	101.5	51.3	64.8	76.5	101.5
Energy from fat (%)	55.9	57.0	57.2	54.1	55.9	57.0	57.2	54.1
Total Fat (g)	65.5	85.9	103.1	122.7	65.5	85.9	103.1	122.7
Saturated fat (g)	15.3	20.0	24.0	28.7	12.3	16.0	19.3	23.2
Trans fat (g)	0.17	0.22	0.27	0.38	0.17	0.22	0.27	0.38
Monounsaturated fat (g)	13.3	17.4	20.8	24.7	44.8	58.8	70.6	83.5
Polyunsaturated fat (g)	36.8	48.4	58.1	68.8	8.3	10.9	13.0	15.5
Omega 3 fatty acid (g)	0.28	0.36	0.43	0.51	0.60	0.79	0.95	1.13
Omega 6 fatty acid(g)	36.6	48.0	57.6	68.3	7.8	10.1	12.1	14.4
Cholesterol (mg)	57.5	75.2	89.8	121.0	57.5	75.2	89.8	121.0
Total fat from intervention oil (%)	88.2	87.3	87.3	85.6	88.2	87.3	87.3	85.6
Fat from intervention oil (g)	57.8	75.0	90.0	105.0	57.8	75.0	90.0	105.0

[1] Daily nutrients delivered through the provided study foods within each treatment and energy tier. Participants were assigned to a kilocalorie tier based on their estimated energy requirements from a resting metabolic rate measurement at the screening visit. Energy tiers are named for the range of total energy requirements of the participants that were assigned to that tier. Energy (kcal) in the first row is the amount of energy actually provided each day from the diet intervention foods. The only difference between treatments was from the different treatment oil used (cottonseed oil vs. olive oil).

Table 3.3. Characteristics of adults with hypercholesterolemia at pre-, mid-, and post-intervention in cottonseed oil or olive oil intervention groups.

	Cottonseed Oil (n=21)			Olive Oil (n=22)		
Characteristic	Week 0	Week 4	Week 8	Week 0	Week 4	Week 8
Female (%)	57	--	--	68	--	--
Age (y)	53 ± 2	--	--	54 ± 2	--	--
Height (cm)	169.1 ± 2.2	168.9 ± 2.2	169.0 ± 2.2	168.3 ± 1.8	168.3 ± 1.8	168.3 ± 1.9
Weight (kg) [2]	78.6 ± 3.6	79.1 ± 3.5	79.6 ± 3.5	78.7 ± 3.5	79.2 ± 3.6	79.7 ± 3.6
Body Mass Index (kg/m^2) [2]	27.3 ± 0.9	27.5 ± 0.9	27.7 ± 0.9	27.7 ± 1.2	27.9 ± 1.2	28.1 ± 1.2
Waist circumference (cm)	91.1 ± 3.1	93.6 ± 3.0	92.3 ± 3.2	90.9 ± 3.0	91.1 ± 3.1	91.3 ± 3.2
Hip circumference (cm)	107.7 ± 1.4	108.3 ± 1.3	107.9 ± 1.4	109.9 ± 2.3	109.8 ± 2.3	110.4 ± 2.3
Waist to hip ratio	0.85 ± 0.02	0.86 ± 0.02	0.85 ± 0.02	0.83 ± 0.02	0.83 ± 0.02	0.83 ± 0.02
Systolic blood pressure (mmHg)	122 ± 3	125 ± 2	124 ± 3	127 ± 3	122 ± 3	122 ± 3
Diastolic blood pressure (mmHg)	77 ± 2	80 ± 2	79 ± 2	80 ± 2	79 ± 3	78 ± 2
Body fat (%)	31.0 ± 2.1	--	32.1 ± 1.9	33.6 ± 2.6	--	34.0 ± 2.6
Total MET (min/wk)	1,759 ± 293	1,420 ± 275	2,129 ± 570	1,129 ± 214	1,363 ± 405	1,563 ± 349
Perceived Stress Scale	11 ± 1	11 ± 1	12 ± 2	10 ± 1	9 ± 1	11 ± 1

[1]All values are mean ± SEM. Anthropometrics were analyzed with a two way (treatment by visit) repeated measures ANOVA. Week 0 is pre-intervention, Week 4 is mid-intervention, and Week 8 is post-intervention.
[2] Indicates significant effect of visit at $p<0.001$.

Table 3.4. Self-reported daily nutrient intake for diets enriched with cottonseed oil or olive oil in adults with hypercholesterolemia.

	Cottonseed Oil (n=21)		Olive Oil (n=22)		P values		
Nutrient	Baseline	Intervention	Baseline	Intervention	Treatment	Visit	Treatment x Visit
Energy (kcal)	2,075 ± 187	2,450 ± 110	2,539 ± 191	2,731 ± 283	0.11	0.09	0.56
Energy from protein (%)	15.6 ± 1.01	10.8 ± 0.27	14.6 ± 0.73	10.9 ± 0.36	0.51	<0.001	0.33
Protein (g)	74.7 ± 4.99	65.7 ± 5.19	86.2 ± 5.86	78.3 ± 13.8	0.20	0.25	0.94
Energy from carbohydrate (%)	46.0 ± 2.16	39.2 ± 0.76	45.7 ± 2.08	38.9 ± 0.97	0.88	<0.001	0.99
Carbohydrate (g)	239 ± 29.7	234 ± 11.6	283 ± 27.2	240 ± 12.5	0.29	0.21	0.30
Fiber (g)	19.8 ± 2.11	14.7 ± 1.12	22.0 ± 2.18	13.1 ± 0.65	0.87	<0.001	0.20
Sugar (g)	93.0 ± 14.9	105 ± 5.46	106 ± 14.3	109 ± 8.10	0.49	0.40	0.56
Energy from fat (%)	35.5 ± 1.85	49.0 ± 0.79	37.2 ± 1.52	48.0 ± 0.82	0.82	<0.001	0.15
Fat (g)	77.9 ± 7.36	128 ± 4.11	103 ± 8.96	146 ± 21.5	0.12	<0.001	0.77
Saturated fat (g)	26.3 ± 2.32	33.9 ± 1.30	33.9 ± 3.74	35.9 ± 6.18	0.25	0.17	0.42
Trans fat (g)	0.9 ± 0.1	0.6 ± 0.1	0.6 ± 0.1	0.7 ± 0.2	0.67	0.32	0.21
Monounsaturated fat (g)	32.7 ± 3.90	31.4 ± 1.77 [2]	41.4 ± 4.45	86.3 ± 11.8 [3]	<0.001	0.001	<0.001
Polyunsaturated fat (g)	18.0 ± 2.27	61.9 ± 1.72 [2, 3]	26.7 ± 2.16	23.3 ± 3.48	<0.001	<0.001	<0.001
Omega 3 fatty acid (g)	1.9 ± 0.3	1.0 ± 0.1	3.0 ± 0.5	1.6 ± 0.2	0.01	<0.001	0.39
Omega 6 fatty acid (g)	16.1 ± 2.08	60.9 ± 1.68 [2, 3]	23.7 ± 1.82	21.7 ± 3.29	<0.001	<0.001	<0.001
Energy from alcohol (%)	2.8 ± 1.0	1.0 ± 0.4	2.5 ± 0.9	2.1 ± 0.7	0.69	0.007	0.06
Alcohol (g)	9.1 ± 3.5	4.4 ± 2.0	10 ± 4.1	9.4 ± 3.1	0.48	0.02	0.12

[1] All values are mean ± SEM (N=43). Baseline represents an average of the two food diaries before the intervention. Intervention represents an average of all food diaries kept during the 8-week intervention. Main and interaction effects were analyzed using a two way (treatment x time) repeated measures ANOVA.

[2] indicates a significant difference between CSO vs. OO during the intervention at $p<0.05$.

[3] indicates significant difference between intervention vs. baseline within a group at $p<0.05$.

Table 3.5. Change from pre-to post-diet intervention in fasting biochemical markers of adults with hypercholesterolemia in cottonseed oil or olive oil groups.

	Mean Change					Percent Change	
	CSO		OO				
Measure	Baseline	Change	Baseline	Change	CSO vs. OO difference	CSO	OO
Total Cholesterol (mg/dL) [3]	230±6.57	-17.0±3.94	228±5.81	-2.18±3.72	-14.8 (-25.2, -4.43)	-7.38±1.77	-0.96±1.77
LDL cholesterol (mg/dL) [3]	162±5.41	-19.7±3.94	162±4.90	-5.72±4.23	-13.9 (-25.0, -2.88)	-12.16±2.52	-3.54±2.72
HDL cholesterol (mg/dL)	56.5±2.79	3.76±1.71	59.7±2.63	4.43±1.28	-0.67 (-4.75, 3.41)	6.66±3.42	7.43±2.62
Triglyceride (mg/dL)	169±22.9	-6.01±9.74	131±10.8	2.87±9.86	-8.87 (-35.4, 17.6)	-3.55±6.70	2.19±7.84
NEFA (mEq/L)	0.43±0.04	-0.04±0.04	0.39±0.04	0.02±0.03	-0.07 (-0.16, 0.03)	-4.67±8.44	6.07±8.33
TC:HDL-c Ratio	4.30±0.27	-0.51±0.10	3.94±0.16	-0.36±0.13	-0.15 (-0.46, 0.16)	-11.9±2.88	-9.10±3.04
Non-HDL cholesterol (mg/dL) [3]	175±7.81	-20.8±4.00	169±4.95	-6.61±4.01	-14.1 (-3.30, -25.0)	-11.9±2.45	-3.93±2.54
LDL-P (nmol/L)	1380±57.4	-26.9±50.0	1460±67.3	-77.3±68.6	50.3 (-112, 212)	-1.95±3.79	-5.31±4.64
LDL small (nmol/L)	215±24.0	3.43±11.2	216±18.2	-18.3±15.6	21.71 (-15.1, 58.5)	1.60±6.19	-8.48±7.01
LDL medium (nmol/L)	296±24.8	-23.1±15.9	341±24.5	-39.3±19.5	16.2 (-31.9, 64.3)	-7.83±5.33	-11.5±5.49
HDL large (nmol/L)	5410±377	443±295	5740±293	-7.23±316	450 (-377, 1280)	8.18±5.86	-0.13±5.61
ApoB (mg/dL) [3]	112±4.48	-11.7±2.37	111±3.86	-3.1±2.99	-8.65 (-15.4, -1.35)	-10.5±2.24	-2.79±2.82
Insulin (μU/mL)	12.4±2.45	-1.26±1.47	15.2±4.19	1.05±0.64	-2.31 (-5.37, 0.75)	-10.1±11.0	6.94±10.0
Glucose (mg/dL)	97.3±2.22	-2.23±2.02	99.1±2.46	1.33±3.48	-3.56 (-11.3, 4.14)	-2.29±2.10	1.34±3.53

[1]Change from baseline values (mean change, percent change) indicate change from pre- to post-intervention visit (baseline to week 8). Those values are presented as mean ± SEM. The difference in change values from pre- to post-visit for CSO (n=21) versus OO (n=22) are presented as mean difference with 95% confidence intervals. All markers are measured in serum except triglycerides, non-esterified fatty acids, insulin, and glucose, which were measured in plasma. Apo-B = apolipoprotein B; CSO = Cottonseed oil; HDL = high-density lipoprotein; LDL = low-density lipoprotein; LDL-P = Low-density lipoprotein particle number; NEFA = non-esterified fatty acids; OO = Olive oil; TC = total cholesterol.
[2]Change values were compared using unpaired T-tests between groups.
[3] indicates significant difference between groups ($p < 0.05$).

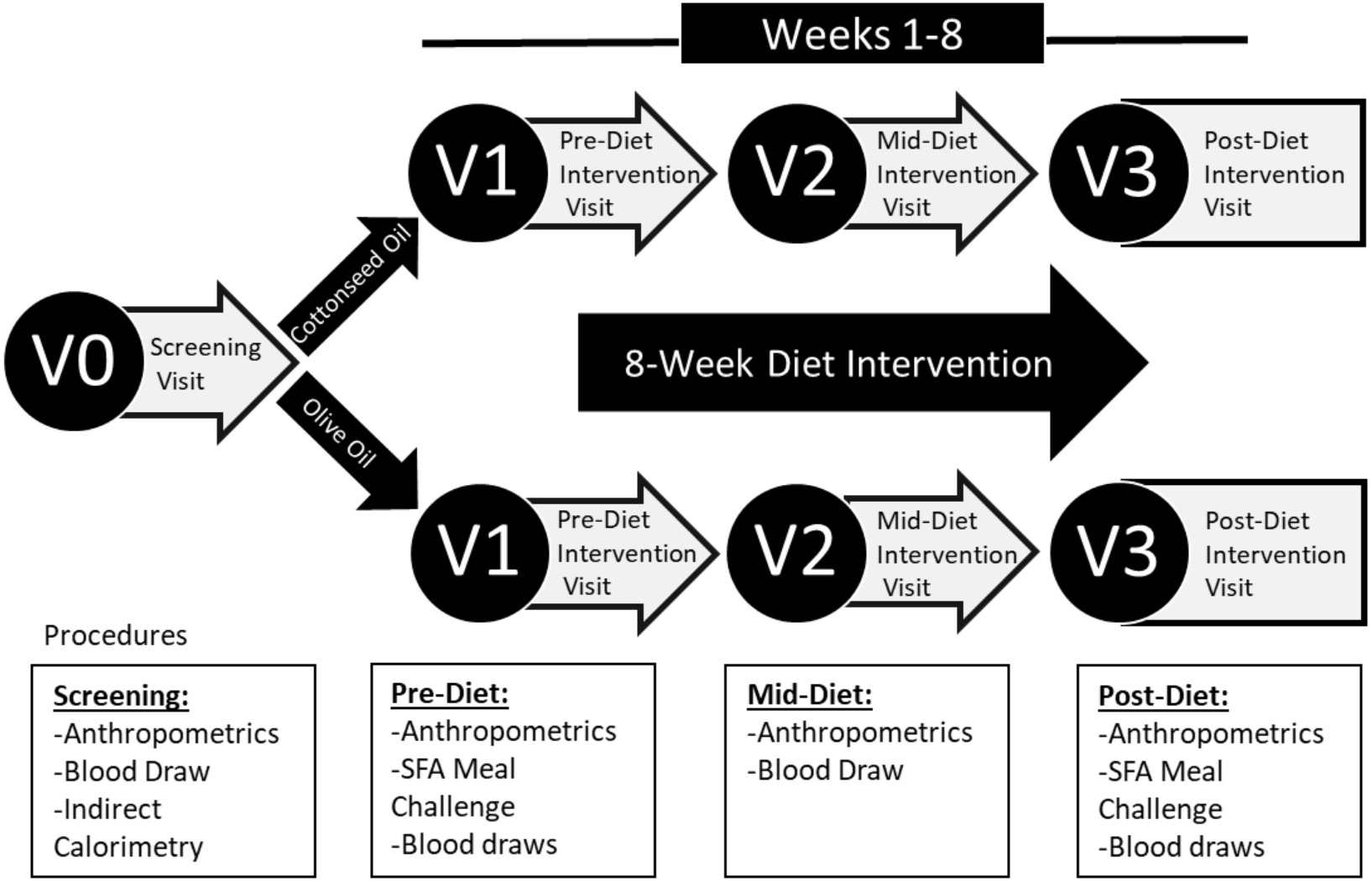
Weeks 1-8
V0
Screening Visit
Cottonseed Oil
Olive Oil
V1
Pre-Diet Intervention Visit
V2
Mid-Diet Intervention Visit
V3
Post-Diet Intervention Visit
8-Week Diet Intervention
V1
Pre-Diet Intervention Visit
V2
Mid-Diet Intervention Visit
V3
Post-Diet Intervention Visit
Procedures
Screening:
-Anthropometrics
-Blood Draw
-Indirect Calorimetry
Pre-Diet:
-Anthropometrics
-SFA Meal Challenge
-Blood draws
Mid-Diet:
-Anthropometrics
-Blood Draw
Post-Diet:
-Anthropometrics
-SFA Meal Challenge
-Blood draws

Figure 3.1. Study Timeline. There were four testing visits including screening (V0), pre-diet intervention (V1), mid-diet intervention (V2), and post-diet intervention (V3) visits. At V0, indirect calorimetry was performed to measure the participant's resting metabolic rate. Anthropometrics included height, weight, body composition, and waist and hip circumference. Questionnaires at V1-V3 were the Perceived Stress Scale (PSS) (29) and International Physical Activity Questionnaire (IPAQ) (30). During the intervention, participants came in for weekly food pickups and turned in compliance logs and food diaries. V = visit, SFA = saturated fatty acid.

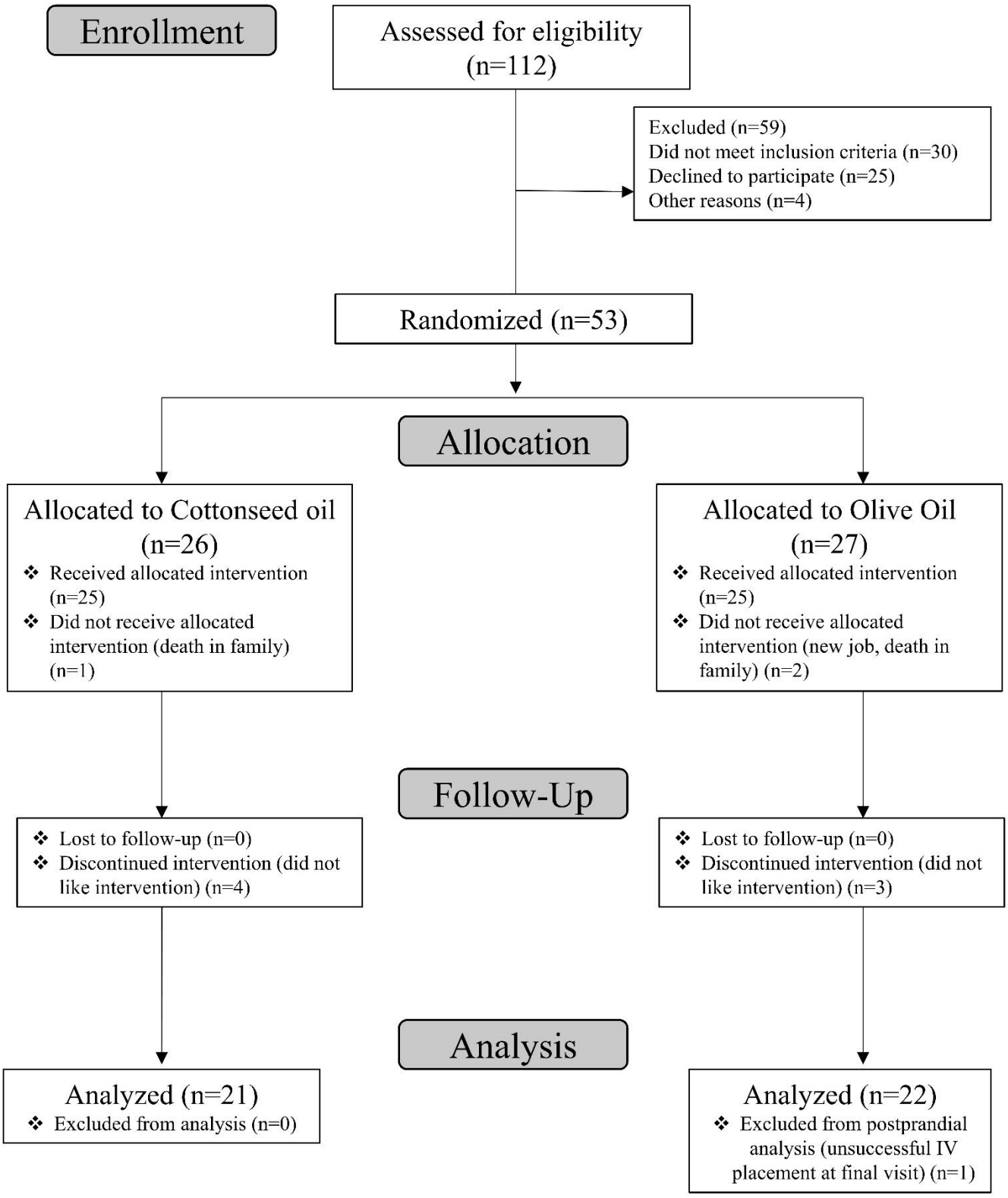

Figure 3.2. CONSORT flow diagram selection of participants.

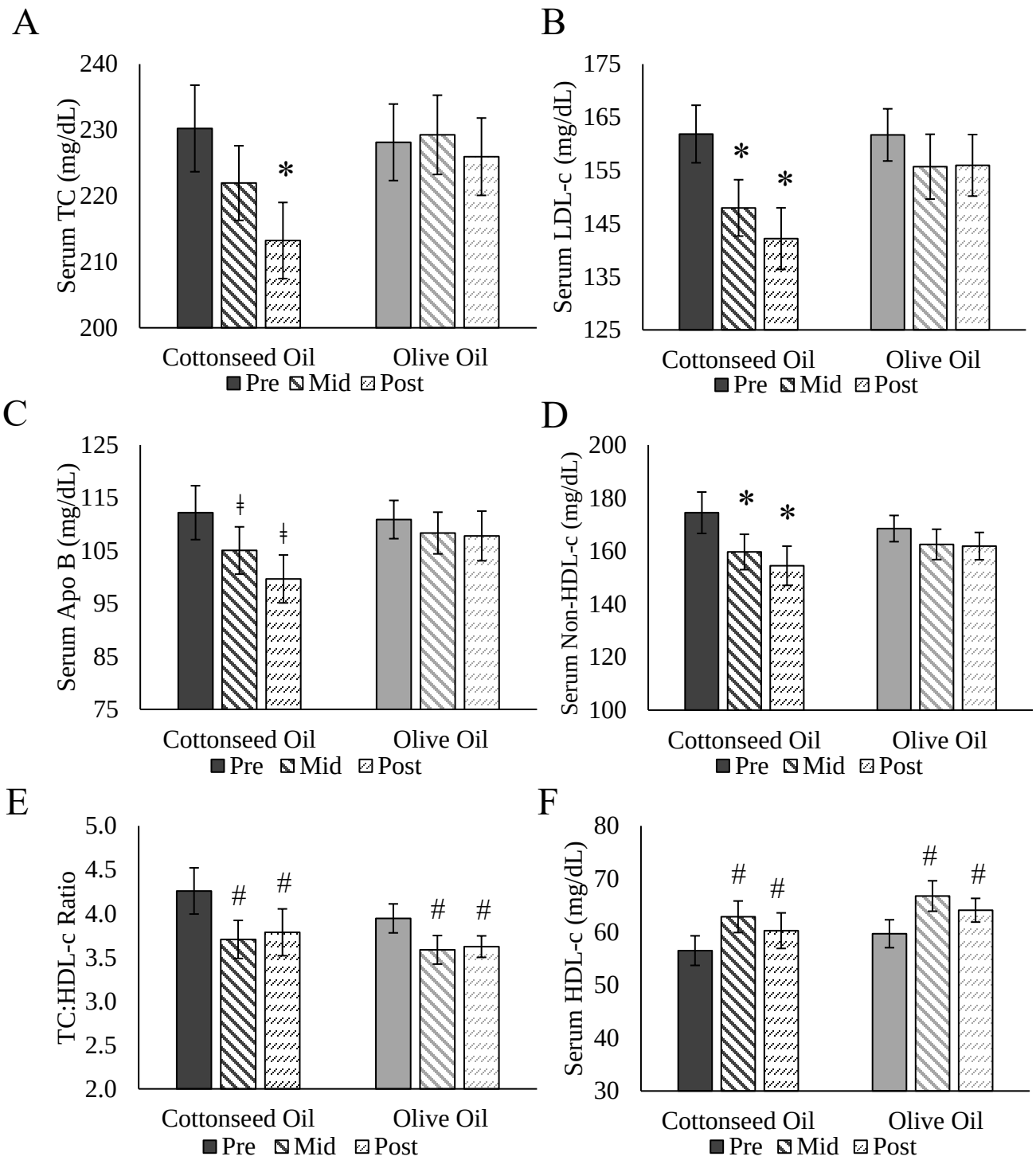
A
Serum TC (mg/dL)
240
230
220
210
200
*
Cottonseed Oil
Olive Oil
Pre Mid Post
B
Serum LDL-c (mg/dL)
175
165
155
145
135
125
*
*
Cottonseed Oil
Olive Oil
Pre Mid Post
C
Serum Apo B (mg/dL)
125
115
105
95
85
75
‡
‡
Cottonseed Oil
Olive Oil
Pre Mid Post
D
Serum Non-HDL-c (mg/dL)
200
180
160
140
120
100
*
*
Cottonseed Oil
Olive Oil
Pre Mid Post
E
TC:HDL-c Ratio
5.0
4.5
4.0
3.5
3.0
2.5
2.0
#
#
#
#
Cottonseed Oil
Olive Oil
Pre Mid Post
F
Serum HDL-c (mg/dL)
80
70
60
50
40
30
#
#
#
#
Cottonseed Oil
Olive Oil
Pre Mid Post

Figure 3.3. Serum fasting (A) total cholesterol, (B) low density lipoprotein cholesterol, (C) apolipoprotein B, (D) non-high density lipoprotein cholesterol, (E) total cholesterol to high density lipoprotein cholesterol ratio, and (F) high density lipoprotein cholesterol from pre- mid- and post-diet intervention visits in adults with hypercholesterolemia. (CSO: n = 21; OO: n = 22). Data were analyzed using a two way (treatment by visit) repeated measures ANOVA.
* indicates significant treatment by visit interaction ($p<0.001$) and a difference from baseline at $p<0.05$. ǂ indicates a trend for a treatment by visit interaction ($p=0.09$) and a difference from baseline at $p<0.01$. # indicates significant visit effect and a difference from baseline regardless of group assignment at $p<0.05$. All values are presented as mean ± SEM. Apo B = Apolipoprotein B; HDL-c = high-density lipoprotein cholesterol; LDL-c = low-density lipoprotein cholesterol; Mid = week 4; TC = total cholesterol; TC:HDL-c Ratio = total cholesterol to high-density lipoprotein cholesterol ratio; Non-HDL-c = non-high-density lipoprotein cholesterol; Post = week 8; Pre= week 0.

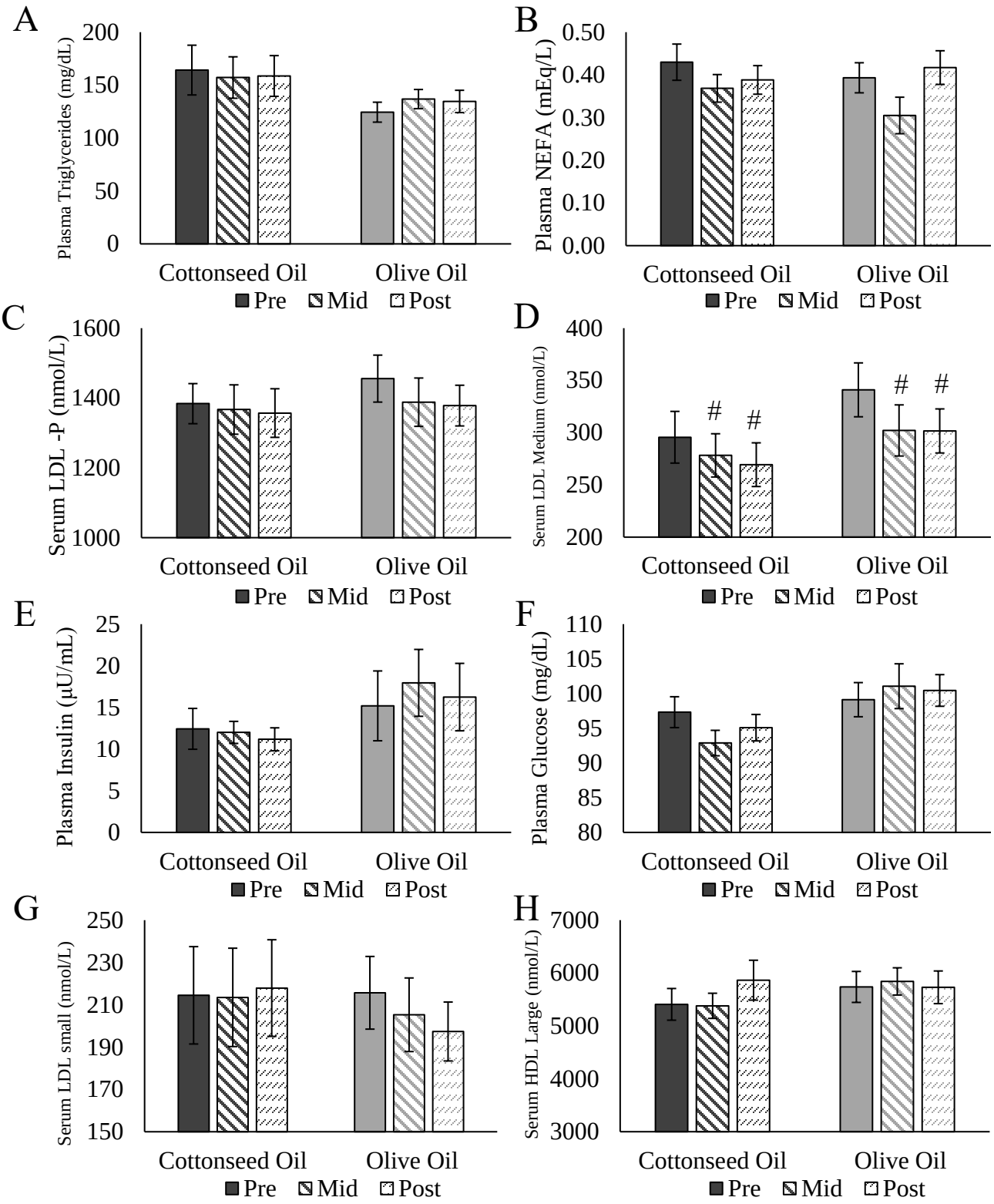
A
Plasma Triglycerides (mg/dL)
200
150
100
50
0
Cottonseed Oil
Olive Oil
Pre
Mid
Post
B
Plasma NEFA (mEq/L)
0.50
0.40
0.30
0.20
0.10
0.00
Cottonseed Oil
Olive Oil
Pre
Mid
Post
C
Serum LDL -P (nmol/L)
1600
1400
1200
1000
Cottonseed Oil
Olive Oil
Pre
Mid
Post
D
Serum LDL Medium (nmol/L)
400
350
300
250
200
#
#
#
#
Cottonseed Oil
Olive Oil
Pre
Mid
Post
E
Plasma Insulin (µU/mL)
25
20
15
10
5
0
Cottonseed Oil
Olive Oil
Pre
Mid
Post
F
Plasma Glucose (mg/dL)
110
105
100
95
90
85
80
Cottonseed Oil
Olive Oil
Pre
Mid
Post
G
Serum LDL small (nmol/L)
250
230
210
190
170
150
Cottonseed Oil
Olive Oil
Pre
Mid
Post
H
Serum HDL Large (nmol/L)
7000
6000
5000
4000
3000
Cottonseed Oil
Olive Oil
Pre
Mid
Post

Figure 3.4. Fasting biochemical markers (A) Plasma Triglycerides, (B) Plasma Non-esterified fatty acids, (C) Serum LDL particle number, (D) Serum LDL Medium, (E) Plasma Insulin, (F) Plasma Glucose, (G) Serum HDL Large, and (H) Serum LDL small, from pre- mid-and post-diet intervention visits in adults with hypercholesterolemia. (CSO: n = 21; OO: n = 22).
Data were analyzed using a two way (treatment by visit) repeated measures ANOVA.
# indicates significant visit effect and a difference from baseline regardless of group assignment at $p<0.05$. All values are presented as mean ± SEM. HDL = high-density lipoprotein; LDL = low-density lipoprotein; LDL-P = LDL particle number; Mid = week 4; NEFA = Non-esterified fatty acids; Post = week 8; Pre = week 0.

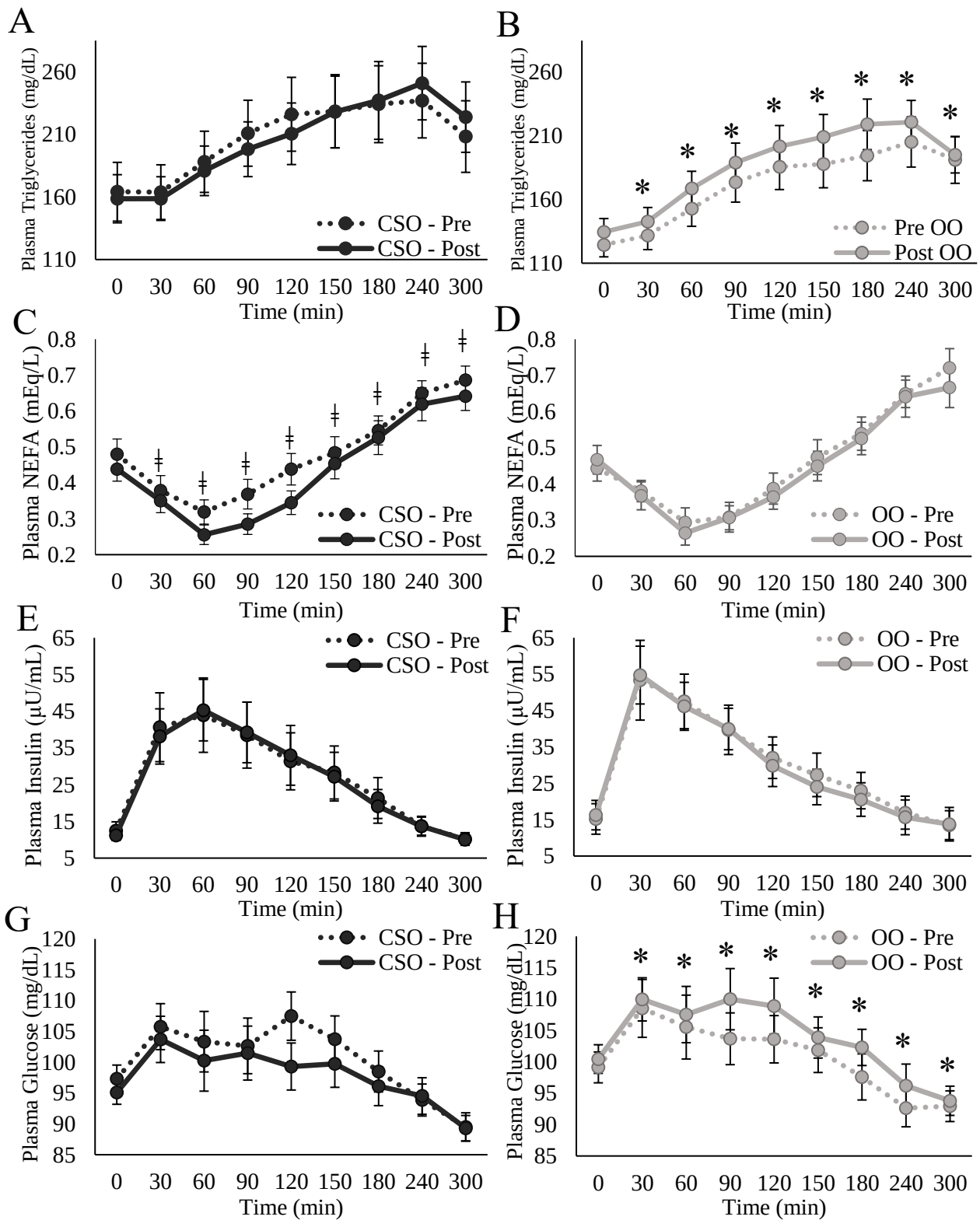
A
Plasma Triglycerides (mg/dL)
110
160
210
260
CSO - Pre
CSO - Post
0 30 60 90 120 150 180 240 300
Time (min)
B
Plasma Triglycerides (mg/dL)
110
160
210
260
Pre OO
Post OO
0 30 60 90 120 150 180 240 300
Time (min)
C
Plasma NEFA (mEq/L)
0.2
0.3
0.4
0.5
0.6
0.7
0.8
CSO - Pre
CSO - Post
0 30 60 90 120 150 180 240 300
Time (min)
D
Plasma NEFA (mEq/L)
0.2
0.3
0.4
0.5
0.6
0.7
0.8
OO - Pre
OO - Post
0 30 60 90 120 150 180 240 300
Time (min)
E
Plasma Insulin (µU/mL)
5
15
25
35
45
55
65
CSO - Pre
CSO - Post
0 30 60 90 120 150 180 240 300
Time (min)
F
Plasma Insulin (µU/mL)
5
15
25
35
45
55
65
OO - Pre
OO - Post
0 30 60 90 120 150 180 240 300
Time (min)
G
Plasma Glucose (mg/dL)
85
90
95
100
105
110
115
120
CSO - Pre
CSO - Post
0 30 60 90 120 150 180 240 300
Time (min)
H
Plasma Glucose (mg/dL)
85
90
95
100
105
110
115
120
OO - Pre
OO - Post
0 30 60 90 120 150 180 240 300
Time (min)

Figure 3.5. Time course for plasma triglycerides (TGs) (A, B), nonesterified fatty acids (NEFAs) (C, D), insulin (E, F), and glucose (G, H) for each treatment at pre- and post-intervention in adults with hypercholesterolemia (CSO: n = 21; OO: n = 21). Participants consumed a high saturated fat breakfast meal immediately after time 0. * indicates significant treatment by visit interaction and a difference between the pre- and post-intervention meal response within a group ($p < 0.05$). ǂ indicates trend for treatment by visit interaction and a difference between the pre- and post-intervention meal response within a group ($p=0.10$). All values are presented as mean ± SEM. CSO = cottonseed oil; OO = olive oil; Post = post-intervention or week 8; Pre=pre-intervention or week 0.

CHAPTER 4

EIGHT WEEKS OF DAILY COTTONSEED OIL INTAKE ATTENUATES POSTPRANDIAL ANGIOPOIETIN-LIKE PROTEINS 3 AND 4 RESPONSES COMPARED TO OLIVE OIL IN ADULTS WITH HYPERCHOLESTEROLEMIA: A SECONDARY ANALYSIS OF A RANDOMIZED CLINICAL TRIAL[2]

[2] Prater M.C., Scheurell A. R., Paton C. M., Cooper J. A. 2024. *Nutrition Research.* 123:88-100.
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Abstract

Angiopoietin-like proteins (ANGPTLs) – 3, -4, and -8 are regulators of lipid metabolism and have been shown to respond to changes in dietary fats. It is unknown how ANGPTLs respond to cottonseed oil (CSO) and olive oil (OO) consumption in a population with hypercholesterolemia. Purpose: to determine the impact of CSO vs. OO consumption on fasting and postprandial ANGPTL responses in adults with hypercholesterolemia. We hypothesized that CSO would have lower fasting and postprandial ANGPTL responses compared to OO. Forty-two adults with high cholesterol completed a single-blind, randomized trial comparing CSO (n=21) vs. OO (n=21) diet enrichment. An 8-week partial outpatient feeding intervention provided ~60% of the volunteers' daily energy expenditure (TEE) (~30% of TEE as CSO or OO). The remaining 40% was not controlled. Fasting blood draws were taken at pre-, mid- and post-intervention visits. Volunteers consumed a high saturated fat meal followed by 5h of blood draws at pre- and post-visits. Fasting ANGPTL3 had a marginally significant treatment by visit interaction ($p=0.06$) showing an increase from pre- to post-intervention in CSO vs. OO (CSO: 385.1±27.7 to 440.3±33.9 ng/ml; OO: 468.2±38.3 to 449.2±49.5ng/ml). Both postprandial ANGPTL3 ($p=0.02$) and ANGPTL4 ($p<0.01$) had treatment by visit interactions suggesting increases from pre- to post-intervention in OO vs. CSO with no differences between groups in ANGPTL8. These data show a worsening (increase) of postprandial ANGPTLs after the OO, but not CSO, intervention. This aligns with previously reported data where postprandial triglycerides were protected from increases compared to OO. ANGPTLs may mediate protective effects of CSO consumption on lipid control. This trial was registered at clinicaltrials.gov (NCT04397055).

Introduction

Cardiovascular disease (CVD) is the leading cause of death in the United States, accounting for 928,713 American deaths in 2020 alone (1). Dyslipidemia has been well established as a risk factor for CVD (2) and is characterized by elevated total cholesterol (TC), low-density lipoprotein cholesterol (LDL-c) and triglycerides (TG) (3). It is reported that approximately 35% of U.S. adults have elevated TC, LDL-c, and/or TG (1). Lowering metrics such as LDL-c as little as 1 mmol/L can reduce the risk of major vascular events (4). Thus, reduction of elevated blood lipids is a public health necessity.

While hydroxymetylgluteryl-coenzyme A (HMG-CoA) reductase is a common drug target for reducing blood lipids (5), angiopoietin-like proteins (ANGPTLs) have more recently come to light as another regulating mechanism and potential target for reducing circulating lipids, especially TG (6). ANGPTL-3, -4, and -8 have been shown to work together to inhibit lipoprotein lipase (LPL) (6). LPL acts at the luminal surface of capillaries where it hydrolyzes TGs transported by lipoproteins, facilitating lipid delivery to tissues (7). Inhibition of LPL is known to produce elevated TGs and dysregulation of lipoprotein metabolism (7).

In concert with these LPL inhibitory actions of ANGPTLs, there are differences in the expression of these proteins depending on the fed vs. fasted state. ANGPTL4 is typically induced in the fasted state and is shown to inhibit LPL, often at adipose tissue, to prevent lipid storage (8). Conversely, ANGPTL-3 and -8 are complexed and secreted from the liver in the fed state inhibiting LPL at the vasculature, which prevents TG delivery to oxidative tissues. In healthy models, free ANGPTL4 is an active inhibitor of LPL, but postprandially expressed ANGPTL8 can bind ANGPTL4 reducing its ability to inhibit LPL (9). Overexpression of ANGPTL-3, -4,

and -8 have all been shown to promote hypertriglyceridemia, while down regulation results in reduction of circulating TGs (8, 10-13).

Dietary fat is known to influence circulating lipoproteins as well as ANGPTLs. It is well established that unsaturated fats tend to reduce circulating lipid profiles compared to saturated fats (SFAs) (14). Yet, it is debated whether monounsaturated fatty acids (MUFAs) or polyunsaturated fatty acids (PUFAs) are more beneficial for lipoprotein metabolism and, by extension, ANGPTL regulation. Olive oil (OO) is a rich source of MUFAs and is commonly recommended to adults with hypercholesterolemia. While diets rich in MUFAs reduce lipid profiles when replacing SFAs in the diet, PUFA-rich diets may have a greater effect on reducing lipid profiles (15). Cottonseed oil (CSO) is a PUFA-rich oil regularly incorporated into commercially available foods. When used in replacement for OO in cooking, CSO has been shown to improve cholesterol profiles more than OO in both healthy (16) and populations with hypercholesterolemia (17). ANGPTL responses to CSO v. OO diets have only been measured in a young healthy male population (18), and showed a reduction in ANGPTL-3 and -4 with CSO and an increase with OO. Therefore, ANGPTLS may be a potential mechanism through which CSO or OO exerts effects on blood lipids. However, few clinical trials have been conducted exploring changes in ANGPTL responses to different types of dietary fat enrichment, especially in a population at-risk for CVD. The purpose of this secondary analysis was to determine both the changes in fasting ANGPTL concentrations, and the changes in postprandial ANGPTL responses to an 8-week CSO vs. OO enriched diet in adults with hypercholesterolemia. We hypothesized that CSO diets would elicit a reduction in fasting and postprandial ANGPTL responses compared to the OO diets. To test this hypothesis, we conducted a parallel partial outpatient feeding trial of CSO and OO enriched diets.

Methods

Study design

This study was a single-blinded, parallel, randomized clinical trial (registered at clinicaltrials.gov: NCT04397055) conducted according to the guidelines in the Declaration of Helsinki. The methods of this trial have been described in detail elsewhere (17). Briefly, the trial involved one screening visit, and three testing visits (pre-, mid-, and post- visits) over an 8-week intervention period. Volunteers were randomized to one of two groups (CSO or OO) using a previously described balanced blocks method with 1:1 allocation (17). Intervention foods were provided over the 8-week period that delivered the test oils (CSO or OO). Recruitment began in May of 2018 and concluded in June 2021 when all primary outcomes were addressed. The primary outcome for this trial was cholesterol and blood lipid responses (presented in (17)). ANGPTL responses are a secondary outcome. This study was approved by the Institutional Review Board for human participants at the University of Georgia (STUDY00005869) and all volunteers provided written informed consent. All testing procedures were completed at the Human Nutrition Lab (HNL).

Volunteers

Sedentary volunteers between 30 and 75 years of age were screened for eligibility based on inclusion and exclusion criteria that have been presented in detail previously (17). Briefly, inclusion criteria included the presence of hypercholesterolemia as defined by at least two blood lipids in a "borderline undesirable/at risk" range, or at least one in an "undesirable" range. The "borderline undesirable/at risk" ranges were defined as TC ≥ 180 mg/dL (4.65 mmol/L), LDL-c ≥ 110 mg/dl (2.84 mmol/L), high-density lipoprotein cholesterol (HDL-c) < 50 mg/dL (1.29 mmol/L), or TG ≥ 130 (1.47 mmol/L). The "undesirable" ranges were defined as TC ≥ 240

mg/dL (6.21 mmol/L), LDL-c ≥ 160 mg/dl (4.14 mmol/L), HDL-c < 40 mg/dL (1.03 mmol/L), or TG ≥ 200 (2.26 mmol/L) (19-22). Exclusion criteria included excessive alcohol use, tobacco use, exercise (>3h/week), weight instability (gain or loss of >5% of body weight in the past 3 months), chronic or metabolic diseases, and medication use (such as lipid-lowering medications, diabetes medications, steroid/hormone therapies, or ADD/ADHD medications).

Protocol

Screening visit

Volunteers completed fasting procedures before all testing visits. Fasting (or visit preparation) procedures included avoiding alcohol and exercise for 24h and fasting (consuming nothing except water) for 8-12h prior to the visit. Anthropometrics (height, weight, and blood pressure) were measured, and a fasting blood draw was taken to assess fasting lipid panels and glucose. Finally, respiratory gasses were measured for 30 min (TrueOne 2400; Parvo Medics, Sandy, Utah, USA) using indirect calorimetry under standard conditions (23). The final 20min of the measurement was used to calculate resting metabolic rate using the Weir equation (24). Total energy expenditure (TEE) were determined by resting metabolic rate *1.65 (25). After the screening visit, volunteers were randomized to either the CSO or OO intervention groups as previously described (17).

Lead-in protocol

Prior to the pre-diet intervention, volunteers completed two-day food records consisting of one week-day and one weekend day as previously described (17). The night before the pre-diet visit, volunteers were provided a standardized meal to consume (energy: 50% carbohydrate,

15% protein, 35% fat). In addition to the standardized dinner meal, volunteers were instructed to complete the same fasting procedures as described for the screening visit.

Pre-diet intervention visit (V1)

Volunteers arrived in the fasted state to the HNL, where anthropometrics were taken and body composition was measured using a BodPod (Cosmed USA, Inc.). An IV catheter was then inserted and a fasting blood sample was collected. Next, volunteers were provided a high SFA meal as described previously (17). Briefly, the meal contained 35% of TEE, and percent TEE from macronutrients are as follows: 25% carbohydrates, 5% protein, 70% fat, with 47% as SFAs. Volunteers were given 10min to consume the meal. For the next 5h, blood was drawn from the IV catheter at the following time points (30 min, 1h, 2h, 3h, 4h, 5h) for the analysis of ANGPTLs. At the end of the visit, volunteers were provided instructions on how to record their compliance with the intervention and were told how many kilocalories were being provided by the study foods. Finally, volunteers were sent home with a one-week supply of meals prepared with their assigned intervention oil.

Partial outpatient feeding intervention

Volunteers were instructed to begin consuming intervention meals the day after the pre-visit. They were blinded to their treatment group (CSO or OO). The intervention was a partial outpatient feeding design. Research personnel provided ~ 60% of the volunteers TEE (**Table 4.1**). Energy tiers were used to administer the partial outpatient feeding meaning volunteers who had similar TEE received the same portion of foods. This design allowed the researchers to deliver approximately 30% of the volunteer's TEE as the assigned intervention oil (CSO or OO). Volunteers were instructed to consume the remaining ~40% TEE ad libitum away from the lab.

This typically translated to one meal of choice per day. Volunteers were given their personal information (TEE from screening visit, and kilocalories provided by intervention foods daily) so they could make their own decisions about filling their remaining TEE.

The recipes for the intervention foods were identical between groups, except for the oils used to prepare the foods (CSO or OO). Two meals and a snack were provided daily using a 7-day rotating menu. Research personnel prepared all intervention meals and snacks as described previously (17). Weekly compliance checklists were used to collect self-reported compliance. In addition to compliance checklists, volunteers kept two-day food records biweekly (end of weeks 2, 4, 6, and 8). Weekly, volunteers returned study materials to the lab and picked up meals for the next week.

Mid-diet intervention visit (V2)

The Mid-diet intervention visit occurred after week 4 of the intervention. Volunteers completed the same lead-in procedures as described above. Only fasting measures, excluding body composition, were collected as describe for V1.

Post-diet intervention visit (V3)

The post-diet intervention visit (V3) was at the end of week 8. All procedures from V1 were repeated at V3.

Sample analyses

Blood samples were collected in EDTA coated vacutainers (Becton, Dickinson, and Company, Franklin Lakes, NJ, USA) and placed on ice before centrifuging. Plasma was aliquoted and stored at -80°C until analysis. ANGPTL3 and ANGPTL4 concentrations were

measured using human DuoSet enzyme-linked immunoassay kits (R&D Systems, Inc., Minneapolis, MN, USA), and ANGPTL8 was measured using the Betatrophin (139-198) enzyme immunoassay kit (Phoenix Pharmaceuticals, Inc., Burlingame, CA, USA).

Statistical analyses

All statistical analyses were conducted using SAS version 9.4 statistical package (SAS Institute, Inc., Cary, NC, USA). All values were reported as mean±SEM unless otherwise stated. Statistical significance was set at $p \leq 0.05$. P values between 0.05 and 0.07 were treated as marginally significant. A sample size of 34 (17/group) was estimated to detect an intermediate effect size for postprandial ANGPTL4 (18) using G*power 3.19.7 assuming at least 80% power and an α of 0.05. This was lower than our sample size calculation for the primary outcome of blood lipids (38 with 19/group) which have been presented previously (17); thus, recruitment continued to satisfy the needs of the primary outcome and this secondary outcome. Per protocol analysis was decided a priori with a compliance requirement of at least 75%.

Change from baseline was calculated as the fasting value subtracted from each postprandial time point for all time course data. Repeated measures linear mixed models for treatment, visit, and time were used to test for between group differences. Incremental area under the curve (iAUC) was calculated using the trapezoid method. Additional repeated measures linear mixed models for treatment and visit were used to test for differences between groups for fasting ANGPTLs, anthropometric data, and iAUCs. Post hoc analyses were completed using Tukey's test. Self-reported food record data was averaged separately for "lead-in"/"baseline" records and then for intervention records. The averages for these two time periods were compared using repeated measures linear mixed models for treatment and time period

(period)(17). Finally, differences in compliance between groups were compared using an unpaired t-test.

Exploratory correlation analyses were done to determine potential relationships between ANGPTLs and blood lipids using Pearson Correlation Coefficients. Absolute values of fasting ANGPTLs, TG and body fat percentage, as well as meal responses for ANGPTLs and TG, were correlated at pre- and post-intervention visits. Additionally, to explore any relationships in the magnitude of change between visits for the meal responses, correlations were run on change data (V3-V1) for ANGPTLs and TG.

Results

Volunteers

One hundred and twelve volunteers were assessed for eligibility. A total of fifty-three adults were randomized to one of the two intervention groups. Of those randomized, only forty-two completed the study and have been included in this analysis **Figure 4.1**. Pre-diet intervention characteristics of the sample population are presented in **Table 4.2**. There were no differences in baseline characteristics between groups. Changes in anthropometrics have been presented previously (17). Of note, there were increases in body weight (kg) and corresponding BMI (kg/m^2) in both CSO and OO groups that were not different between groups. Self-reported compliance was not different between groups (CSO 91±2% vs. OO 92±1%), and no volunteer fell below the 75% compliance threshold for per protocol compliance.

Self-reported dietary intake

Self-reported daily nutrient intake averages before and during the interventions are displayed in **Table 4.3**. Overall, during both interventions, participants consumed a lower percent of their

daily energy from protein and carbohydrate and a greater percent of daily energy from fats compared to their pre-intervention diets with no differences between groups. There was an increase in percent of fat from MUFA in the OO group and an increase in % fat from PUFA and n-6 PUFA in the CSO group. These are expected changes and reflect the fatty acid profiles of the intervention oils. There was a greater reduction in percent fat from *trans*-fat in CSO that was driven by a higher percent fat from *trans*-fat at baseline in CSO and both groups reducing percent fat from *trans*-fat during the interventions. There were no other differences in self-reported nutrient intakes.

Fasting ANGPTLs

Fasting ANGPTL-3, -4, and -8 values are presented in **Table 4.4**. There were no differences between groups in fasting ANGPTL4 or ANGPTL8 values. There was a marginally significant difference between groups across the intervention for ANGPTL3 values ($p=0.06$). This difference was shown to be driven by an increase in the CSO group that was not significant after Tukey's correction ($p=0.12$).

Postprandial ANGPTLs

The time course for the meal responses for ANGPTL-3, -4, and -8 are presented in **Figure 4.2.** For both ANGPTL-3, and -4, there were significant treatment by visit interactions. Post-hoc analyses revealed an increase in the postprandial responses for the OO group ($p=0.006$, $p<0.001$) and no change in the CSO group ($p=0.99$, $p=0.99$) for ANGPTL-3 and -4, respectively. The iAUCs for ANGPTL-3, -4, and -8 are presented in **Figure 4.3**. There was a significant treatment by visit interaction for the ANGPTL4 iAUC driven by an increase from pre to post in OO (V1 -25.5 ± 12.1 to V3 34.8 ± 16.2 ng/ml/5h $p<0.01$) with no changes in CSO (V1 6.6 ± 7.8

to V3 7.9 ± 10.2 ng/ml/5h p=0.99). For ANGPTL3 iAUC, there was an effect of treatment (CSO V1 -76.6±60.1 to V3 -73.7±38.2 ng/ml/5h vs. OO V1 -261.6±60.7 to V3-136.9±63.4 ng/ml/5h, p=0.03) but no other main or interaction effects. Finally, there was a marginally significant difference (p=0.06) for a visit effect with greater postprandial ANGPTL8 at V3 vs. V1 in both groups, with no other main effects or interactions. ANGPTL8 also had no main or interaction effects in iAUC (CSO V1 0.07±2.70 to V3 1.30±1.84 ng/ml/5h; OO V1 -4.88±2.52 to V3 -1.38±2.09 ng/ml/5h, p=0.59).

Exploratory correlations

Pearson correlations of fasting and postprandial ANGTPLs with previously reported TG and body composition data [17] were conducted and are presented in **Tables 4.5 and 4.6**. Fasting correlations were first conducted separately by diet intervention groups for each visit. No significant associations were found likely due to low sample number; thus, both treatment groups were combined and analyzed by visit. Fasting ANGPTL3 was associated with body fat percentage at V1 (r=0.37 p=0.019) but not V3 (r=0.27, p=0.088). Conversely, Fasting ANGPTL8 was negatively associate with body fat percentage at V3 (r=-0.32, p=0.046) but not V1 (r=-0.13, p=0.426) (**Table 4.5**). With respect to the high SFA meal challenge, there were no associations between absolute values of postprandial TG and the ANGPTL meal responses for either group or at either visit (data not shown); however, there were associations with the magnitude of change in TG postprandial responses and the magnitude of change in ANGPTL meal responses between visits (V1 meal response subtracted from V3 meal response) (**Table 4.6**). Specifically, regardless of treatment group, there was a negative association between the change in postprandial ANGPTL8 and TG (r=-0.21, p=0.001) as well as a positive association between the change in ANGPTL3 and TG (r=0.16, p=0.015). Both relationships still existed when isolating the OO

group (TG v. ANGPTL3 r=0.27, p=0.003; TG v. ANGPTL8 r=-0.28, p=0.002) but not when isolating the CSO group (TG v. ANGPTL3 r=0.03, p=0.749; TG v. ANGPTL8 r=-0.16, p=0.09). Finally, when just comparing ANGPTL responses to one another, there was an association between the changes in ANGPTL3 and ANGPTL4 (r=0.16, p=0.016). When the OO group was isolated, there were relationships between the change in ANGPTL8 and ANGPTL4 (r=0.25, p=0.014), and ANGPTL3 with ANGPTL4 (r=0.22, p=0.028). When CSO was isolated, there was a similar relationship between AGNPTL4 and ANGPTL8 (r=-0.20, p=0.033); however, there was also a correlation between ANGPTL3 and ANGPTL8 (r=0.25, p=0.007) but not ANGPTL3 with ANGPTL4.

Discussion

In this secondary analysis comparing ANGPTL responses to CSO vs. OO enriched diets in adults with hypercholesterolemia, we found that eight weeks of daily OO (a high MUFA oil) dietary enrichment resulted in increased ANGPTL-3 and -4 meal responses vs. CSO (a high PUFA oil) with no significant between group differences for ANGPTL8. Conversely, we observed non-significant increases in fasting ANGPTL3 in the CSO group and reductions in fasting ANGPTL8 at V2 that recovered by V3 regardless of group. This data supports the hypothesis of differing postprandial responses after CSO vs. OO diets, but does not support the hypothesis of differing fasting responses. Further exploratory correlations revealed associations between postprandial ANGPTL and TG responses as well as body fat percentage and fasting ANGPTLs.

While it was hypothesized that CSO and OO would elicit different ANGPTL responses in this population, there were some unexpected aspects to the results. Specifically, the marginally significant increase in fasting ANGPTL3 with the CSO diet was not expected. Although this did

not reach the level of statistical significance (p=0.06), this difference could be important. An increase in ANGPTL3 could correspond to increases in circulating TG levels through its LPL inhibiting functions (10). The previous studies that assessed ANGPTL responses to PUFA-rich diets found that fasting ANGPTL3 either did not change (18) or decreased (26). With CSO being a high PUFA oil, it is unexpected that this data would be in opposition to both previous trials, but could be explained by differences in study populations. Both of those previous trials were in young healthy adults where they also observed decreases in fasting TG, while the current trial in a population at-risk for CVD had no such changes in fasting TG while the current trial in a population at-risk for CVD had no such changes in fasting TG (17). Importantly though, ANGPTL3 is thought of as being more active in the postprandial state (27), and ANGPTL3 alone must be at supraphysiologic levels to exert TG increasing affects (28, 29). Thus, the marginally significant increase in fasting ANGPTL3 within the CSO group may have low activity, which could diminish the clinical relevance. Further, our exploratory association between body composition and fasting ANGPTL3 at V1 suggests that higher body fat percentages may be related to higher fasting ANGPTL3 levels, which may relate to the already established association between higher adiposity and elevated TG (30). This relationship did not maintain significance a V3 suggesting the intervention may have modified the relationship of ANGPTL3 and body fat percentage.

While the lack of significant between group differences for fasting ANGPTL data was somewhat surprising, the postprandial responses between groups were interesting with differences driven by changes in the OO group. Our design, including high-fat meal challenges (lipemia tests), was intentional to be able to stress the lipid metabolism system in order to observe potential effects that might otherwise not be present in the fasting state. Similarly,

postprandial TG are increasingly being used in research due to their strong associations to CVD that are in some cases, better predictors of CVD than fasting TG (31-34). With this context, it is not surprising that we observed more changes in the postprandial state than in the fasted state. The observed postprandial ANGPTL3 increases with OO, and marginally significant increases in ANGPTL8, align with a previously proposed ANGPTL-3/-8 mechanism (35). In the postprandial state, ANGPTL-3 and -8 are combined in a 3:1 ratio (9) and secreted from the liver. Combined, ANGPTL-3/-8 are more active than either protein alone (29), and are believed to inhibit LPL, preventing lipid uptake at oxidative tissues (such as skeletal muscle) (35). This inhibition of LPL could be slowing TG clearance from circulation, which aligns with the higher postprandial TG levels previously reported for the OO group following the intervention (17). Our exploratory correlations confirmed a positive relationship in the OO group with increases in ANGPTL3 and TG meal responses, which follows the proposed function of ANGPTL3. However, the negative association of ANGPTL8 and TG meal response contradicts the previously proposed ANGPTL-3/-8 mechanism. Due to the exploratory nature of these associations more work would be required to confirm and understand this phenomenon.

The increase in ANGPTL4 with OO postprandially compared to CSO is additionally of interest in that it aligns with previous observations of OO enriched diets (26). However, ANGPTL4 is usually considered more active in the fasted state partly because postprandially secreted ANGPTL8 has the ability to bind ANGPTL4 in a 1:1 ratio, lowering the LPL inhibitory function of ANGPTL4 (9, 35). Since we observed an increase in ANGPTL4 only, it suggests an increase in free ANGPTL4 in circulation, that could inhibit LPL thereby slowing TG clearance from circulation after the meal (36). Therefore, the increase in free circulating ANGPTL4 may

have also contributed to the previously observed increase in postprandial TG in the OO group (17).

It has been shown that elevated postprandial TG levels are associated with increased risk for CVD events (37-39). If reduction/control of ANGPTLs can lower/control postprandial TG levels, it follows that they may be appropriate targets for CVD risk reduction. Data from genetic studies supports this logic, estimating that loss of function of ANGPTL3 could be associated with a 40% reduction in the odds of atherosclerotic CVD in part due to the lipid lowering that is possible with the reduction of ANGPTL3 (40, 41). Further, a high dose of atorvastatin (lipid lowering statin) was found to reduce ANGPTL3 mRNA by ~25% (42), which may contribute to the CVD risk reduction associated with statin use (43). If daily food choices, such as CSO consumption, could impact or help regulate ANGPTL responses, they have the potential to offer meaningful protection from CVD in at-risk adults.

It is still unknown why CSO and OO elicit different ANGPTL-3 and -4 responses from one another. We propose that this older population with already poor cholesterol profiles was potentially in some metabolic stress due to the high-fat nature of the intervention diets (50% fat) and observed slight weight gain in both groups (17). It has long been reported that high-fat feeding can lead to hyperlipidemic responses (44, 45). One previous study utilizing an OO-enriched diet that provided 50% fat diets reported increases in post-meal ANGPTL-3 and -4 with OO (18); however, a different intervention only utilized a 35% fat diet and reported no increased ANGPTLs in the control group (moderate MUFA diet) (26). While the OO intervention potentially caused TG regulation disruption, it is possible that the presence of a high-fat and/or slight energy surplus of the diet intervention was necessary, and that those factors worsened lipid metabolism that OO was not able to prevent. Conversely, CSO appears to be offering some

protection in this state of energy surplus and high-fat diet. PUFAs are already known to regulate transcription factors that control genes related to lipid metabolism including peroxisome proliferator activated receptors (PPARs) (46). Included among genes regulated by PPARs are ANGPTLs (47). Since CSO is rich in PUFAs, it may be able to counteract increases in expression of ANGPTLs that would otherwise be stimulated by a high-fat diet via regulation of PPARS. Whether this protection would be similar among other PUFA-rich foods, or is unique to CSO, is yet to be determined in humans.

If the high-fat diets would otherwise increase TG circulation, and the CSO group did not gain more fat mass than OO, the next curiosity relates to the fate of TG with CSO consumption. A potential explanation was presented by Son et al (48) who found that CSO tends to increase the mRNA expression and protein concentrations of ACOX1, a key rate-limiting enzyme of peroxisomal fatty acid β-oxidation (49) in addition to increasing PPAR expression. This suggests that CSO may be promoting utilization of lipid by fatty acid oxidation via regulation of ACOX1 in the liver, thus lowering lipid levels. However, this present study was not designed to determine mechanistic insights or draw tissue specific conclusions of ANGPTL-3, -4, or -8 functioning. Therefore, we are unable to confirm the exact mechanisms by which CSO may be eliciting protective effects on high-fat diet inducement of ANGPTLs. The presented data provides insight into future work aimed at discerning functional differences in habitual fat intake for the purpose of informing dietary choices for people at increased risk of CVD.

There were some limitations to this study. First, results from the use of a high-fat diet may not translate to lower fat diet patterns. A high-fat diet was chosen to match the dose of CSO used in previous studies (16, 50) but limits our ability to determine the extent of CSO or OO diet enrichment at lower fat intakes. Further, self-reported compliance and dietary intake measures

were used which are susceptible to over- and under-reporting. This trial was also not designed to be a mechanistic study. Tissue specific expression of ANGPTLs, concentrations of ANGPTL-3/-8 or ANGPTL-4/-8 complexes, and LPL enzyme activities were not measured which limits the conclusions that can be drawn from this data. Lastly, this trial was a secondary analysis and was not designed to evaluate differences based on sex, race, or age which warrants further work to determine.

In conclusion, we have shown that high-fat diets rich in OO increase circulating postprandial ANGPTL-3 and -4 compared to CSO in adults with hypercholesterolemia. This increase in ANGPTLs in the OO group may contribute to postprandial hyperlipidemia, an increasingly recognized CVD risk factor. The lack of increase in ANGPTL responses from the CSO group following eight weeks of a high-fat diet may have clinical significance in controlling or preventing increases in CVD risk. Additional work on the effects of these oils in other populations as well as with lower doses/fat intake is warranted. This data is valuable in its ability in power future studies to test hypotheses to determine if the phenomenon found in this secondary analysis can be confirmed.

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Table 4.1. Nutrient breakdown of provided intervention foods for each kilocalorie tier.

	Cottonseed Oil				Olive Oil			
Intervention Tiers (kcal)	<1,600	1,600-2,299	2,300-3,000	>3,000	<1,600	1,600-2,299	2,300-3,000	>3,000
Energy (kcal)	1,090	1,402	1,678	2,107	1,090	1,402	1,678	2,107
Energy (KJ)	4,561	5,866	7,021	8,816	4,561	5,866	7,021	8,816
Energy from protein (%)	7.2	6.7	6.5	6.8	7.2	6.7	6.5	6.8
Protein (g)	19.1	23.0	26.8	35.2	19.1	23.0	26.8	35.2
Energy from carbohydrates (%)	36.9	36.3	36.3	39.0	36.9	36.3	36.3	39.0
Carbohydrates (g)	98.0	124.0	148.5	200.6	98.0	124.0	148.5	200.6
Fiber (g)	3.2	4.1	5.1	7.9	3.2	4.1	5.1	7.9
Sugar (g)	51.3	64.8	76.5	101.5	51.3	64.8	76.5	101.5
Energy from fat (%)	55.9	57.0	57.2	54.1	55.9	57.0	57.2	54.1
Total Fat (g)	65.5	85.9	103.1	122.7	65.5	85.9	103.1	122.7
Saturated fat (g)	15.3	20.0	24.0	28.7	12.3	16.0	19.3	23.2
Trans fat (g)	0.17	0.22	0.27	0.38	0.17	0.22	0.27	0.38
Monounsaturated fat (g)	13.3	17.4	20.8	24.7	44.8	58.8	70.6	83.5
Polyunsaturated fat (g)	36.8	48.4	58.1	68.8	8.3	10.9	13.0	15.5
Omega 3 fatty acid (g)	0.28	0.36	0.43	0.51	0.60	0.79	0.95	1.13
Omega 6 fatty acid(g)	36.6	48.0	57.6	68.3	7.8	10.1	12.1	14.4
Cholesterol (mg)	57.5	75.2	89.8	121.0	57.5	75.2	89.8	121.0
Total fat from intervention oil (%)	88.2	87.3	87.3	85.6	88.2	87.3	87.3	85.6
Fat from intervention oil (g)	57.8	75.0	90.0	105.0	57.8	75.0	90.0	105.0

Daily nutrients delivered through the provided study foods within each treatment and energy tier. Participants were assigned to a kilocalorie tier based on their estimated energy requirements from a resting metabolic rate measurement at the screening visit. Energy tiers are named for the range of total energy requirements of the participants that were assigned to that tier. Energy (kcal in the first row and (KJ) in the second row) is the amount of energy actually provided each day from the diet intervention foods. The only difference between treatments was from the different treatment oil used (cottonseed oil vs. olive oil). <1,600 kcal = <6,694 KJ; 1,600-2,299 kcal = 6,694-9,619 KJ; 2,300-3,000 kcal = 9,623-12,552 KJ; >3,000 kcal = >12,552 KJ.

Table 4.2. Pre-diet intervention demographics and clinical characteristics of adults with hypercholesterolemia in cottonseed oil or olive oil diet groups

	Cottonseed Oil (n=21)	Olive Oil (n=21)	p values
	V1	V1	Treatment
Age (y)	53±2	54±2	0.73
Sex (% female)	57.1%	66.7%	0.53
Ethnicity (% Hispanic or Latino)	4.8%	0.0%	0.33
Race (% white)	100%	95.2%	0.33
Height (cm)	169.1±2.2	168.0±1.9	0.73
Weight (kg)	78.6±3.6	77.9±3.6	0.88
Body Mass Index (kg/m^2)	27.3±0.9	27.6±1.2	0.87
Waist to Hip ratio	0.85±0.02	0.82±0.02	0.33
Body Fat Percent	31.0±2.1	32.9±2.6	0.65
Total Cholesterol (mg/dL)	230±6.57	228±5.81	0.73
LDL cholesterol (mg/dL)	162±5.41	162±4.90	0.99
HDL cholesterol (mg/dL)	56.5±2.79	59.7±2.63	0.52
Triglyceride (mg/dL)	169±22.9	131±10.8	0.13
ApoB (mg/dL)	112±4.48	111±3.86	0.83
ANGTPL3 (ng/ml)	385±28	468±38	0.08
ANGPTL4 (ng/ml)	66.6±13.0	70.6±14.4	0.83
ANGPTL8 (ng/ml)	9.13±1.31	10.85±1.61	0.40

Values are presented as mean ± SEM or percent of total.
Analyzed using a two-sample t-test.
To convert total cholesterol, LDL cholesterol and HDL cholesterol measures from mg/dL to mmol/L multiply by 0.026.
To convert triglyceride measures from mg/dL to mmol/L multiply by 0.011
To convert ApoB from mg/dL to g/L multiply by 0.01

Table 4.3. Self-reported daily nutrient intake for diets enriched with cottonseed oil or olive oil in adults with hypercholesterolemia.

	Cottonseed Oil (n=21)		Olive Oil (n=21)		P values		
Nutrient	Baseline	Intervention	Baseline	Intervention	Treatment	Period	Treatment x Period
Energy (kcal)	2,075 ± 187	2,450 ± 110	2,563 ± 199	2,538 ± 124	0.11	0.17	0.12
Energy from protein (%)	15.6 ± 1.01	10.8 ± 0.27	14.6 ± 0.76	10.9 ± 0.36	0.50	<0.01	0.38
Energy from carbohydrate (%)	46.0 ± 2.16	39.2 ± 0.76	46.0 ± 2.17	39.3 ± 0.88	0.99	<0.01	0.97
Fiber (g)	19.8 ± 2.11	14.7 ± 1.12	22.0 ± 2.18	13.1 ± 0.68	0.80	<0.01	0.17
Sugar (g)	93.0 ± 14.9	105 ± 5.46	108 ± 14.8	109 ± 8.44	0.43	0.44	0.53
Energy from fat (%)	35.5 ± 1.85	49.0 ± 0.79	36.8 ± 1.54	47.6 ± 0.75	0.97	<0.01	0.16
Saturated fat (% fat)	34.3 ± 1.33	26.3 ± 0.41	32.6 ± 1.19	23.7 ± 0.43	0.04	<0.01	0.58
Trans fat (% fat)	1.1 ± 0.2	0.4 ± 0.1 *	0.7 ± 0.1#	0.4 ± 0.0	0.05	<0.01	0.03
Monounsaturated fat (% fat)	41.8 ± 2.06	24.3 ± 0.69 *	39.9 ± 1.35	59.9 ± 0.62 *^	<0.01	0.24	<0.01
Polyunsaturated fat (% fat)	22.8 ± 1.84	48.7 ± 0.89 *	26.8 ± 1.63	15.8 ± 0.55 *^	<0.01	<0.01	<0.01
Omega 3 fatty acid (% fat)	2.4 ± 0.3	0.8 ± 0.1	3.0 ± 0.5	1.2 ± 0.1	0.15	<0.01	0.75
Omega 6 fatty acid (% fat)	20.3 ± 1.64	47.9 ± 0.91 *	23.8 ± 1.21	14.7 ± 0.50 *	<0.01	<0.01	<0.01
Energy from alcohol (%)	2.8 ± 1.0	1.0 ± 0.4	2.6 ± 1.0	2.3 ± 0.7	0.60	<0.01	0.06

All values are mean ± SEM (N=42). Baseline represents an average of the two food diaries before the intervention. Intervention represents an average of all food diaries kept during the 8-week intervention. Main and interaction effects were analyzed using a repeated measures linear mixed model for treatment and period.

^ indicates a significant difference between CSO vs. OO during the intervention at $p<0.05$.

* indicates significant difference between intervention vs. baseline within a group at $p<0.05$.

# indicates significant difference between CSO vs. OO at baseline at $p<0.05$.

Table 4.4. Fasting ANGPTLs at pre-, mid- and post-diet intervention visits for eight weeks of cottonseed oil or olive oil enriched diets in adults with hypercholesterolemia.

	Cottonseed Oil (n=21)			Olive Oil (n=21)			p values		
	V1	V2	V3	V1	V2	V3	Treatment	Visit	Interaction
ANGTPL3 (ng/ml)	385±28	415±37	440±34	468±38	472±34	449±29	0.32	0.25	0.06
ANGPTL4 (ng/ml)	66.6±13.0	62.2±13.9	71.0±16.8	70.6±14.4	65.3±12.1	63.0±12.2	0.90	0.83	0.21
ANGPTL8 (ng/ml)	9.13±1.31	7.86±0.87	8.16±1.15	10.85±1.61	8.63±1.14	10.44±1.95	0.40	0.01	0.32

Values are presented as means±SEM.

Analyzed using repeated measures linear mixed models for treatment and visit.

ANGPTL - Angiopoietin-like protein, V1 – Pre-Intervention visit, V2 – Mid-Intervention visit, V3 – Post-Intervention visit

Table 4.5. Correlation matrix for fasting ANGPTLs, triglycerides, and body fat percentage in adults with hypercholesterolemia

	ANGPTL 3	ANGPTL 4	ANGPTL 8	Triglycerides	Body fat percentage
Pre-diet intervention visit					
ANGPTL 3					
ANGPTL 4	r: 0.12 p: 0.461				
ANGPTL 8	r: 0.11 p: 0.471	r: -0.07 p: 0.656			
Triglycerides	r: -0.12 p: 0.437	r: -0.14 p: 0.418	r: -0.20 p: 0.195		
Body fat percentage	**r: 0.37** **p: 0.019**	r: 0.08 p: 0.641	r: -0.13 p: 0.426	r: 0.12 p: 0.455	
Post-diet intervention visit					
ANGPTL 3					
ANGPTL 4	r: 0.18 p: 0.275				
ANGPTL 8	r: -0.07 p: 0.667	r: -0.16 p: 0.326			
Triglycerides	r: -0.5 p: 0.750	r: -0.15 p: 0.375	r: -0.26 p: 0.095		
Body fat percentage	r: 0.27 p: 0.088	r: 0.23 p: 0.179	**r: -0.32** **p: 0.046**	r: 0.28 p: 0.080	

Pearson correlation matrix of fasting ANGPTLs, triglycerides, and body fat percentage for both cottonseed oil and olive oil groups combined.
r – Pearson correlation coefficient, ANGPTL – angiopoietin-like protein.

Table 4.6. Correlation matrix for the magnitude of change between visits in ANGPTLs and triglycerides in adults with hypercholesterolemia

Both oil groups	ANGPTL 3	ANGPTL 4	ANGPTL 8	Triglycerides
ANGPTL 3				
ANGPTL 4	**r: 0.16** **p: 0.016**			
ANGPTL 8	r: 0.10 p: 0.113	r: 0.05 p: 0.440		
Triglycerides	**r: 0.16** **p: 0.015**	r: -0.02 p: 0.725	**r: -0.21** **p: 0.001**	
Cottonseed oil				
ANGPTL 3				
ANGPTL 4	r: -0.04 p: 0.698			
ANGPTL 8	**r: 0.25** **p: 0.007**	**r: -0.20** **p: 0.034**		
Triglycerides	r: 0.03 p: 0.750	r: 0.02 p: 0.795	r: -0.16 p: 0.095	
Olive oil				
ANGPTL 3				
ANGPTL 4	**r: 0.22** **p: 0.028**			
ANGPTL 8	r: -0.07 p: 0.476	**r: 0.25** **p: 0.014**		
Triglycerides	**r: 0.27** **p: 0.003**	r: 0.03 p: 0.772	**r: -0.28** **p: 0.002**	

Pearson correlation matrix for ANGPTLs and triglycerides for all subjects and by the cottonseed oil and olive oil diet enrichments separately. Magnitude of change calculated as V3-V1 meal response.
r – Pearson correlation coefficient, ANGPTL – angiopoietin-like protein, V1 - pre-diet intervention visit, V3 – post-diet intervention visit.

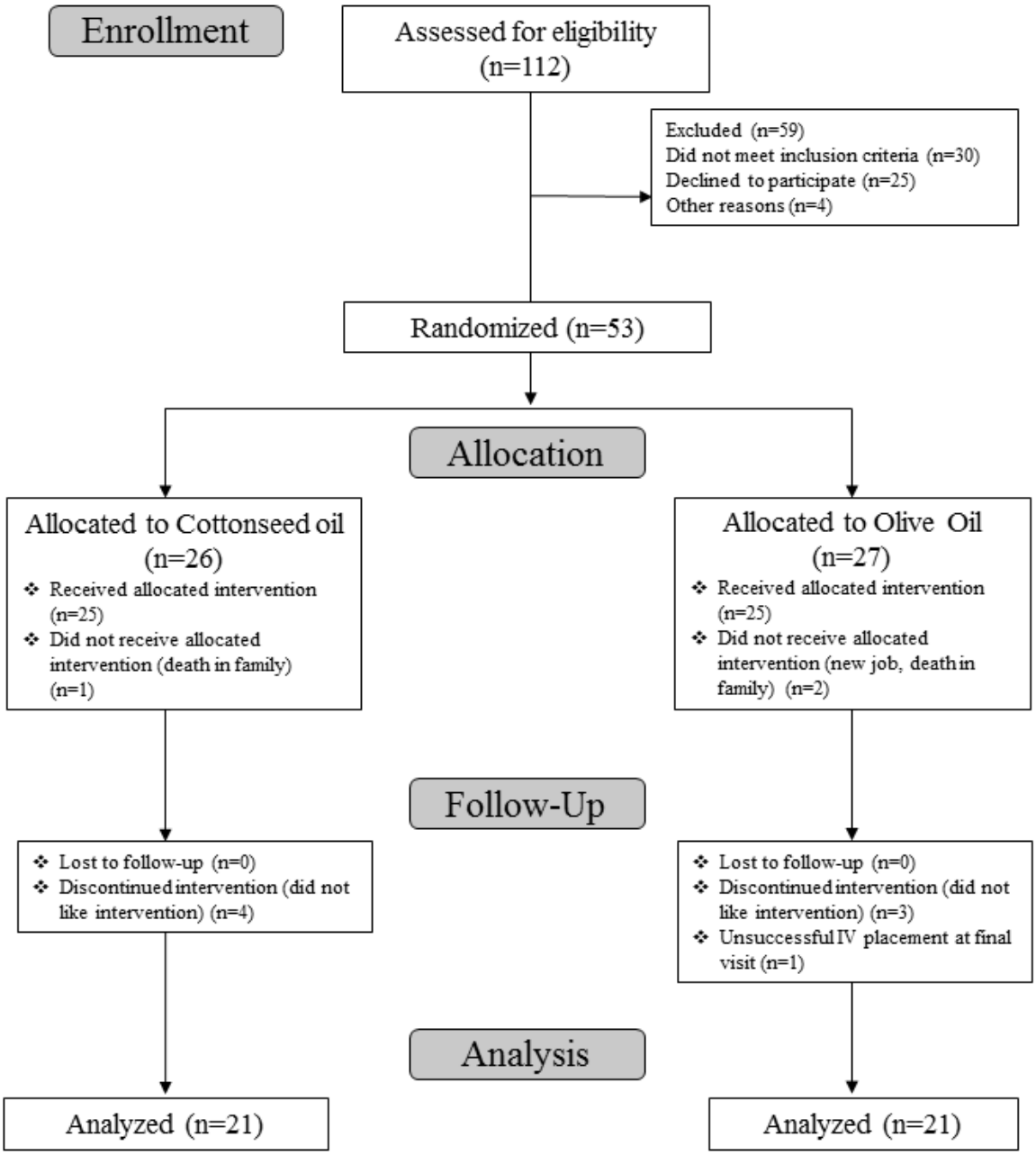

Figure 4.1. CONSORT flow diagram determining the eligibility of participants for a partial outpatient feeding trail comparing cottonseed oil to olive oil enriched diets in adults with hypercholesterolemia.

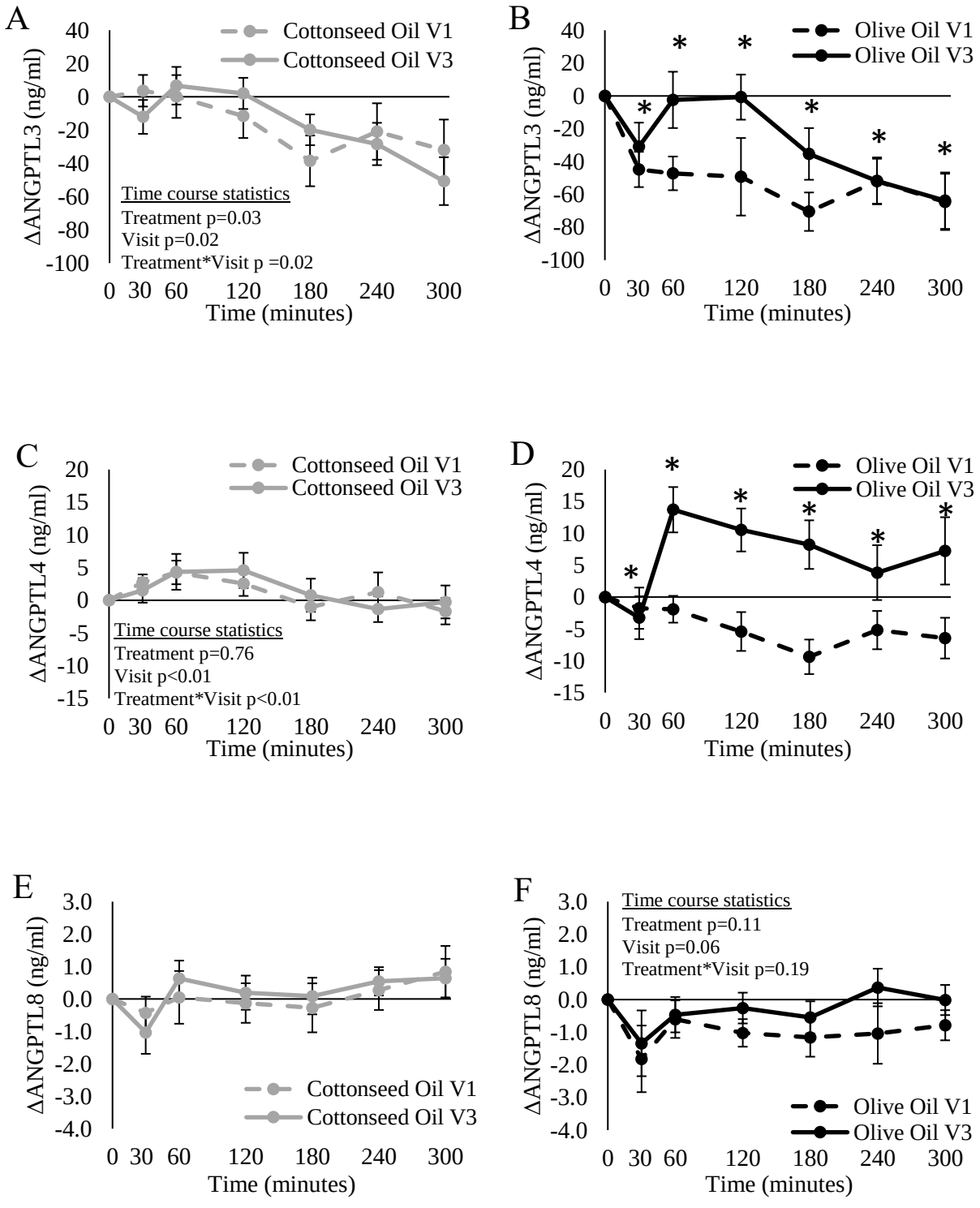
A
ΔANGPTL3 (ng/ml)
Cottonseed Oil V1
Cottonseed Oil V3
Time course statistics
Treatment p=0.03
Visit p=0.02
Treatment*Visit p =0.02
Time (minutes)
B
ΔANGPTL3 (ng/ml)
Olive Oil V1
Olive Oil V3
Time (minutes)
C
ΔANGPTL4 (ng/ml)
Cottonseed Oil V1
Cottonseed Oil V3
Time course statistics
Treatment p=0.76
Visit p<0.01
Treatment*Visit p<0.01
Time (minutes)
D
ΔANGPTL4 (ng/ml)
Olive Oil V1
Olive Oil V3
Time (minutes)
E
ΔANGPTL8 (ng/ml)
Cottonseed Oil V1
Cottonseed Oil V3
Time (minutes)
F
ΔANGPTL8 (ng/ml)
Time course statistics
Treatment p=0.11
Visit p=0.06
Treatment*Visit p=0.19
Olive Oil V1
Olive Oil V3
Time (minutes)

Figure 4.2. Time course of ANGPTL3 (A, B), ANGPTL4 (C, D) and ANGPTL8 (E, F) concentrations in CSO and OO groups. Data were analyzed using a repeated measures linear mixed model for treatment, time, and visit. Main effects of time were all significant at $p<0.01$ and the meal response for ANGPTLs is already established, thus it is not displayed in the figure. Main effects of treatment, and visit were found for ANGPTL3. ANGPTL4 had a main effect of visit and ANGPTL8 had a marginally significant visit effect. Treatment*visit effects show a greater increase in post meal ANGPTL-3 and -4 from V1 to V3 in OO vs. CSO. Δ - change from baseline, ANGPTL3 - angiopoietin like protein 3, ANGPTL4 - angiopoietin like protein 4, ANGPTL8 - angiopoietin like protein 8, V1 - pre-diet intervention visit, V3 – post-diet intervention visit, CSO – cottonseed oil, OO – olive oil. * indicates significant treatment*visit interaction.

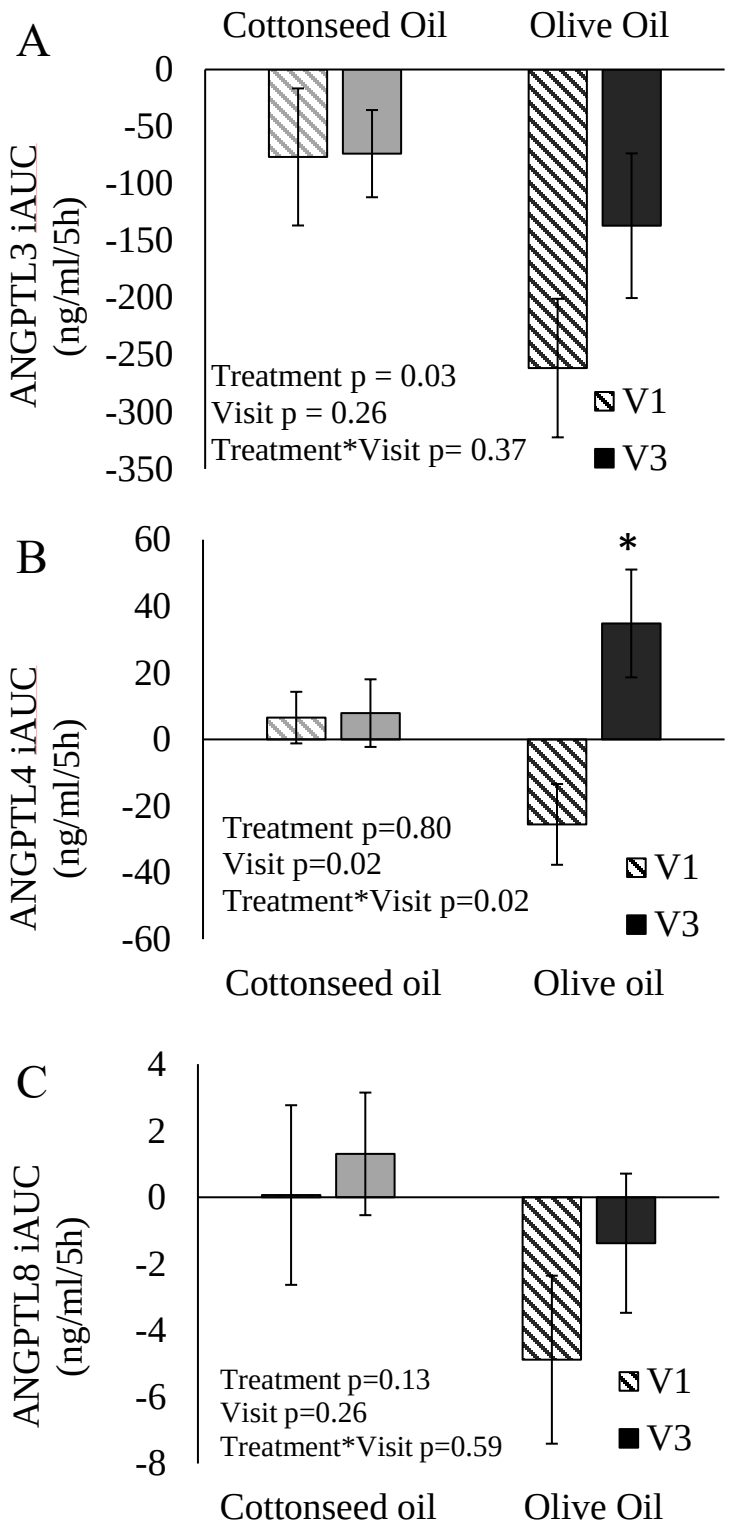
A
Cottonseed Oil
Olive Oil
ANGPTL3 iAUC (ng/ml/5h)
0
-50
-100
-150
-200
-250
-300
-350
Treatment p = 0.03
Visit p = 0.26
Treatment*Visit p= 0.37
V1
V3
B
ANGPTL4 iAUC (ng/ml/5h)
60
40
20
0
-20
-40
-60
*
Treatment p=0.80
Visit p=0.02
Treatment*Visit p=0.02
V1
V3
Cottonseed oil
Olive oil
C
ANGPTL8 iAUC (ng/ml/5h)
4
2
0
-2
-4
-6
-8
Treatment p=0.13
Visit p=0.26
Treatment*Visit p=0.59
V1
V3
Cottonseed oil
Olive Oil

Figure 4.3. Incremental area under the curve (iAUC) of ANGPTL3 (A), ANGPTL4 (B), and ANGPTL8 (C) concentrations in CSO and OO groups. Data were analyzed using a repeated measures linear mixed model for treatment and visit. There was a treatment effect for lower ANGPTL3 in OO vs. CSO regardless of visit. There was a visit effect and a treatment*visit interaction for greater increases in ANGPTL4 in OO vs. CSO group. There were no main or interaction effects for ANGPTL8. ANGPTL3 - angiopoietin like protein 3, ANGPTL4 - angiopoietin like protein 4, ANGPTL8 - angiopoietin like protein 8, V1 - pre-diet intervention visit, V3 – post-diet intervention visit, CSO – cottonseed oil, OO – olive oil. * indicates significant treatment*visit interaction.

CHAPTER 5

METABOLIC RESPONSES TO 8 WEEKS OF CONSUMING COTTONSEED OIL VERSUS OLIVE OIL IN ADULTS WITH DYSLIPIDAEMIA: A RANDOMISED TRIAL[3]

[3] Prater M.C., Scheurell A. R., Paton C. M., Cooper J. A. 2023. *Journal of Human Nutrition and Dietetics*. 36(3):1079-89. Reprinted here with permission of the publisher

Abstract

Background: Differences in metabolic responses between diets rich in monounsaturated (MUFA) and polyunsaturated fats (PUFA) could affect energy balance and weight maintenance. The present study was a secondary analysis to investigate 8-week diet interventions rich in either PUFA (cottonseed oil (CSO)) or MUFA (olive oil (OO)) on metabolic responses in adults with dyslipidemia.

Methods: Forty-one adults with dyslipidemia completed this randomized trial consisting of an 8-week partial-outpatient feeding trial. Provided foods accounted for ~60% of their daily energy needs, with ~30% of energy needs provided by CSO (n=20) or OO (n=21). At pre- and post-diet intervention visits, participants consumed a high saturated fat (SFA) meal (35% daily energy needs, 47.9% from SFA), and fasting and 3.5h postprandial indirect calorimetry was used to measure energy expenditure (EE) and substrate oxidation.

Results: No changes were observed in fasting measures. The OO group had greater increases in postprandial EE (p=0.002); however, there were no differences in substrate oxidation between groups. A lack of metabolic flexibility was found in both groups, which was partially explained by changes in insulin sensitivity (homeostasis model assessment of insulin resistance (HOMA-IR)).

Conclusions: The results of the present study show OO, but not CSO, diet enrichment improves EE following the occasional high SFA meal, which may improve weight maintenance over time. Registered at clinicaltrials.gov (NCT04397055).

Introduction

It is well established that U.S. adults gain an average of 0.5 to 1 kg of weight per year throughout middle age which can contribute to overweight and obesity over time (1, 2). The current rate of obesity is over 40% in the United States (3) and may be driven, in part, by over-consumption of energy dense foods, often rich in fat (4). Obesity is a major risk factor for cardiovascular disease (CVD) and other chronic diseases (5). While this disease is multifactorial in cause, it can be promoted or exacerbated by an energy surplus where energy intake (EI) exceeds energy expenditure (EE) (6, 7). Thus, there is a need to identify how different dietary fats affect EE and substrate utilization because small changes can be clinically relevant for weight maintenance.

Prior research suggests that unsaturated fatty acids are more metabolically active/beneficial than saturated fatty acids (SFA)(8). Both acute high-fat (HF) meal challenges and longer-term dietary interventions have shown increases in diet induced thermogenesis (DIT) (9-11) and fat oxidation (10-12) for unsaturated fat compared to SFA (8). Lower measures of EE and fat oxidation with high SFA meals and/or diets compared to unsaturated fats (9, 10, 13-15) has led to their association with acute energy surplus and macronutrient imbalance, which can contribute to fat and weight gain. Polyunsaturated fatty acid (PUFA)-rich diets seem to be particularly beneficial for improving postprandial fat oxidation compared to SFA-rich diets (15-18). Unfortunately, the literature on differences between MUFA vs. PUFA, is limited. Stable isotope studies reveal that individual MUFAs tend to be more highly oxidized than n-6 PUFAs (19, 20); however, n-3 PUFA is more highly oxidized than MUFA and n-6 PUFA (19). To further complicate the issue, whole body substrate utilization as measured by indirect calorimetry

has not shown acute differences between MUFA- vs. PUFA-rich meals (13, 14, 21). In those studies, DIT was increased with either PUFA only (13) or MUFA and PUFA equally (14).

Cottonseed oil (CSO) is a plant oil that has been in the U.S. food supply for over 100 years, primarily as part of vegetable oil blends. It is commonly used in salad dressings, snack items, and for frying foods in restaurants. It is rich in n-6 PUFA (57.0%) with a moderate amount SFA (22.7%), and lower amounts of MUFA (19.9%) and n-3 PUFA (0.4%) (22). Despite its relatively high level of SFA, CSO has been shown to improve fasting and postprandial lipid metabolism, including relatively large improvements in total and LDL cholesterol (22-24). While this is important for chronic disease risk reduction, other effects of CSO consumption on health, and whole body metabolism in particular, are largely unknown. Conversely, numerous studies have assessed the acute and chronic effects of olive oil (OO) consumption on energy metabolism (10, 11, 25, 26). Olive oil is commonly used worldwide and is a rich source of MUFA (70.9%) but contains lower amounts of SFA (17.5%) and PUFA (n-6: 10.8%, n-3: 0.8%) (22). To date, only one study has compared diets enriched in OO vs. CSO (27), which showed favorable improvements in substrate utilization (RER, carbohydrate and fat oxidation), but not EE, in young men with CSO diet enrichment only; however, this intervention was just 5 days in duration and included only young healthy men. Therefore, the purpose of the present study was to complete a secondary analysis in which we assessed the impact of 8-week diets enriched with CSO vs. OO on fasting energy metabolism and substrate utilization, as well as postprandial metabolic responses to a SFA-rich meal challenge, in adults with dyslipidemia. We hypothesized that the CSO-enriched diet would improve fasting and postprandial EE and substrate utilization (higher fat oxidation) more than OO diet enrichment.

Methods

This was a single-blind, randomized, parallel trial (clinicaltrials.gov: NCT04397055) involving an 8-week partial outpatient feeding intervention conducted at the University of Georgia. Recruitment began in May 2018 and final testing took place in June 2021 when participant testing was completed to address all primary and secondary outcomes. The protocol included a screening visit and two testing visits (pre- and post-diet intervention). Subjects were randomly assigned using balanced blocks stratified by total cholesterol (TC), low-density lipoprotein cholesterol (LDL-c), and body mass index (BMI) to either an OO or CSO intervention arm in 1:1 allocation ratio (22). Both groups were provided meals enriched with the assigned oil in a partial outpatient feeding trial controlling ~60% of the participants energy requirements as estimated from resting metabolic rate (RMR) measurement at screening. This study was conducted according to the guidelines laid down by the Declaration of Helsinki and the Institutional Review Board for human subjects approved all procedures involving human subjects. Informed written consent was obtained from each participant prior to testing.

Participants

Fifty-three sedentary adults between the ages of 30 and 75y with dyslipidemia were recruited for the study. We included men and women whose blood lipids at the screening were "borderline undesirable" or "at risk" in two blood lipid measures or "undesirable" in one. "Borderline undesirable" or "at risk" was defined as TC 180-239 mg/dL, LDL-c 110-159 mg/dL, HDL-c 40-50 mg/dL, or triglycerides (TG) 130-200 mg/dL while "undesirable" were defined as TC $\geq$ 240, LDL $\geq$ 160, HDL-c $<$ 40, or TG $\geq$ 200 (3, 28-30).

To rule out individuals with familial hypercholesterolemia, participants with LDL-c levels greater than the 95th percentile or HDL-c levels lower than the 20th percentile were excluded as recommended by the lifelines cohort study (31). Complete exclusion criteria have

been previously provided (22). Briefly, chronic or metabolic diseases, gastrointestinal surgeries or conditions, regular exercise (>3h/wk), medication or supplement use affecting metabolism, weight instability, nicotine use, or excess alcohol use resulted in exclusion.

Screening visit

Participants reported to the Human Nutrition Laboratory (HNL) following an 8-12h overnight fast and 24h abstaining from exercise and alcohol. A fasting blood sample was collected for a basic blood lipid panel and glucose. Anthropometrics were collected prior to the 30-min indirect calorimetry measurement (TrueOne 2400; Parvo Medics) under standard conditions (32). The final 20 min of respiratory gasses were used to calculate RMR using the Weir equation (33). Estimated total daily energy needs were calculated as participant's RMR multiplied by 1.65, an average U.S. physical activity factor (34). This estimated energy need was used for determination of volume of test meal to provide, as well as assignment to energy tiers for the diet intervention. Additionally, habits of alcohol consumption were assessed by the Alcohol Use Disorders Identification Test (35). Qualifying participants were then randomized to either the OO or CSO diet intervention group by a researcher who was not involved in the collection or analysis of these data using a random-number generator. Groups were balanced for total TC, LDL-c, and BMI.

Pre-diet intervention visit (V1)

Participants completed a 2-day food record containing 1 week day and 1 weekend day prior to the pre-diet intervention visit (V1) (36). A lead-in meal was consumed the night before V1 consisting of a frozen meal and ready to eat snack bar that provided 50% of total energy from carbohydrate, 15% from protein and 30% from fat. Participants then arrived at the HNL

following an 8-12h overnight fast and 24h without exercise and alcohol. A Bod Pod (Cosmed USA, Inc.) was used to measure body composition. Additional fasting measures included height, weight, waist and hip circumference, blood pressure and RMR.

Following fasting measures, participants consumed a SFA-rich, HF test meal, which provided 35% of total daily energy needs. The meal was made from an original, milk-chocolate, ready-to-drink shake (Ensure, Abbott Laboratories, Inc.), unsalted butter, red palm oil, coconut oil, soy lecithin granules, and powdered chocolate drink mix. Furthermore, this meal had 5.0% energy from protein, 25.0% from carbohydrate, and 70% from dietary fat (46.9% SFA, 15.7% MUFA, 6.9% PUFA). The meal was designed to provide the maximum amount of SFA using common fat sources while maintaining palatability of the meal. Participants had 10 minutes to drink the meal after which, 4 oz of water was used to rinse out the container, which was then also consumed. High-fat meal challenges are commonly provided as a liquid rather than solid meal (37-39), likely due to their ability to be consumed quickly and easily, and have been shown to be comparable for measuring circulatory markers of lipid metabolism and metabolic function parameters in previous studies (40, 41). A SFA-rich meal challenge was used to observe how diets enriched with CSO and OO influence metabolic responses to an occasional unhealthy meal. Following the SFA-rich meal, indirect calorimetry was measured in 20 min increments followed by 10 min breaks for 3.5 h. Four ounces of water was provided once every hour.

Partial Outpatient Feeding Protocol

Participants began the 8-week diet intervention (either CSO or OO enriched) the day following V1. Participants were blinded to their group allocation and codes were used on all study materials to maintain the single blind. A detailed description of procedures used to prepare study meals has been previously reported (22). Briefly, the two groups had identical recipes with

the only difference being the oil used. Provided foods were designed to provide about 60% of the participants energy needs which was accomplished by assigning participants to a kilocalorie tier based on their RMR measured at screening as previously described (22). A partial outpatient feeding protocol was chosen to allow the results from this trial to be more applicable to free living conditions while still ensuring the delivery of the intended amount of intervention oils. Foods were prepared in research kitchens by research personnel who weighed all ingredients to the 0.01g. Two meals and snacks were provided each day in a 7-day rotating menu pattern including breakfast and lunch/dinner entrée. Weekly responsibilities included meal compliance checklists for self-reported compliance, and two-day food diaries completed every other week including one week day and one weekend day. Participants were instructed to report all consumed foods including self-selected items. Compliance cut off was set at reporting consumption of at least 75% of provided foods. The a priori decision to remove any participants not meeting this compliance cut-off was in place; however, all participants that completed the trial met this compliance requirement. The Food Processor SQL software (ESHA Research; version 10.12.0) was used to analyze self-reported nutrient intakes from food diaries. Once per week participants returned to the HNL to pick up weekly meals, as well as turn in compliance checklists and food diaries.

Post-diet intervention visit (V2)

At the end of the 8-week partial outpatient feeding protocol, participants returned to the HNL for the post-intervention visit (V2). At V2, anthropometric measures, fasting metabolism measures, high SFA meal challenge and postprandial metabolism measures were repeated in an identical fashion as described above for V1.

Calculations

The final 20 min of the 30 min fasted indirect calorimetry measurements at screening, V1, and V2, was used to calculate RMR via the Weir equation (33). At V1 and V2, the final 15 min from each 20 min postprandial measurement was used to calculate EE (32, 33) and respiratory exchange ratio (RER). Diet induced thermogenesis (DIT) was calculated by subtracting postprandial EE from baseline EE to isolate the thermogenic effect of the meal challenge. Macronutrient utilization was calculated with equations developed by Frayn et al (42). Methanol burns were conducted for the development of correction factors that were applied to the collected data (43). The average correction factors were 99.3% and 98.6% for O_2 and CO_2, respectively. Macronutrient averages from food records were used to calculate food quotient (FQ) (44, 45) from the two respective periods (baseline, intervention). As proxy measures of metabolic flexibility, defined as the ability to respond or adapt to conditional changes in metabolic demand including the ability to appropriately utilize available fuels, the fasting RER:FQ ratio was calculated as explained in Carstens et al (45) as well as the difference between RER and FQ. To accompany our analysis of metabolic flexibility, the homeostasis model assessment of insulin resistance (HOMA-IR) was calculated as an indication of insulin resistance status (46) from fasting plasma glucose and insulin (22).

Statistical analysis

SAS version 9.2 (SAS Institute Inc., Cary, NC) was used for statistical analyses. All values were reported as mean ± SEM unless otherwise noted. Statistical significance was set at $p \leq 0.05$. A sample size of 38 (19 per group) was estimated post hoc using a Cohen's F of 0.238 to detect a difference in postprandial fat oxidation based on the results from Polley et al (27). Sample size was calculated using G*power 3.19.7 assuming at least 80% power and an α of 0.05. This is in agreement with our sample size for primary outcomes of fasting blood lipids (22). The

decision to use per-protocol analyses was made a priori. A repeated measures (RM) linear mixed model for treatment (CSO v. OO) and time (visit) was used to determine between and within group differences for fasting metabolic data, metabolic flexibility score, HOMA-IR, and anthropometric data. Two averages of self-reported dietary intake were calculated from the collected food diaries: one was the average of the two-day baseline food record, and the other was the average of the two-day records collected 4 times during the intervention. Averages of dietary intake and FQ were analyzed using a similar RM linear mixed model as fasting data. Postprandial data was assessed for between and within group differences using a RM linear mixed model for treatment (CSO v. OO), time (visit), and time point. In all linear mixed models, participants were modeled as random effects. When significance was found, post hoc analyses were done using Tukey's test. Unpaired t-tests were used to assess differences in self-reported compliance.

Finally, exploratory multiple regression analyses were conducted to determine predictors for the change in metabolic flexibility score (RER:FQ ratio). Scatter plot matrices of baseline and change variables were used to select which variables to include in the model to avoid collinearity between predictor variables. Factors included in the model were baseline body fat percentage, waist circumference, and TG (22), change in weight, change in HOMA-IR, change in LDL (22), age, sex (0=woman, 1=man), and treatment (0=CSO, 1=OO). To determine predictors, four different model selection methods were used. Best subsets methods were used to select models that minimized Mallows Cp (47), Akaike Information Criteria (AIC) (48), and Bayesian Information Criteria (BIC) (49). Two-way stepwise inclusion technique utilized alpha to enter and alpha to stay at 0.10 for both (50). The models selected by the different criteria were compared to determine the most appropriate model to continue using in this analysis (51, 52).

Multiple regression analysis was used to model the change in metabolic flexibility score (fasting RER:FQ ratio) with the predictors obtained from the model selection methods.

Results

Participants

Fifty-three participants were allocated to either the CSO or OO group but twelve participants never received the intervention, discontinued their participation, or could not complete the metabolic measurements at both visits (**Figure 5.1**). Thus, forty-one participants (CSO: 11 women and 9 men, OO: 14 women and 7 men) completed the study and were included in the final per protocol analysis. There were no differences in self-reported compliance (CSO: 91.0 ± 1.6%; OO: 92.5 ± 1.2%; p= 0.47). Participant characteristics at pre- and post- intervention visits are presented in **Table 5.1**. There were no differences between groups at pre-intervention for any anthropometric or metabolic variables. Regardless of group assignment, there were increases in weight ($p<0.001$), BMI ($p<0.001$), and waist circumference ($p=0.04$) from pre- to post-intervention; however, hip circumference, waist to hip ratio, or body fat percentage did not change.

Average self-reported intakes of baseline and during the intervention are presented in **Table 5.2.** At baseline, the only difference between groups was higher reported *trans*-fat intake in CSO vs. OO ($p<0.001$). Regardless of group assignment, there were reported decreases in protein, carbohydrates, fiber, SFA, *trans* fat, n-3, alcohol, ($p<0.001$ for all) as well as cholesterol and sodium ($p=0.007$ for both) from baseline to intervention. Conversely, there were increases in total fat ($p<0.001$) in both groups although the changes differed based on fatty acid type. There

were greater increases in MUFA for OO vs. CSO ($p<0.001$) while there were greater increases in PUFA and n-6 for CSO vs. OO ($p<0.001$ for both).

Fasting metabolism measures

Fasting RMR, RER, fat oxidation, and carbohydrate oxidation are presented in **Table 5.3.** There were no significant differences in fasting metabolic measurements including RMR, RER, fat oxidation, or carbohydrate oxidation from pre- to post-intervention.

Postprandial metabolism measures

Time course data for EE, RER, fat oxidation, and carbohydrate oxidation in response to the high SFA meal challenge are presented in **Figures 5.2 and 5.3**. The DIT is also presented in **Figure 5.2**. There was an increase from pre- to post-diet (change of 8.22±4.99 kcal over 3.5h) in postprandial EE in OO ($p=0.003$) with no changes in CSO (change of -2.36±4.05 kcal over 3.5h, $p=0.99$) (**Figure 5.2 A, B**). Similarly, there was a greater increase in DIT from pre- to post-visit (change of 10.0±4.46 kcal over the 3.5h period) in OO only (OO: $p<0.001$; CSO change of 0.39±5.25 kcal over 3.5h, $p=0.99$) (**Figure 5.2 C, D**). There were no changes observed in postprandial RER (**Figure 5.3 A, B**), fat oxidation (**Figure 5.3 C, D**), or carbohydrate oxidation for either treatment group from pre- to post-intervention (**Figure 5.3 E, F**).

Exploratory Regression Analysis

Although there were no changes in fasting RER for either group, there was a decrease from pre-to post-intervention in calculated FQ, regardless of group assignment, indicating the increase in dietary fat intake from baseline to intervention. However, there were no differences in the metabolic flexibility score or HOMA-IR (**Table 5.3**). Based on our lack of change in substrate utilization after placing adults on HF diets, but variation among subjects in the change

of metabolic flexibility score, exploratory best subsets and two-way stepwise multiple regression model selection approaches were employed to determine predictors of the change in metabolic flexibility score. The best model selection method for Mallow's Cp, BIC, as well as the two-way stepwise inclusion method indicated the best model included the change in HOMA-IR only. AIC selected a model that included both the change in HOMA-IR and the change in LDL; however, we chose the model that only included change in HOMA-IR since a majority of the model selection methods selected that variable only. When the regression model was analyzed, the change in HOMA-IR (β=0.03; p=0.0036) explained 25.5% of the variability in the change in metabolic flexibility (Full Model: intercept=0.01; p=0.0036).

Discussion

While several trials have shown the metabolic benefits of meals or diets rich in unsaturated fatty acids, diets enriched with CSO and OO have never been compared in adults with dyslipidemia. Contrary to our hypothesis, we have shown OO diet enrichment increased postprandial measures of EE and DIT while CSO had no effect on these measures of energy metabolism. Additionally, contrasting to our hypothesis, there were no changes in fasting metabolic measures (RMR, substrate oxidation) nor postprandial substrate oxidation (RER, carbohydrate or fat oxidation), regardless of treatment.

The only other published study comparing CSO to OO diet enrichment, previously done in healthy, young adult men, also saw no changes in fasting measures after either treatment. They did, however, observe postprandial changes in substrate utilization that suggested a CSO-enriched diet improves fat oxidation (27). It is difficult to compare those improvements to our study results since the test meals in Polley et al included CSO or OO and ours was rich in SFAs without any OO or CSO. We are only aware of one other study that used SFA-rich meal

challenges after a high PUFA diet, and they observed improvements in postprandial substrate oxidation (18). That high PUFA diet was rich in both n-3 and n-6 PUFA while CSO is only rich in n-6 PUFA. N-3 PUFA may be more highly oxidized than MUFA and n-6 PUFA resulting in those observed improvements in substrate utilization (18, 19). Additionally, the current study participants were older men and women who were less healthy than those of the previous studies, which may have affected their ability to metabolically adapt or respond to the oil-enriched diets. Further our protocol was truncated compared to previous studies to reduce participant burden of older, less healthy adults, potentially reducing our ability to observe differences late in the meal response.

Our observations that OO diet enrichment improved measures of EE and DIT may be due to its high MUFA content. Stable isotope studies have shown that MUFAs are more readily oxidized than n-6 PUFAs (20), but not n-3 PUFAs (19). Previous acute meal challenges made with OO have also shown greater DIT responses than sunflower oil (n-6 PUFA) and flaxseed oil (n-3 PUFA) rich meals (26). Conversely, this observation was not supported by other acute studies comparing MUFA- vs. PUFA-rich meals (13, 14). It is possible that the metabolic changes are more complicated than just the general fatty acid class (PUFA, MUFA), such as the specific fat sources used and other bioactive components of those sources, or the sex, age, or health status of the participants.

The clinical relevance of the observed increase in DIT becomes more apparent when we look at it in terms of magnitude of change. The increase in DIT in the OO group was 31.6% compared with 2.7% in the CSO group. This increase in DIT in the OO group only corresponds to a 10 kcal increase over the 3.5h postprandial period, which is small compared to total daily EE. This small change is to be expected as DIT is the smallest component of total EE, and has

been estimated to account for only 10% of total EE when one is in energy balance (53). However, we contest that this difference is still clinically relevant. With the knowledge that Americans consume more SFA on the weekends (54), and that the 0.5 to 1 kg annual weight gain corresponds to an 10-20 kcal daily energy surplus (55), the comparatively small increase in DIT from an OO-enriched diet may be able to curb reductions in DIT to SFA meals (8) promoting energy balance and weight maintenance.

With the overall reduction in FQ for both groups during the eight-week intervention, the lack of change in substrate oxidation was unexpected based on what we know about the time it takes adults to adapt to a HF diet (56-58). Metabolic flexibility is the ability to respond or adapt to conditional changes in metabolic demand which can refer to the ability to appropriately utilize available fuels (56). Despite finding no significant differences in metabolic flexibility score between groups, we observed variation in the change in metabolic flexibility scores suggesting that some people failed to adapt to the change in macronutrient content of their diets; however, not enough to drive statistical significance.

Our exploratory regression analyses showed that changes in HOMA-IR score explained 25.5% of the variation in metabolic flexibility score changes. This suggests that a worsening in insulin resistance may contribute more to one's ability to adapt to a HF diet than the type of fat provided. This is not the first time that insulin resistance has been related to metabolic inflexibility (45, 59). While we found no differences within or between groups for HOMA-IR score, it is of note that both groups were determined to be insulin resistant since the averages were well above the 1.95 HOMA-IR cutoff for insulin resistance (45). This conclusion should be interpreted with caution as it is exploratory in nature and warrants further investigation to

determine the relationship between insulin resistance and metabolic flexibility as it pertains to HF diet adaptation.

This study is not without limitations. We did not present the main outcomes analyzed by sex since this was not the intended study design, and we subsequently had low enrollment of men. However, it was included as a factor in our exploratory regression analyses and found not to explain variation in our metabolic flexibility data. Another limitation could be the generalizability of findings since the relatively high dose of OO or CSO resulted in subjects following a HF diet. However, HF diets, like the ketogenic diet, are gaining popularity showing the potential need for consideration of healthy HF diet patterns. We also used a 2-day baseline food diary to reduce participant burden but this may be less sensitive than a longer food record. This trial was also single blinded and we did not have a true control arm receiving no intervention. These could limit the strength of the design; however, we contest this comparative design was better to directly answer the question of how MUFA- vs. PUFA- rich oils (or more specifically OO vs. CSO) affect metabolic responses differently. Also, by design, we utilized a shorter indirect calorimetry protocol to reduce potential participant discomfort or burden which could have reduced our ability to observe differences late in the meal response. Finally, we used self-reported measures of intake and compliance, which introduces some degree of under- or over- reporting, and we did not have direct measures or biomarkers to assess compliance.

In conclusion, we have shown that an OO-enriched diet improved measures of postprandial EE and DIT in middle-aged adults with dyslipidemia. Given our unique study design of employing high SFA test meals instead of OO- or CSO-rich meals, these findings may be more clinically relevant. They suggest that OO diet enrichment can offer protection against an occasional, unhealthy HF meal, potentially improving energy balance and weight maintenance.

Our exploratory analyses showed that changes in insulin resistance are important in predicating the ability of adults with dyslipidemia to adapt to HF diets, and the lack of metabolic changes between MUFA vs. PUFA may have been overshadowed by the insulin resistant state of the subject population. Future studies are needed comparing CSO and OO diet enrichment that are powered to account for insulin resistance status, sex, age, and race. It would also be important to investigate these oils at lower doses, for longer durations, and in various disease states.

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Table 5.1. Characteristics of adults with dyslipidemia consuming cottonseed oil or olive oil enriched diets.

	Cottonseed Oil (n=20)		Olive Oil (n=21)		P values		
Characteristic	Week 0	Week 8	Week 0	Week 8	Treatment	Time (Visit)	Interaction
Age (y)	53 ± 3	--	54 ± 2	--	--	--	--
Weight (kg)	78.1 ± 3.8	79.2 ± 3.7	77.9 ± 3.6	78.9 ± 3.7	0.96	<0.001	0.59
BMI (kg/m^2)	27.2 ± 1.0	27.6 ± 0.9	27.6 ± 1.2	27.9 ± 1.2	0.83	<0.001	0.55
Waist circumference (cm)	91.2 ± 3.3	92.6 ± 3.3	90.0 ± 3.0	90.4 ± 3.2	0.71	0.04	0.27
Hip circumference (cm)	107.3 ± 1.4	107.5 ± 1.4	109.3 ± 2.4	109.7 ± 2.3	0.44	0.37	0.71
Waist to Hip Ratio	0.85 ± 0.02	0.86 ± 0.03	0.82 ± 0.02	0.82 ± 0.02	0.33	0.18	0.27
Systolic blood pressure (mmHg)	123 ± 3	124 ± 3	127 ± 3	122 ± 3	0.85	0.31	<0.05
Diastolic blood pressure (mmHg)	77 ± 2	78 ± 2	80 ± 2	77 ± 2	0.88	0.14	0.08
Body fat (%)	30.3 ± 2.1	31.5 ± 1.8	32.8 ± 2.6	33.2 ± 2.6	0.50	0.16	0.40

All values are mean ±SEM. Main and interaction effects were analyzed with repeated measures linear mixed models for treatment and time (visit). Week 0 is the pre-intervention visit and week 8 is the post-intervention visit. BMI – body mass index.

Table 5.2. Self-reported intake for baseline and 8-week intervention averages.

	Cottonseed Oil (n=20)		Olive Oil (n=21)		P values		
Nutrient	Baseline	Intervention	Baseline	Intervention	Treatment	Time (Visit)	Interaction
Energy (kcal)	2,134 ± 186	2,466 ± 114	2,563 ± 199	2,538 ± 124	0.16	0.24	0.17
Energy from protein (%)	15.7 ± 1.07	10.8 ± 0.28	14.6 ± 0.76	10.9 ± 0.36	0.50	<0.001	0.35
Energy from carbohydrate (%)	46.1 ± 1.98	39.3 ± 0.79	46.0 ± 2.17	39.3 ± 0.88	0.98	<0.001	0.99
Fiber (g)	20.6 ± 2.08	15.0 ± 1.12	22.3 ± 2.27	13.1 ± 0.68	0.97	<0.001	0.23
Sugar (g)	94.3 ± 15.6	105 ± 5.75	108 ± 14.8	110 ± 8.44	0.46	0.50	0.59
Energy from fat (%)	35.3 ± 1.93	49.0 ± 0.82	37.2 ± 1.52	47.6 ± 0.75	0.94	<0.001	0.15
Saturated fat (% fat)	34.3 ± 1.40	26.2 ± 0.41	32.6 ± 1.19	23.7 ± 0.43	0.04	<0.001	0.64
Trans fat (% fat)	1.16 ± 0.19	0.44 ± 0.07†	0.65 ± 0.10 *	0.40 ± 0.05	0.27	<0.001	0.02
Monounsaturated fat (% fat)	41.2 ± 2.07	24.0 ± 0.65†	34.1 ± 3.92	59.9 ± 0.62 *†	<0.001	0.19	<0.001
Polyunsaturated fat (% fat)	23.3 ± 1.85	49.1 ± 0.84†	26.8 ± 1.63	15.8 ± 0.55 *†	<0.001	<0.001	<0.001
Omega 3 fatty acid (% fat)	2.52 ± 0.33	0.80 ± 0.06	3.00 ± 0.54	1.16 ± 0.06	0.20	<0.001	0.84
Omega 6 fatty acid (% fat)	20.8 ± 1.66	48.3 ± 0.87†	23.8 ± 1.21	14.7 ± 0.50 *†	<0.001	<0.001	<0.001
Energy from alcohol (%)	3.0 ± 1.0	1.0 ± 0.4†	2.6 ± 1.0	2.2 ± 0.7	0.68	0.006	<0.05
Cholesterol (mg)	281 ± 43.7	226 ± 21.0	333 ± 53.5	216 ± 14.6	0.60	0.007	0.30
Sodium (mg)	3300 ± 301	2832 ± 156	3984 ± 510	2878 ± 137	0.29	0.007	0.25

All values are mean ± SEM (n=41). Baseline represents an average of the two food diaries before the intervention. Intervention represents an average of all food diaries kept during the intervention. Participants were instructed to report both provided foods and self-selected foods. All consumed foods were analyzed together regardless of whether they were provided or self-selected. Main and interaction effects were analyzed using a two way (treatment x time) repeated measures linear mixed model.

* indicates a significant difference between CSO vs. OO at baseline or during the intervention at $p<0.05$.

†indicates significant difference between intervention vs. baseline within a group at $p<0.05$.

Table 5.3. Fasting metabolic measurements

	Cottonseed Oil (n=20)		Olive Oil (n=21)		P Values		
	Week 0	Week 8	Week 0	Week 8	Treatment	Time (Visit)	Interaction
RMR (kcal/d)	2,332±108.7	2,329±110.1	2,283±89.52	2,247±104.3	0.64	0.46	0.54
Fat oxidation (g/15 min)	0.79±0.08	0.85±0.07	0.68±0.05	0.74±0.07	0.19	0.24	0.97
Carbohydrate oxidation (g/15 min)	1.95±0.14	1.85±0.14	2.20±0.18	1.97±0.21	0.35	0.22	0.65
RER	0.85±0.01	0.84± 0.01	0.86±0.01	0.85±0.01	0.23	0.17	0.86
FQ*	0.85±0.01	0.82±0.00	0.85±0.01	0.82±0.00	0.87	<0.001	0.98
Difference of RER and FQ	0.00±0.01	0.01±0.01	0.02±0.01	0.03±0.01	0.21	0.29	0.88
Metabolic flexibility score (RER:FQ)†	1.00±0.02	1.01±0.01	1.02±0.01	1.03±0.01	0.24	0.18	0.77
HOMA-IR	2.57±0.42	2.35±0.0.2	2.85±0.51	3.15±0.49	0.33	0.86	0.19

All values are mean ± SEM (N=41). Main and interaction effects were analyzed using a repeated measures linear mixed model for treatment and time (visit). Week 0 is the pre-intervention visit; week 8 is the post-intervention visit.

*FQ is calculated from averaged food diaries (week 0 calculated from baseline average, week 8 from intervention average)

Food quotient = FQ; gram = g; Homeostasis model assessment of insulin resistance = HOMA-IR; Kilocalorie = kcal; minute = min; Respiratory exchange ratio = RER; Resting metabolic rate = RMR.A

†A metabolic flexibility score of >1 suggests that fat oxidation is lower relative to the fat intake of the diet.

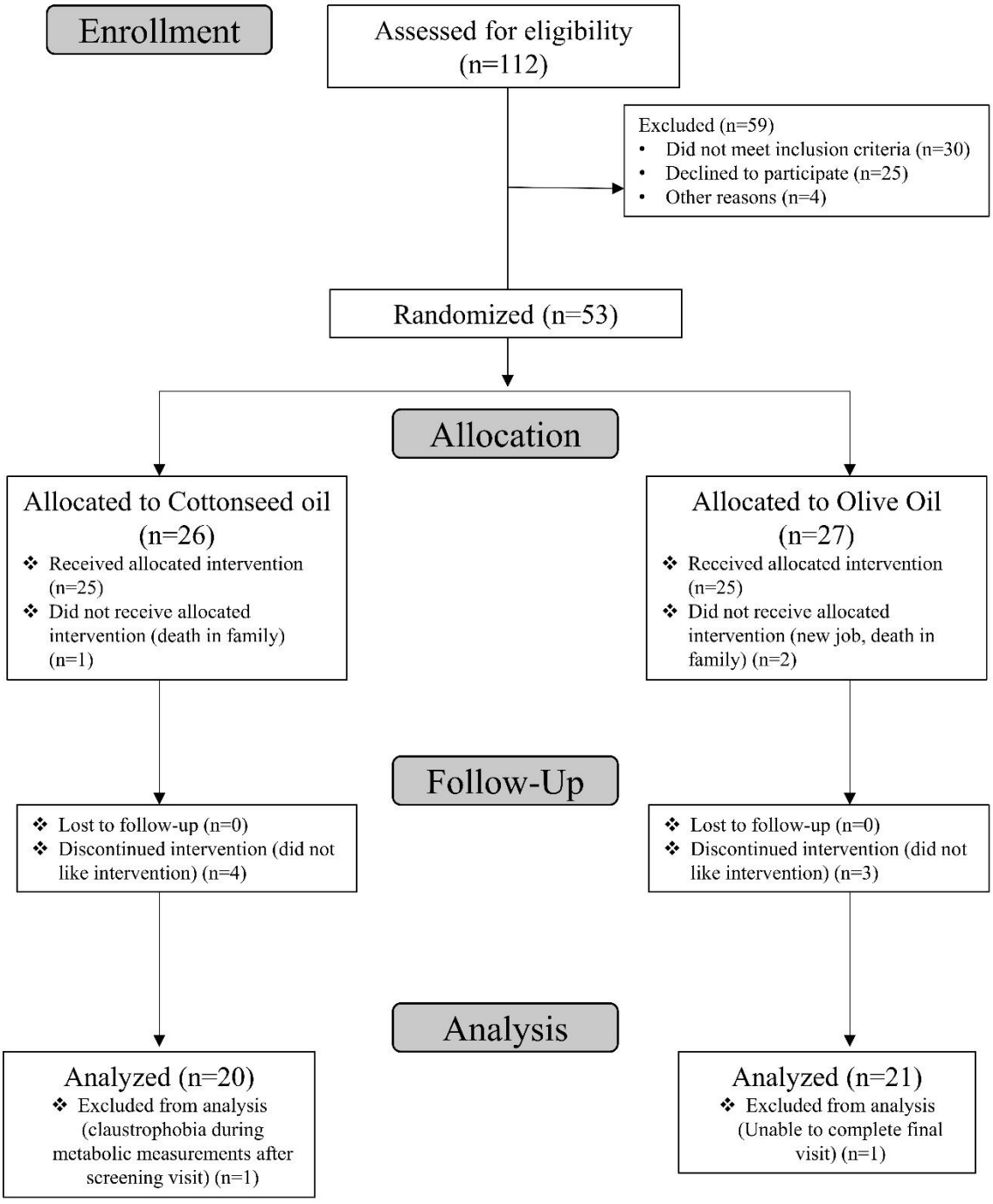

Figure 5.1. CONSORT flow diagram selection of participants.

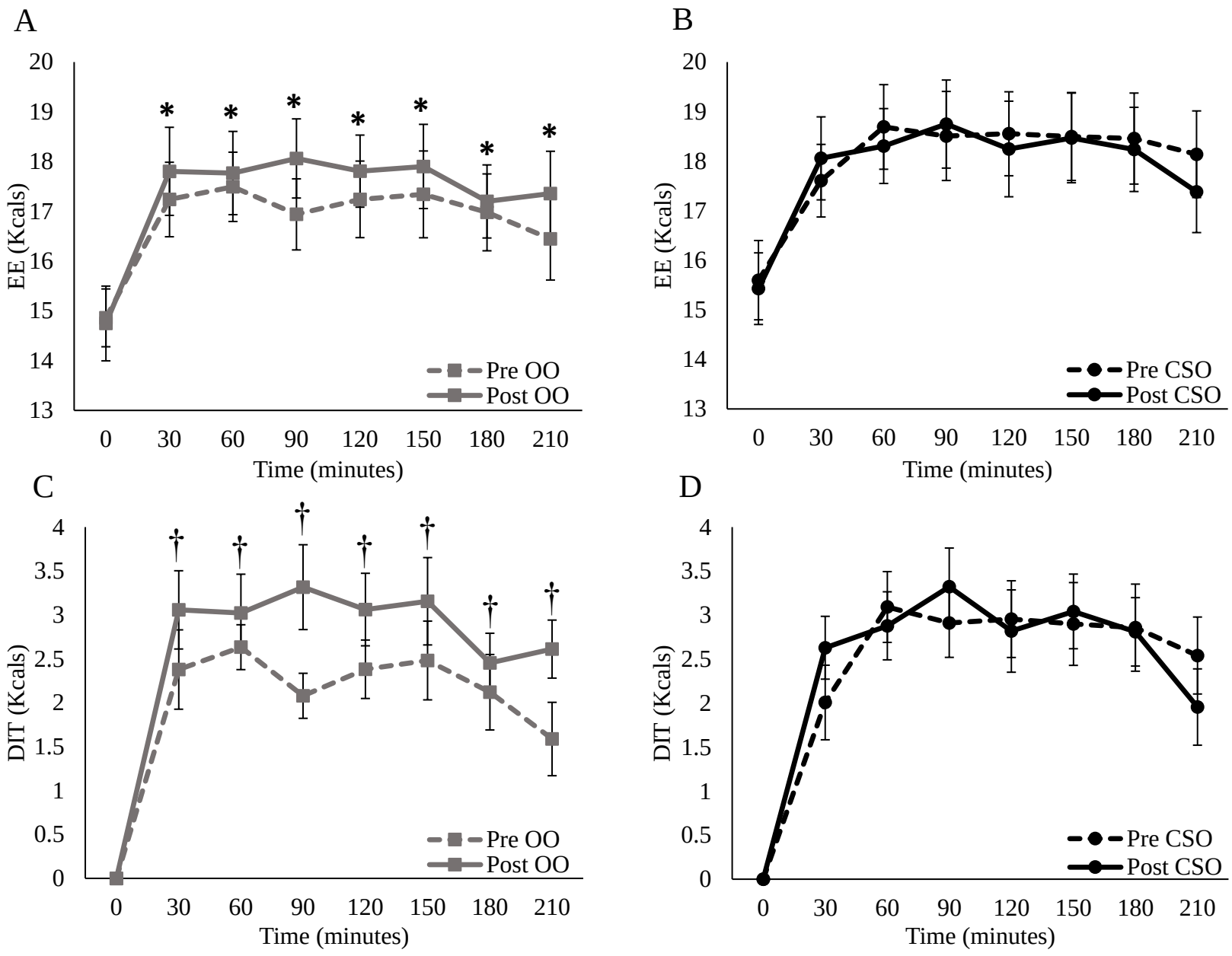
A
EE (Kcals)
20
19
18
17
16
15
14
13
*
Pre OO
Post OO
0
30
60
90
120
150
180
210
Time (minutes)
B
EE (Kcals)
Pre CSO
Post CSO
Time (minutes)
C
DIT (Kcals)
4
3.5
3
2.5
2
1.5
1
0.5
0
†
Pre OO
Post OO
Time (minutes)
D
DIT (Kcals)
Pre CSO
Post CSO
Time (minutes)

Figure 5.2. Time course for energy expenditure (EE) (A, B), and diet induced thermogenesis (DIT) (C, D). There were main effects of time point ($p<0.001$; $p=0.001$) for both EE and DIT, respectively. There was a main effect of time (visit) ($p=0.001$) for DIT and a trend for a time effect for EE ($p=0.07$), but no treatment effects. There were treatment by time (visit) interactions ($p=0.002$; $p=0.003$) for both EE and DIT, respectively, which was for an increase in both variables from pre- to post-intervention in OO only. Kcals = kilocalories; OO = cottonseed oil; CSO = cottonseed oil.

*indicates increase from pre- to post- intervention visit for postprandial EE ($p=0.003$).

† indicate increased DIT from pre- to post- intervention visit ($p<0.001$).

.

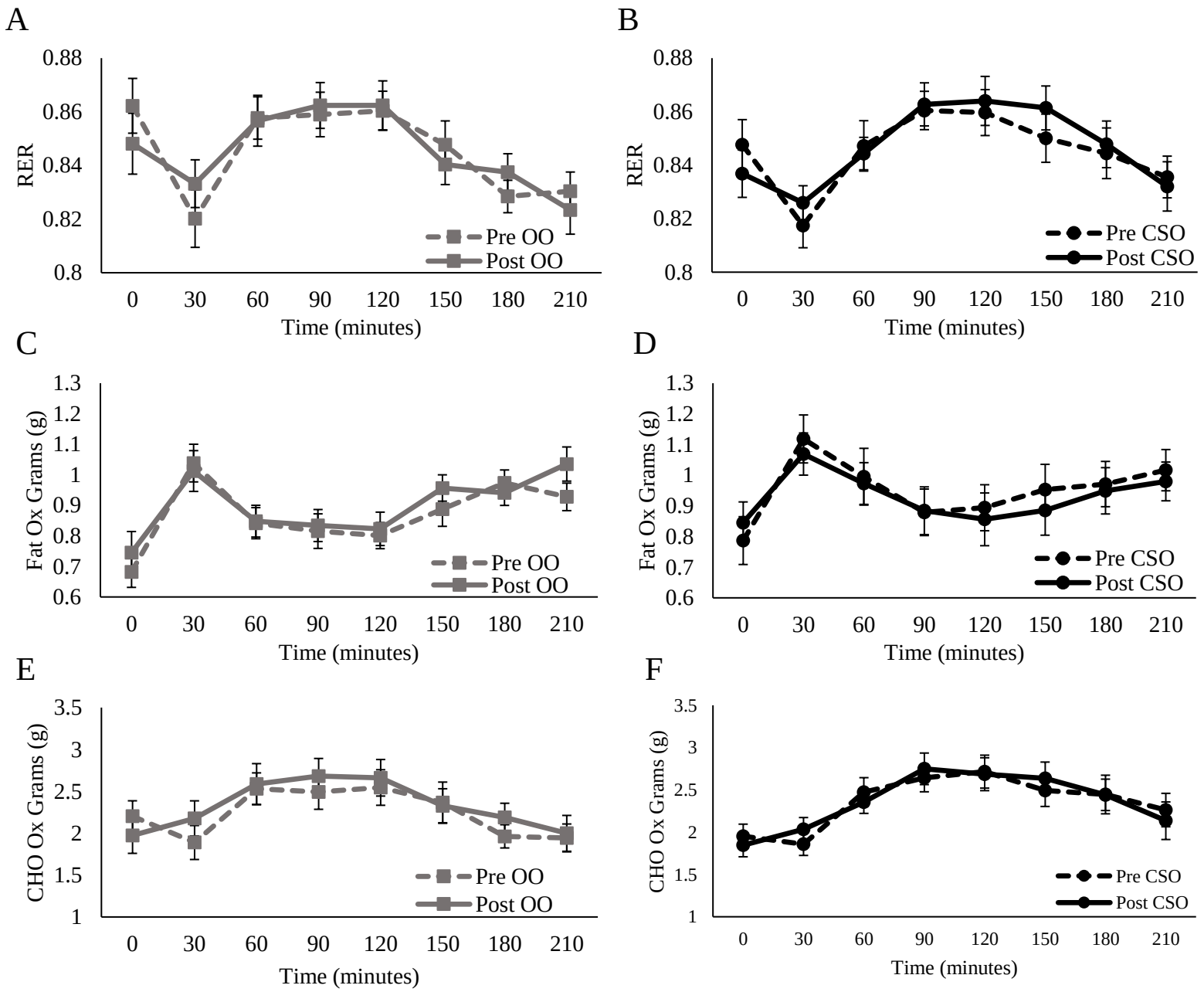
A
RER
0.88
0.86
0.84
0.82
0.8
Pre OO
Post OO
0 30 60 90 120 150 180 210
Time (minutes)
B
RER
0.88
0.86
0.84
0.82
0.8
Pre CSO
Post CSO
0 30 60 90 120 150 180 210
Time (minutes)
C
Fat Ox Grams (g)
1.3
1.2
1.1
1
0.9
0.8
0.7
0.6
Pre OO
Post OO
0 30 60 90 120 150 180 210
Time (minutes)
D
Fat Ox Grams (g)
1.3
1.2
1.1
1
0.9
0.8
0.7
0.6
Pre CSO
Post CSO
0 30 60 90 120 150 180 210
Time (minutes)
E
CHO Ox Grams (g)
3.5
3
2.5
2
1.5
1
Pre OO
Post OO
0 30 60 90 120 150 180 210
Time (minutes)
F
CHO Ox Grams (g)
3.5
3
2.5
2
1.5
1
Pre CSO
Post CSO
0 30 60 90 120 150 180 210
Time (minutes)

Figure 5.3. Time course for respiratory exchange ratio (A, B), fat oxidation (C, D), and carbohydrate oxidation (E, F). For all measures of substrate oxidation, there were main effects of time point ($p<0.001$ for all), but no treatment or time (visit) effects and no treatment by time interactions. CHO = carbohydrate; Ox = oxidation; RER = respiratory exchange ratio; OO=olive oil; CSO = cottonseed oil.

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CHAPTER 6

HUNGER AND SATIETY RESPONSES TO DIETS ENRICHED WITH COTTONSEED OIL VS. OLIVE OIL[4]

[4] Prater M.C., Scheurell A. R., Paton C. M., Cooper J. A. 2023.
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Abstract

Studies suggest that the type of dietary fat consumed habitually may modulate appetite and further influence weight management. The purpose of this study was to evaluate the impact of an 8-week diet intervention enriched with either cottonseed oil (CSO; polyunsaturated fat-rich) or olive oil (OO; monounsaturated fat-rich) on appetite responses in adults with high cholesterol. This was a parallel design, randomized partial outpatient feeding trial designed to provide approximately 60% of participants daily energy needs with ~30% of energy needs as CSO (n=21, BMI 27.3±0.92kg/m^2, age 53±2y) or OO (n=21, BMI 27.6±1.20 kg/m^2, age 54±2y). A high saturated fat meal challenge was completed at pre- and post-intervention visits with 5h postprandial blood draws and visual analog scales (VAS) for cholecystokinin (CCK), peptide YY (PYY), ghrelin, and subjective appetite, respectively. Participants also completed VAS questionnaires hourly and recorded dietary intake after leaving the lab for the remainder of the day. There was a greater increase in fasting CCK (CSO: 0.54±0.03 to 0.56±0.04; OO: 0.63±0.07 to 0.60 ±0.06 ng/ml $p=0.05$), a greater suppression of postprandial ghrelin ($p<0.01$), and a greater increase in postprandial VAS fullness ($p=0.04$) in CSO compared to OO. Additionally, there was a greater decrease in self-reported energy intake in CSO compared to OO (CSO: 2,464±123 to 2,115±123; OO: 2,263±147 to 2,434±184 kcal/d $p=0.02$). Only postprandial VAS prospective consumption showed greater suppression ($p=0.03$) in OO vs. CSO. Altogether, these data show that CSO has a greater effect on appetite suppression than OO diet enrichment and may be beneficial for weight maintenance, especially in a population at-risk for chronic disease.

Registered at clinicaltrials.gov: NCT04397055

Introduction

Obesity is a growing problem on the global scale (1, 2) and is a risk factor for other chronic diseases (3). Therefore, it is essential to identify weight management practices to serve as primary prevention of weight gain. One effective method may be through enhancement of appetite regulation (4). Accepted approaches to assessing appetite include physiologic (gastrointestinal hormones), subjective, or applied measurements. Ghrelin is the only known "hunger hormone" and is released from the stomach in low energy states to activate orexigenic neurons, stimulating hunger and promoting feeding behavior (5). Released from the proximal and distal gut in response to a meal, cholecystokinin (CCK) and peptide YY (PYY) are satiety hormones that stimulate anorexigneic neurons and inhibit orexigenic neurons leading to satiation and satiety (6, 7). Additionally, CCK is known to stimulate the release of some hormones including PYY and suppress the release of ghrelin (8). Subjective feelings or ratings of appetite are commonly measured using visual analog scales (VAS) anchored to questions about hunger and fullness (9). Finally, applied methods of measuring appetite most commonly involve assessments of energy intake (EI) and/or macronutrient intake (10).

While high-fat (HF) diets are typically associated with weight gain (11) due to its highly palatable nature (12), the fatty acid (FA) composition of the diet may differentially influence appetite responses, and potentially impact long-term weight management (13-15). Some acute meal challenge studies measuring subjective and applied appetite found no differences based on FA composition (16-22), while other studies do report differences (23-26). Our lab has previously shown that single meals rich in polyunsaturated fat (PUFA) elicit a stronger PYY response when compared to a monounsaturated fat (MUFA) rich meal (22, 26). Additionally, a 7-day, PUFA-rich diet has been shown to increase fasting and postprandial PYY, and decrease

fasting ghrelin (27). These limited data suggest PUFA may have a greater effect on physiologic measures of appetite than MUFA, but is insufficient to make strong, or longer-term, conclusions.

There are very few dietary intervention studies focusing on FA composition, specifically PUFA vs. MUFA, and appetite (27-29), and even fewer using a whole foods approach. Consumers recognize olive oil (OO) as a "heart healthy" MUFA-rich oil, primarily based on research on the Mediterranean Diet (30). While not as familiar to consumers, cottonseed oil (CSO), which is rich in PUFAs, is present in the food supply and has been linked to improvements in lipid metabolism and chronic disease risk reduction (31-34). Only one previous study has examined CSO vs. OO on appetite (35). They demonstrated the ability of CSO-rich diets and meals to improve physiologic and subjective measures of appetite, even when compared to OO (35). However, that trial only included healthy males and was a 5-day diet protocol. The purpose of this study was to examine the impact of an 8-week, HF diet enriched with CSO or OO on fasting and postprandial appetite measures in a parallel design in adults at-risk for chronic disease (36). We evaluated subjective ratings of appetite, self-reported EI, and biological hunger and satiety hormones in response to a partial outpatient feeding protocol. We hypothesized that CSO would elicit more favorable changes in physiologic and subjective measures of appetite with no differences in EI compared to OO based on the previous short-term CSO trial (35).

Methods

Study Design

This was a single-blind, randomized, parallel trial (clinicaltrials.gov: NCT04397055) involving an 8-week partial outpatient feeding intervention. Recruitment began in May 2018 and

final testing took place in June 2021 when all primary and secondary outcomes were addressed. The protocol included a screening visit and two testing visits (pre- and post-intervention). Subjects were randomly assigned using balanced blocks to either an OO or CSO intervention arm as previous described (36). This study was conducted according to the guidelines laid down by the Declaration of Helsinki and the Institutional Review Board for human subjects at the University of Georgia approved all procedures involving human subjects (STUDY00005869). Informed written consent was obtained from each participant prior to any testing procedures.

Participants

One hundred and twelve sedentary adults between the ages of 30 and 75y were assessed for eligibility (**Figure 6.1**). Inclusion and exclusion criteria were measured at the screening visit and has been described previously (36). Briefly, exclusion criteria included excessive alcohol use, tobacco use, exercise (>3h/week), weight instability (gain or loss >5% of body weight in the past 3mo), lipid-lowering medications, diabetes medications, steroid/hormone therapies, and ADD/ADHD medications and chronic/metabolic diseases.

Protocol

Screening visit

Prior to arriving at the Human Nutrition Lab (HNL) on the morning of the screening visit, participants completed an 8-12h overnight fast and 24h abstaining from exercise and alcohol. Following anthropometric measures, a 30-min indirect calorimetry measurement (TrueOne 2400; Parvo Medics, Sandy, Utah, USA) was taken under standard conditions (37). The Weir equation was applied to the final 20 min of respiratory gasses to calculate resting metabolic rate (RMR) (38). Participants' RMRs were multiplied by 1.65, an average U.S. physical activity factor (39),

to estimate total daily energy needs. This was used to calculate the test meal, as well as assignment to diet intervention energy tiers. Using a random number generator and stratified balanced blocks (36), a researcher who was not involved in the collection or analysis of these data assigned qualifying participants to either the OO or the CSO intervention groups.

Pre-intervention visit

Prior to the pre-intervention visit, participants completed a two-day food record consisting of one week day and one weekend day (40). For this visit, participants arrived between 0700 – 0800h to the HNL in the fasted state (8-12h fast) after consuming a standardized dinner meal and snack (energy: 50% carbohydrate, 15% protein, 35% fat) the evening before the visit, which was provided by research personnel. Participants were instructed to consume enough of the standardized meal to be comfortably full. Height, weight, waist and hip circumference, blood pressure, and body composition were measured upon arrival. Body composition was measured by Bod Pod (Cosmed USA, Inc.). Next, participants completed a 100 mm visual analog scale (VAS) for hunger, fullness, prospective consumption, desire to eat to measure subjective appetite (41). An intravenous catheter was placed in the antecubital vein and a fasting blood sample was collected. Saline was used to keep the IV-line patent throughout the protocol.

Once fasting measures were completed, participants consumed a high saturated fat (SFA) test meal which has been previously described (36). Briefly, 35% of individual estimated daily energy needs were provided with 5%, 25%, and 70% of energy provided as protein, carbohydrate and fat, respectively. The FA breakdown was 47%, 16%, and 7% of total energy from SFA, MUFA, and PUFA, respectively. A high SFA meal was chosen to assess how changes in the fat composition of the chronic diet may influence appetite responses to an occasional “unhealthy” (high SFA) meal, which is commonly observed with weekend eating habits (42). Four ounces of

water was used to rinse the container and ingested to ensure the entire meal was consumed. A validated sensory questionnaire was administered to assess sensory modalities of the high SFA meal(43, 44). This questionnaire used a modified 9-point hedonic scale with '1' indicating 'dislike extremely' and '9' indicating 'like extremely'. Following the SFA-rich test meal, blood samples were collected, and VAS questionnaires were administered, intermittently for 5h (time points: 30, 60, 90, 120, 150, 180, 240, 300 min). VAS measures taken at these time points were denoted as in lab (LAB) measures. Four ounces of water was provided hourly. After 5h, the IV was removed and participants were instructed to eat a self-selected lunch within 1h of leaving the lab and a self-selected dinner 4h later. Participants were also provided with VAS questionnaires to complete once per hour for the next 7h (starting at 360 min post SFA test meal). VAS measures taken at these time points were denoted as at-home (HOME) measures. In addition to our record of the SFA test meal, participants recorded all food and drink for the rest of the day on a food record. Participants were instructed to record everything consumed including condiments and beverages with attention to ingredients in recipes and brands of products. Estimated quantities were recorded in standard measures such as cups and tablespoons. Food records were analyzed using The Food Processor SQL software (version 10.12.0). The combined analysis of the SFA test meal and self-reported intake made up the total EI data.

Intervention protocol

The day following the pre-intervention visit, participants began the 8-week diet intervention. Participants were assigned to either CSO or OO intervention groups but were blinded to their assignment. The partial outpatient feeding of the intervention was designed to provide approximately 60% of energy needs (actual range was 56-87% of energy needs, with an average of 66±2% for CSO and 67±2% for OO) was utilized to allow for control of most of the diet,

while allowing participants to maintain some of their usual diet. Participants were sent home with a one-week supply of daily meals and snacks that corresponded to their assigned group. They were informed of their estimated daily energy needs, how many kilocalories were being provided by the study foods, and how many kilocalories were left for them to fill with their own foods.

With the exception of the intervention oils (CSO or OO), the two diets were identical. All study foods were prepared by research personnel, and ingredients were weighted to the 0.01g. A 7-day rotating menu was used to provide two meals plus snacks daily including breakfast and lunch/dinner entrée. This allowed for one self-selected meal daily. The meals provided included soups, sautéed vegetables with rice, turkey meatloaf with mashed potatoes and baked products. Breakfast shakes were provided 5 days per week as one of the two meals. Participants were instructed to prepare this breakfast shake by mixing proportioned shake mix with milk of choice and designated amount of the assigned intervention oil. The rest of the meals were pre-portioned by research personnel, packaged in microwave and freezer safe containers, and frozen. Participants were instructed on safe reheating practices.

Kilocalorie tiers, as described previously (36), were used to determine the quantity of food to provide participants. The intention of the tiers were to provide some (approximately 60%; actual range of 56-87%) of the estimated energy needs as foods from the study, leading to about 30% of their total energy needs being provided by the assigned oil (CSO or OO). Kilocalorie tier assignment was non-random and dependent on their estimated energy requirement, derived from their RMR at screening. Tier intervals are presented in **Table 6.1**. Fatty acid analyses of CSO and OO are presented in **Table 6.2**.

During the intervention, participants completed weekly compliance checklists as a self-reported measure of compliance, bi-weekly two-day food records including a week day and a weekend day, and the sensory evaluation questionnaire the first time they ate any of the provided foods. Participants returned to the HNL on a weekly basis to pick up meals and turn in study materials.

Post-intervention visit

Participants returned to the HNL for their post-intervention visit after completing the 8-week partial outpatient feeding. All procedures from the pre-intervention visit were repeated at the post-intervention visit.

Plasma sample analyses

All blood samples were drawn into EDTA vacutainers (Becton, Dickinson and Company, Franklin Lakes, NJ) with 10 μl/ml whole blood each of 4-benzenesulfonyl fluoride hydrochloride (AEBSF) and Dipeptidyl Peptidase IV (DPP IV) inhibitor (Sigma Aldrich, St. Louis, MO, USA), immediately placed on ice, and then centrifuged for 15 min at 4°C. The plasma was aliquoted and stored at -80°C until analysis. Total PYY and active ghrelin were measured using radioimmunoassays (Millipore, Billerica, MA, USA), and total CCK was measured using an extraction free enzyme immunoassay (Phoenix Pharmaceuticals, Burlingame, CA, USA). Each participant's total number of samples were run within the same assay.

Statistical analysis

SAS version 9.2 (SAS Institute Inc., Cary, NC, USA) was used for statistical analyses. The Food Processor SQL software (version 10.12.0) was used to assess all food record data. All values are reported as mean ± SEM unless otherwise noted. Statistical significance was set at

$p \leq 0.05$. A sample size of 40 (20 per group) was estimated using a Cohen's F of 0.233 to detect a difference in postprandial ghrelin based on the results from Polley et al. (35). A second sample size calculation estimated a sample size of 24 (12 per group) using a Cohen's F of 0.312 based on the postprandial hunger VAS ratings presented also in Polley et al. (35). Sample size was calculated using G*power 3.19.7 assuming at least 80% power and an α of 0.05. The decision to use per-protocol analyses was made a priori. A repeated measures (RM) linear mixed model for treatment and visit was used to determine between and within group differences for fasting hormone and VAS data, energy and macronutrient intake on test days anthropometric data, and sensory evaluation data of SFA test meals. Postprandial hormone and VAS data was assessed for between and within group differences using a RM linear mixed model for treatment, visit, and time point. All VAS data (incremental area under the curve (iAUC) and time course) was analyzed with all time points (0 – 720 min), as well as LAB (time points 0 – 300 min) and HOME (time points 360 – 720 min) subgroups separately using similar linear mixed models. In all linear mixed models, participants were modeled as random effects. When significance was found, post hoc analyses were done using Tukey's test. Finally, unpaired t-tests were used to assess differences between groups in self-reported compliance and sensory evaluation data of intervention foods.

Results

Participants

Fifty-three participants were allocated to an intervention group, but eleven did not start or complete the intervention and/or testing visits (**Figure 6.1**). Thus, forty-two individuals (12 women and 9 men in CSO and 14 women and 7 men in OO) completed the study and were included in the final analysis. Participant anthropometrics at pre- and post-intervention visits are

presented in **Table 6.3**. Weight (kg) and BMI (kg/m^2) increased in both groups but this increase was not different between groups. No other anthropometric characteristic changed during the intervention. Compliance of subjects averaged at 91 ± 2% and 92 ± 1% for CSO and OO groups, respectively, with no differences in compliance between groups.

Physiologic responses

Fasting plasma CCK, PYY, and ghrelin levels are presented in **Table 6.4**. There was a treatment by visit interaction ($p<0.05$) with an increase in fasting CCK in CSO and a decrease in OO from pre- to post-intervention visit. There were no changes in fasting PYY or ghrelin. The time course of the meal responses and corresponding iAUC for CCK, PYY, and ghrelin are presented in **Figure 6.2**. There was a visit effect ($p=0.03$) for higher postprandial CCK (**Figure 6.2 A, B**) from pre- to post-intervention visit in both groups, and this increase was not different between groups. Similarly, the PYY meal response (**Figure 6.2 D, E**) was increased from pre- to post-intervention in both groups (visit effect $p=0.006$), but this change was not different between groups. For ghrelin (**Figure 6.2 G, H**), there was a significant treatment by visit interaction ($p=0.005$) which was driven by greater postprandial ghrelin suppression in CSO vs. OO, although post hoc analyses did not reach significance. No significant differences between groups were observed for iAUC measures (**Figure 6.2 C, F, I**).

Subjective ratings of appetite

There were no differences between groups for any fasting subjective measure of appetite (**Table 6.4**). The time course of meal responses and corresponding iAUC at pre-and post-intervention visits for VAS are presented in **Figures 6.3** and **6.4** and **Table 6.5**. For the appetite score time course data (**Figure 6.3 A, B**), there was a visit effect ($p=0.004$) for an increase from

pre- to post-intervention, but no difference between groups. When analyzed further, this 'all day' visit effect was driven by the LAB measures (visit effect $p<0.001$) but not the HOME measures (visit effect $p=0.44$).

Despite the time course composite appetite score (**Figure 6.3 A, B**) changing in similar ways in both groups, there were differences between groups for individual VAS questions. There was a treatment by visit interaction ($p=0.04$) for fullness (**Figure 6.3 C, D**). Post hoc analyses revealed greater increases in fullness in the CSO group ($p=0.02$) vs. the OO group ($p=1.00$). After further analysis, the HOME (interaction $p = 0.04$) rather than the LAB measures (interaction $p=0.46$) drove the fullness interaction. Conversely, prospective consumption ratings (**Figure 6.4 A, B**) ("how much do you think you could eat right now?") were lower at the post- vs. pre-intervention in both groups (visit effect $p=0.003$), but there was a greater decrease in OO vs. CSO group (treatment by visit interaction $p=0.03$; OO $p=0.003$; CSO $p=0.92$). The LAB measures were found to mirror the visit ($p<0.001$) and interaction effects (interaction $p=0.02$; OO $p<0.001$; $p=0.48$) but the HOME measures were not significant. Time course ratings of hunger (**Figure 6.4 C, D**) decreased regardless of group assignment (visit effect $p<0.001$). After further analysis, this decrease was present for both LAB (visit effect $p<0.001$) and HOME measures (visit effect $p=0.009$). There were no differences between groups for desire to eat (**Figure 6.4 E, F**). Finally, for iAUC measures, appetite score and hunger showed visit effects for LAB (**Table 6.5**) but no other main or interaction effects for iAUC were observed.

Applied responses

Total energy and macronutrient intake from the pre- and post-intervention visit days based on the analysis of the food diaries and the provided SFA meal are presented in **Figure 6.5**. There was a significant difference in total EI (treatment by visit interaction $p=0.02$) driven by a

trend for decrease in the CSO group (p=0.11) compared to non-significant increase in the OO group (p=0.66). This reduction in total EI in the CSO group and increase in the OO group was driven by corresponding changes in energy from protein (treatment by visit interaction p<0.001; CSO p=0.015; OO p=0.011)) and carbohydrate (treatment by visit interaction p=0.002; CSO p=0.02; OO p=0.34). There were no changes in EI from fat or alcohol.

Sensory evaluation

Sensory evaluations of the SFA meal and the intervention foods are presented in **Table 6.6**. There were no significant differences within or between groups for any characteristic of the SFA test meal. Additionally, there were no differences between groups for overall acceptance of the CSO or OO-enriched foods provided during the intervention.

Discussion

For the first time, we have shown that an 8-week, CSO-enriched diet leads to better improvement in appetite control, compared to an OO-enriched diet in adults at risk for chronic disease. Specifically, CSO diet enrichment led to increases in fasting CCK, increased postprandial feelings of fullness, and suppression of postprandial ghrelin when compared to the OO-enriched diet. The only measure where OO was better than CSO was for greater improvements in postprandial desire to eat ratings. Similar between group improvements were observed in postprandial feelings of hunger, appetite score, CCK and PYY. Finally, and possibly most importantly, the aforementioned greater improvements in subjective and physiologic measures of appetite with CSO diet enrichment resulted in reductions in EI in the CSO, but not OO group.

Of the appetite hormones measured, CCK and ghrelin appear to be consistently responsive to CSO consumption either in fasting or postprandial measures (35). Our results demonstrated more favorable fasting CCK responses with CSO compared to OO diets; however, the previous CSO v. OO study by Polley et. al. found CSO meals to elicit greater postprandial CCK responses (35). Additionally, postprandial ghrelin is consistently suppressed whether CSO is consumed daily (and not part of the test meal), or as part of the test meal (35). While Polley et. al. did not find changes in ghrelin responses from pre- to 5-day post-diet intervention, they were able to show that consuming CSO meals suppressed ghrelin more than OO meals (35). It is possible that the longer intervention and lack of CSO and OO in test meals used in the present study is responsible for the slight differences in hormone responses between trials. Of note, there is no established reference range for changes in these hormones that would be considered clinically relevant for appetite suppression. The work of others has demonstrated that increases in CCK and decreases in ghrelin are associated with subjective feelings of appetite and decreased energy intake (45, 46). However, these examples observed more robust changes in those hormones than presented here. Importantly, though, others have observed changes in these hormones postprandially in similar magnitude as what was observed in the current study but without reductions in energy intake (47-49). Furthermore, increases in fasting CCK are not commonly observed in response to diet interventions; yet, small differences in basal CCK (<5 pmol/L) (similar to our observed changes) are suggested to be a driver of reduced appetite and energy intake associated with old age (50). Taken together, this highlights that small changes in fasting or postprandial appetite hormones are difficult to interpret at this time but may still be physiologically relevant. The clinical relevance of our results may lie more in the concurrence of

improvements in physiologic and subjective measures of appetite than either hormone changes independently, along with the subsequent changes in self-reported energy intake.

For VAS, the changes in fullness we observed in the CSO group were similar to 4 weeks of a PUFA supplement study that resulted in improvements in fullness ratings and a reduction in EI compared to a control diet (51). Conversely, Polley et. al. (35) reported decreased hunger ratings with CSO, but similar improvements in fullness for CSO and OO. Our study design was different from these two studies, and most other FA composition and appetite literature, in that we included "HOME" measures of VAS. While this gives us a more complete picture of subjective appetite ratings across the entire day, it is difficult to compare our results to previous studies. Some of our results were driven by the HOME measures, demonstrating the importance for obtaining this data outside of laboratory visits.

While our measure of EI is self-reported and should be interpreted with some caution, it is not common to find differences in EI based on FA composition acutely or with diet interventions (16-21, 25, 26, 35). This highlights the importance of our data showing suppressed EI with CSO vs. OO diet enrichment. It also suggests there may be additional characteristic besides the FA composition of CSO that is suppressing appetite, or the FA composition of CSO is uniquely able to suppress EI compared to other oils. Unlike most other PUFA-rich oils, CSO also contains a relatively high amount of SFA (52). Since SFAs have been shown to have a greater effect on postprandial appetite hormones and VAS in some studies (13, 23, 26, 53), it is possible that the combination of high PUFA and moderately high SFA of CSO has a more potent effect on appetite regulation.

While CSO was more favorable than OO in many appetite-related outcomes, we also observed several visit effects in both subjective and physiologic measures that could have been a

result of multiple factors. First, there was a 1kg average weight gain across the intervention that was the same in both groups. Weight gain has known implications on appetite and may explain some of the improvements observed in both groups from pre- to post-intervention. However, the fact that this weight gain was consistent between groups preserves the validity of the between group differences we have observed. Second, most studies that are looking at the impact of FA composition on appetite regulation are incorporating HF meals and/or diets (22, 26-28, 54). It is possible that simply being on a HF diet could have driven some of our visit effects. Lastly, differences in the rate of absorption of FA have been hypothesized to drive differences in appetite hormone responses observed in acute meal challenge studies (55). Saturated FAs are absorbed more slowly than unsaturated FAs suggesting that having the same high SFA meal challenge could have driven some of the visit effects and overrode the daily diet FA composition differences. However, our use of an SFA-rich meal was intentional. Since it has already been shown that acute CSO consumption more favorably alters appetite hormone levels compared to OO (35), we sought to determine whether daily CSO consumption could be protective, from an appetite standpoint, against an occasional unhealthy (high SFA) meal.

The implications of reduced EI during an afternoon and evening of self-selected meals becomes more apparent in the context of weight management. Traditional weight loss recommendations are to reduce intake by 500 to 1000 kcal/day (2,092-4,184 KJ/day) to observe 1-2 lbs. of weight loss per week (56, 57). While this is an oversimplification of the complicated physiologic mechanisms controlling weight loss (58, 59) , it is of note that our observed reduction of 349 kcal (1,460 KJ) corresponds to 69.7% of this 500 kcal/day (2,092 KJ/day) deficit recommendation. This was achieved on a day when the breakfast test meal was a fixed percentage of energy needs thus magnifying the 349 kcal (1,460 KJ) reduction for the remaining

meals of the day. Furthermore, it is well known that appetite increases proportional to weight loss (60) making adherence to weight loss and weight maintenance programs difficult. Therefore, the reduction in EI with CSO diet enrichment, along with the improvements in physiologic and subjective appetite measures, demonstrates the potential usefulness of CSO in curbing appetite, supporting weight loss, and encouraging weight maintenance. This requires additional research with CSO incorporation as part of a weight loss or weight maintenance protocol,

This study is not without limitations. A completely controlled feeding trial may have been better to ensure weight maintenance, but our partial outpatient feeding trial shows that even with some variation in food choice we are still able to observe improvements between the two intervention groups. Along those lines, the use of kilocalorie tiers limited the precision of the proportion of energy we provided but were employed for practicality of batch cooking of the intervention. Importantly the percent energy provided by study foods was not different between groups, therefore preserving comparability between groups. Secondly, we measured total PYY (PYY $_{1\text{-}36}$ and PYY $_{3\text{-}36}$) and CCK (CCK 33) rather than active forms. The biologically active forms of both of these peptides are associated with appetite control (61, 62), and by measuring the total peptide we may be missing changes in the concentration of the active forms specifically. However, the measurement of these total forms has been shown to track similarly with their active counterparts in postprandial responses (63, 64). Another limitation is our use of self-reported compliance and intake. Self-reported measures are known to introduce under- and over-reporting (65, 66) which may have influenced our results. Furthermore, the use of a relatively high amount of oil may have limited the application of our results to people following lower fat diets. While HF diets are gaining popularity (e.g. ketogenic diet), further research is needed to determine the effects of lower doses of CSO. Additionally, the use of an average physical

activity factor for estimated energy expenditures of all participants may have limited our ability to apply individual activity levels to our estimates, or to account for limitations in physical activity due to COVID-19 restrictions. However, we do believe this average activity factor still appropriately described our generally sedentary population. Finally, this study was not designed to evaluate the effect of sex, race, or BMI; thus, future work is required to elucidate these interactions.

In conclusion, this is the first study to find improvements in appetite control with CSO diet enrichment in adults at risk for cardiovascular disease. Fasting CCK and subjective feelings of fullness were higher, and postprandial ghrelin and EI were suppressed, with CSO diet enrichment compared to OO diet enrichment. The OO diet enrichment did improve subjective desire to eat but there were no other measures where OO enrichment was more favorable. Improvements in postprandial CCK, PYY, subjective ratings of hunger and appetite score were not different between groups and may be attributable to the HF diets or the slight energy surplus (based on 1kg weight gain over 8 weeks) rather than characteristics of the individual oils. Future research should focus on the potential use of CSO in the context of weight management including diets with lower doses of CSO.

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Table 6.1. Nutrient breakdown of provided intervention foods for each kilocalorie tier.

	Cottonseed Oil				Olive Oil			
Intervention Tiers (kcal)	<1,600	1,600-2,299	2,300-3,000	>3,000	<1,600	1,600-2,299	2,300-3,000	>3,000
Energy (kcal)	1,090	1,402	1,678	2,107	1,090	1,402	1,678	2,107
Energy (KJ)	4,561	5,866	7,021	8,816	4,561	5,866	7,021	8,816
Energy from protein (%)	7.2	6.7	6.5	6.8	7.2	6.7	6.5	6.8
Protein (g)	19.1	23.0	26.8	35.2	19.1	23.0	26.8	35.2
Energy from carbohydrates (%)	36.9	36.3	36.3	39.0	36.9	36.3	36.3	39.0
Carbohydrates (g)	98.0	124.0	148.5	200.6	98.0	124.0	148.5	200.6
Fiber (g)	3.2	4.1	5.1	7.9	3.2	4.1	5.1	7.9
Sugar (g)	51.3	64.8	76.5	101.5	51.3	64.8	76.5	101.5
Energy from fat (%)	55.9	57.0	57.2	54.1	55.9	57.0	57.2	54.1
Total Fat (g)	65.5	85.9	103.1	122.7	65.5	85.9	103.1	122.7
Saturated fat (g)	15.3	20.0	24.0	28.7	12.3	16.0	19.3	23.2
Trans fat (g)	0.17	0.22	0.27	0.38	0.17	0.22	0.27	0.38
Monounsaturated fat (g)	13.3	17.4	20.8	24.7	44.8	58.8	70.6	83.5
Polyunsaturated fat (g)	36.8	48.4	58.1	68.8	8.3	10.9	13.0	15.5
Omega 3 fatty acid (g)	0.28	0.36	0.43	0.51	0.60	0.79	0.95	1.13
Omega 6 fatty acid(g)	36.6	48.0	57.6	68.3	7.8	10.1	12.1	14.4
Cholesterol (mg)	57.5	75.2	89.8	121.0	57.5	75.2	89.8	121.0
Total fat from intervention oil (%)	88.2	87.3	87.3	85.6	88.2	87.3	87.3	85.6
Fat from intervention oil (g)	57.8	75.0	90.0	105.0	57.8	75.0	90.0	105.0

Daily nutrients delivered through the provided study foods within each treatment and energy tier. Participants were assigned to a kilocalorie tier based on their estimated energy requirements from a resting metabolic rate measurement at the screening visit. Energy tiers are named for the range of total energy requirements of the participants that were assigned to that tier. Energy (kcal in the first row and (KJ) in the second row) is the amount of energy actually provided each day from the diet intervention foods. The only difference between treatments was from the different treatment oil used (cottonseed oil vs. olive oil). <1,600 kcal = <6,694 KJ; 1,600-2,299 kcal = 6,694-9,619 KJ; 2,300-3,000 kcal = 9,623-12,552 KJ; >3,000 kcal = >12,552 KJ.

Table 6.2. Fatty Acid Distribution of Oils.

Fatty acids	Name	CSO (%)	OO (%)
Total SFA		22.73	17.46
14:0	tetradecanoic acid (myristic)	0.78	0
16:0	hexadecanoic acid (palmitic)	18.83	14.30
18:0	octadecanoic acid (stearic)	2.68	2.37
20:0	eicosanoic acid	0.27	0.45
21:0	Methyl heneicosanoate	0	0.08
22:0	docosanoic acid (behenic)	0.18	0
Total MUFA		19.94	70.85
16:1	*cis*-9-hexadecenoic acid (palmitoleic *n-7)*	0.57	1.30
18:1	*cis*-9-octadecenoic acid (oleic *n-9)*	19.27	68.95
20:1	*cis*-11-eicosenoic acid (gadoleic *n-9*)	0.10	0.32
Total PUFA		57.33	11.69
18:2	*cis*-9,12-octadecadienoic acid (linoleic *n-6)*	56.97	10.67
18:3	*cis*-9,12,15,-octadecatrienoic acid (ALA *n-3)*	0.36	0.74
20:2	cis-11,14,-eicosadienoic (n-6)	0	0.09
20:4	Arachidonic acid (n-6)	0	0.10
20:5	Eicosapentaenoic acid (n-3)	0	0.10

CSO = cottonseed oil; OO = olive oil; SFA = saturated fatty acids; MUFA = monounsaturated fatty acids; PUFA = polyunsaturated fatty acids;

Table 6.3. Population characteristics.

	Cottonseed Oil (n=21)		Olive Oil (n=21)		p-values		
	Pre-intervention	Post-intervention	Pre-intervention	Post-intervention	Treatment	Visit	Interaction
Anthropometric Characteristics							
Age (y)	53±2	--	54±2	--	0.73	--	--
Height (cm)	169.0±2.2	169.0±2.2	168.0±1.9	168.0±1.9	0.72	0.66	0.56
Weight (kg) *	78.6±3.6	79.6±3.5	77.9±3.6	78.9±3.7	0.89	<0.001	0.79
Body Mass Index (kg/m^2) *	27.3±0.9	27.7±0.9	27.6±1.2	27.9±1.2	0.86	<0.001	0.74
Waist to Hip ratio	0.85±0.02	0.85±0.02	0.82±0.02	0.82±0.02	0.39	0.24	0.34
Body Fat (%)	31.0±2.1	32.2±1.9	32.9±2.6	33.2±2.6	0.65	0.17	0.43

Values are presented as mean±SEM. Age was analyzed using a two-sample t-test. All other characteristics were analyzed using a linear mixed model for treatment and visit.

*indicates visit effect for increase from pre-intervention to post-intervention regardless of group assignment.

Table 6.4. Fasting hormones and subjective appetite.

	Cottonseed Oil (n=21)		Olive Oil (n=21)		p-values		
	Pre-intervention	Post-intervention	Pre-intervention	Post-intervention	Treatment	Visit	Interaction
CCK (ng/ml) *	0.54±0.03	0.56±0.04	0.63±0.07	0.60±0.06	0.40	0.59	0.05
PYY (pg/ml)	96.5±7.05	93.6±6.37	97.5±9.78	97.7±9.75	0.82	0.61	0.56
Ghrelin (pg/ml)	125±14.3	128±13.3	134±14.6	131±15.7	0.98	0.11	0.44
Hunger (mm)	46.6±4.8	51.4±7.1	48.1±5.7	51.2±5.7	0.93	0.24	0.81
Fullness (mm)	28.7±5.4	21.5±4.4	17.1±4.4	16.3±5.0	0.17	0.15	0.25
Prospective Consumption (mm)	49.3±4.6	47.4±4.9	50.8±4.2	52.0±4.4	0.59	0.92	0.59
Desire to Eat (mm)	51.8±5.7	49.1±5.5	52.6±5.0	50.0±5.7	0.89	0.43	0.99
Appetite Score (mm)	54.7±4.3	56.6±4.8	58.6±4.0	59.2±4.6	0.57	0.58	0.79

All values are presented as mean±SEM. All fasting data was analyzed using linear mixed model for treatment and time. * indicates a significant difference between groups (treatment by visit interaction at $p<0.05$). Tukey's test reveals this difference was driven by slight increases in CCK in CSO and slight decreases in OO. CCK = Cholecystokinin, CSO = cottonseed oil, mm = millimeter, OO = Olive oil, PYY = Peptide YY

Table 6.5. Incremental area under the curve subjective appetite.

	Cottonseed Oil (n=21)		Olive Oil (n=21)		p-values		
	Pre-intervention	Post-intervention	Pre-intervention	Post-intervention	Treatment	Visit	Interaction
Hunger							
All day (mm/d*h)	-9.9±3.8	-16.3±5.8	-13.2±4.7	-18.6±4.8	0.65	0.06	0.87
LAB (mm/d*h) #	-5.6±4.3	-13.7±6.4	-6.3±4.6	-14.9±5.0	0.89	0.01	0.94
HOME (mm/d*h)	-13.8±4.4	-18.9±6.6	-16.9±6.3	-23.1±6.0	0.62	0.13	0.87
Fullness							
All day (mm/d*h)	17.1±4.6	22.1±4.8	28.2±4.7	27.8±4.7	0.16	0.40	0.33
LAB (mm/d*h)	9.4±5.2	15.9±5.8	17.3±4.5	22.9±5.5	0.27	0.06	0.89
HOME (mm/d*h)	22.9±4.8	28.2±4.9	37.3±5.6	33.4±5.0	0.12	0.84	0.19
Prospective Consumption							
All day (mm/d*h)	-9.8±4.0	-10.2±4.9	-13.3±4.6	-17.4±4.0	0.33	0.39	0.50
LAB (mm/d*h)	-4.0±4.1	-6.6±5.3	-6.1±4.6	-14.3±4.1	0.38	0.07	0.33
HOME (mm/d*h)	-14.3±4.9	-12.4±5.7	-17.5±5.9	-21.0±5.2	0.39	0.81	0.40
Desire to Eat							
All day (mm/d*h)	-13.6±4.9	-10.9±4.6	-16.9±4.3	-17.0±5.0	0.43	0.65	0.62
LAB (mm/d*h)	-8.1±5.5	-8.0±5.4	-10.9±4.1	-13.8±4.9	0.49	0.63	0.62
HOME (mm/d*h)	-18.4±5.7	-13.5±5.3	-20.5±6.0	-21.6±6.3	0.48	0.58	0.40
Appetite Score							
All day (mm/d*h)	-12.6±3.4	-14.9±4.6	-17.6±3.8	-19.9±4.1	0.33	0.26	0.99
LAB (mm/d*h) #	-6.8±4.2	-11.1±5.4	-9.9±3.6	-16.2±4.2	0.46	0.03	0.66
HOME (mm/d*h)	-17.3±3.8	-18.2±5.2	-23.1±5.3	-24.8±5.1	0.33	0.60	0.88

All values presented as mean±SEM. These data were analyzed using linear mixed models for treatment and visit. # indicates a significant visit effect $p<0.05$. Regardless of group, "LAB" hunger and appetite score were reduced at post-intervention vs. pre-intervention. All day = measures taken time points 0-720 min, LAB = VAS measures taken time points 0-300 min, HOME = VAS measures taken time points 360 – 720 min, VAS = visual analog scale, mm = millimeters

Table 6.6. Sensory evaluation.

	Cottonseed Oil (n=21)		Olive Oil (n=21)		p-values		
	Pre-intervention	Post-intervention	Pre-intervention	Post-intervention	Treatment	Visit	Interaction
Sensory evaluation of SFA meal							
Appearance	5.5±0.4	5.7±0.4	5.2±0.4	5.6±0.4	0.71	0.36	0.68
Taste/Flavor	6.8±0.3	6.6±0.4	6.2±0.4	6.0±0.5	0.23	0.33	1.00
Texture/Consistency	5.9±0.4	5.8±0.4	5.6±0.4	5.7±0.4	0.60	0.77	0.77
Aroma/Smell	6.4±0.4	6.4±0.4	6.0±0.4	5.6±0.4	0.20	0.56	0.44
Overall Acceptability	6.9±0.3	6.5±0.3	6.0±0.4	6.0±0.4	0.10	0.29	0.29
Sensory evaluation of intervention food							
Appearance	6.6±0.2	--	6.9±0.3	--	0.50	--	--
Taste/Flavor	7.0±0.2	--	7.4±0.2	--	0.14	--	--
Texture/Consistency	6.7±0.2	--	7.1±0.2	--	0.16	--	--
Aroma/Smell	6.8±0.2	--	7.2±0.2	--	0.23	--	--
Overall Acceptability	7.0±0.2	--	7.2±0.2	--	0.32	--	--

Values presented as mean±SEM. Ratings are based on a 9-point hedonic liking scale with 1 indicating “extremely dislike”, 5 indicating “neither like or dislike”, and 9 indicating “extremely like”. Sensory evaluation of SFA meal was analyzed using a linear mixed model for treatment and visit while sensory evaluation of intervention food was analyzed using a two-sample t-test. There were no differences within or between groups for sensory evaluation of the SFA meal or the intervention foods. SFA= saturated fatty acid

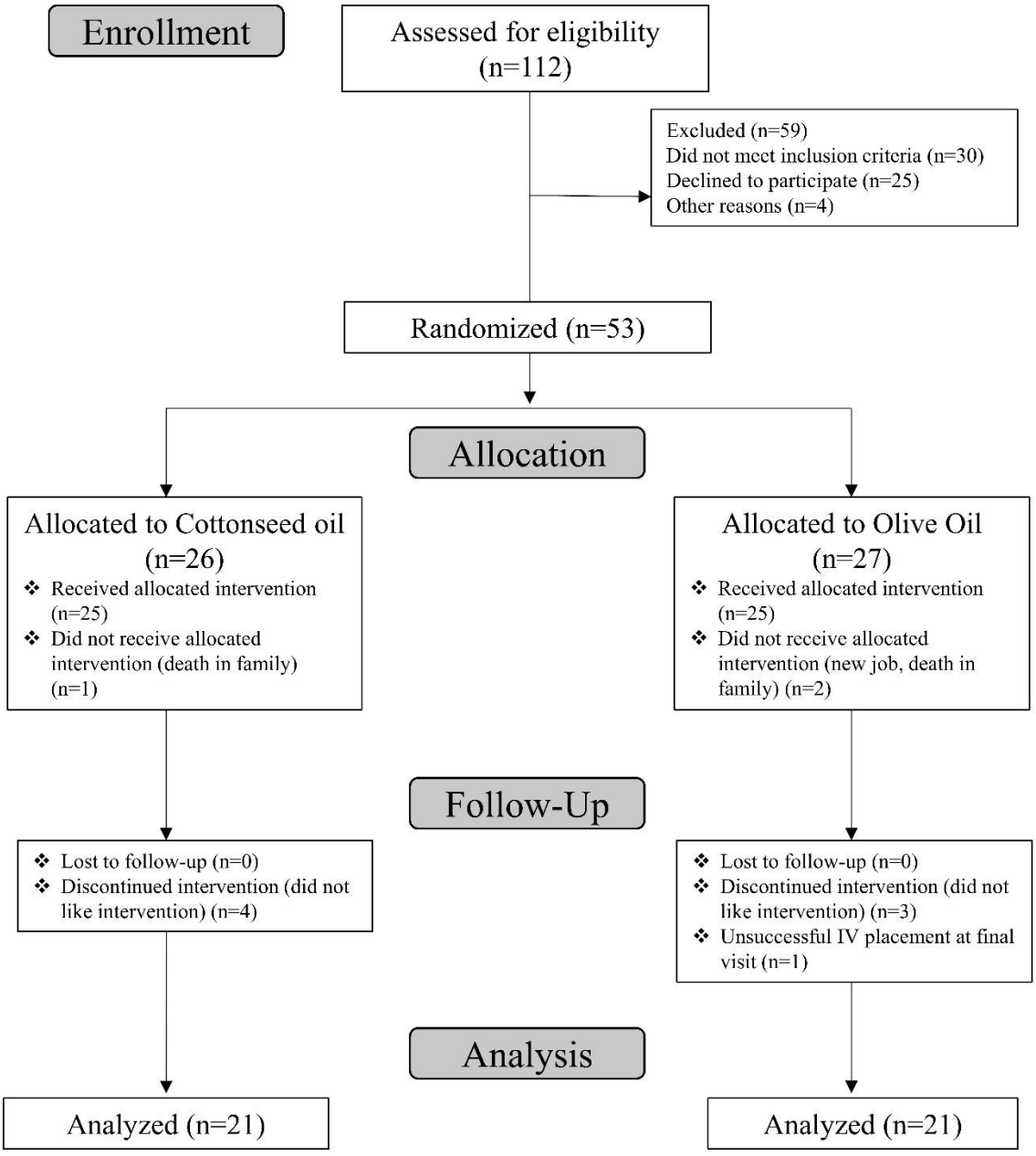


Figure 6.1. CONSORT flow diagram selection of participants.

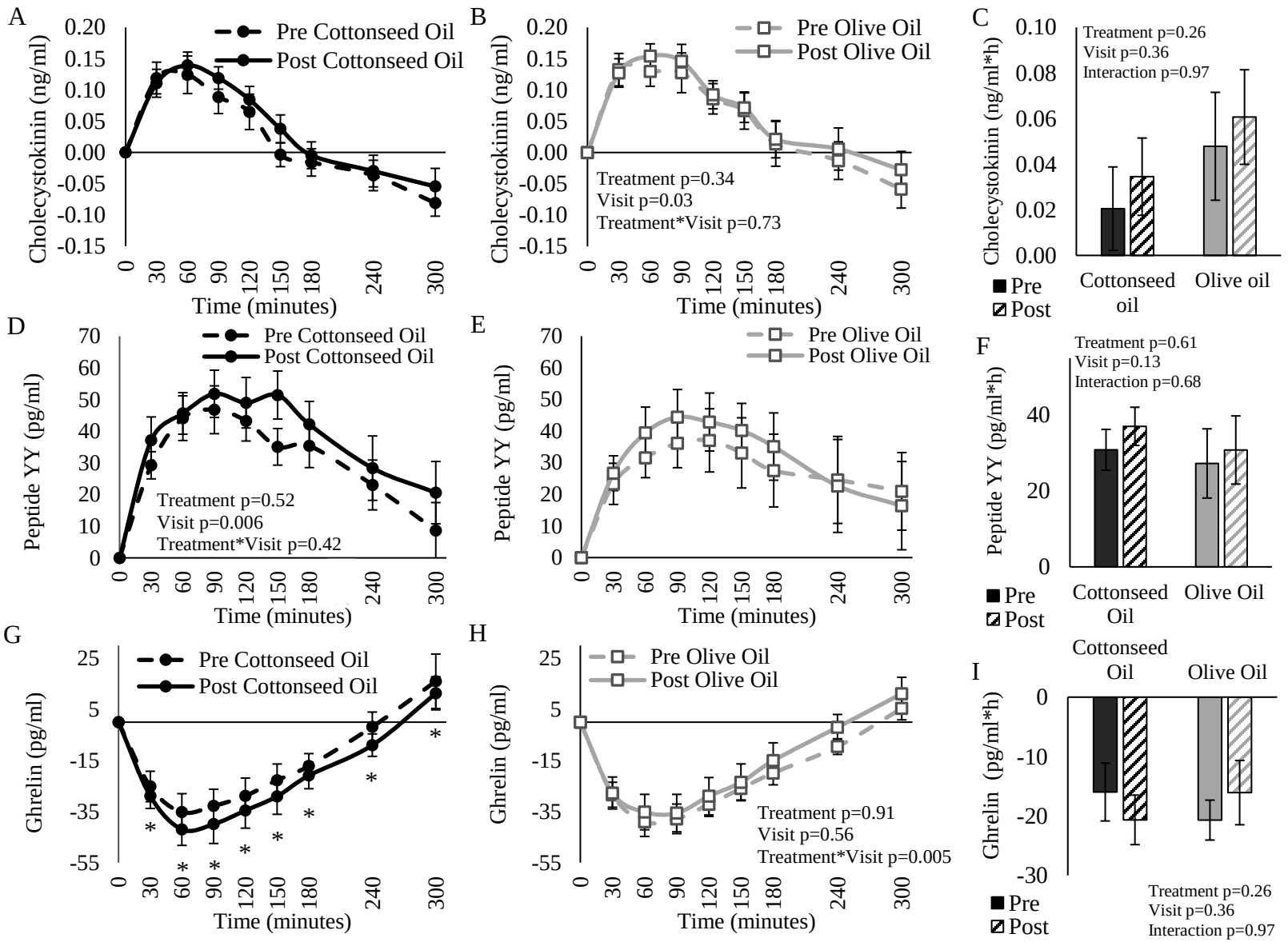
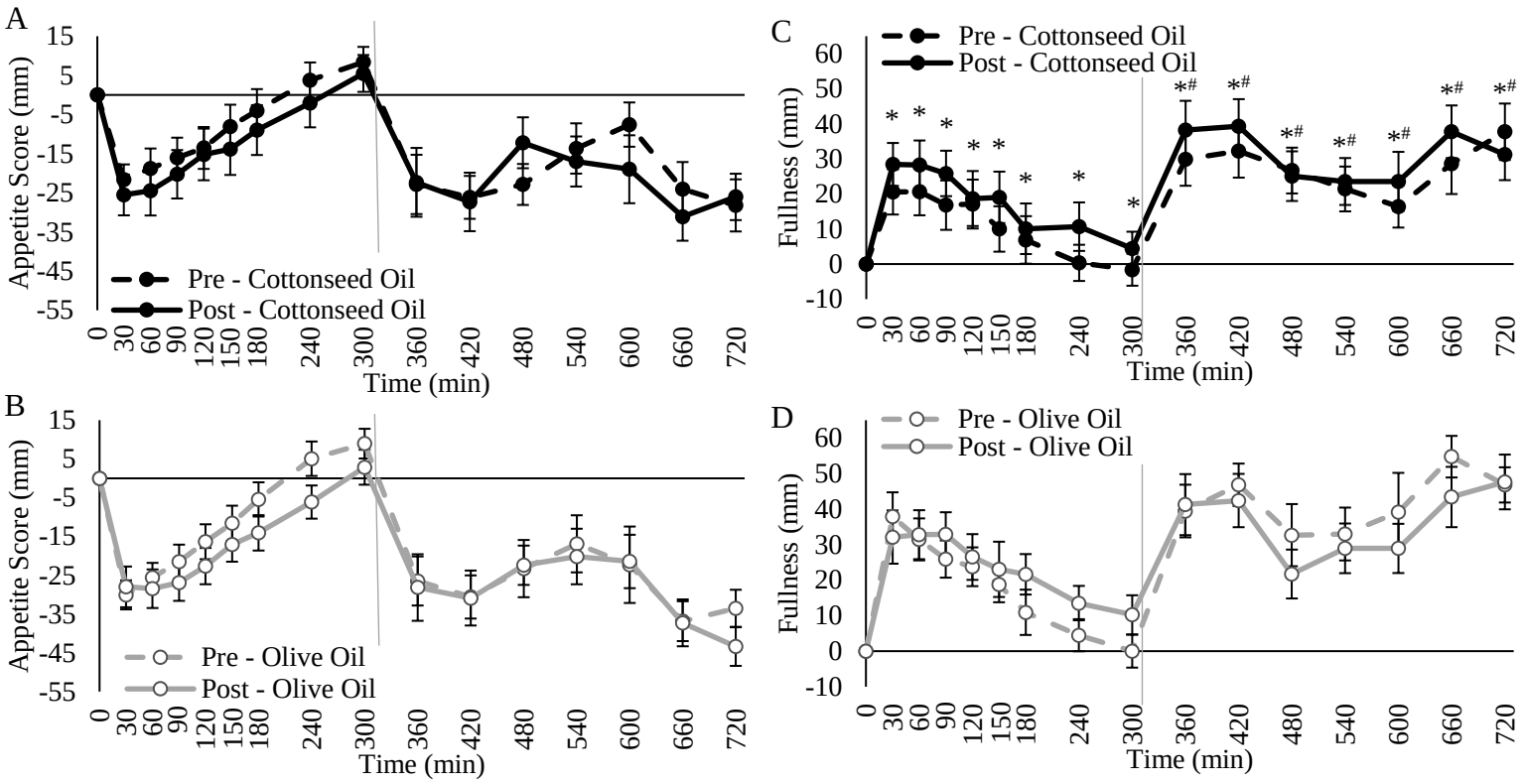

A
Cholecystokinin (ng/ml)
Pre Cottonseed Oil
Post Cottonseed Oil
Time (minutes)
B
Cholecystokinin (ng/ml)
Pre Olive Oil
Post Olive Oil
Treatment p=0.34
Visit p=0.03
Treatment*Visit p=0.73
Time (minutes)
C
Cholecystokinin (ng/ml*h)
Treatment p=0.26
Visit p=0.36
Interaction p=0.97
Cottonseed oil
Olive oil
Pre
Post
D
Peptide YY (pg/ml)
Pre Cottonseed Oil
Post Cottonseed Oil
Treatment p=0.52
Visit p=0.006
Treatment*Visit p=0.42
Time (minutes)
E
Peptide YY (pg/ml)
Pre Olive Oil
Post Olive Oil
Time (minutes)
F
Peptide YY (pg/ml*h)
Treatment p=0.61
Visit p=0.13
Interaction p=0.68
Cottonseed Oil
Olive Oil
Pre
Post
G
Ghrelin (pg/ml)
Pre Cottonseed Oil
Post Cottonseed Oil
Time (minutes)
H
Ghrelin (pg/ml)
Pre Olive Oil
Post Olive Oil
Treatment p=0.91
Visit p=0.56
Treatment*Visit p=0.005
Time (minutes)
I
Cottonseed Oil
Olive Oil
Ghrelin (pg/ml*h)
Pre
Post
Treatment p=0.26
Visit p=0.36
Interaction p=0.97

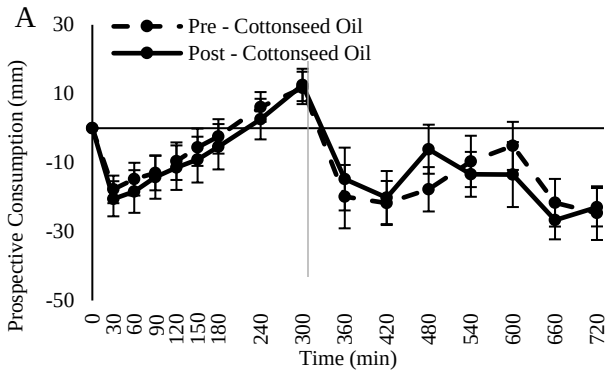
Figure 6.2. Plasma postprandial change from baseline cholecystokinin (CCK) (A, B), peptide YY (PYY) (D, E), and ghrelin (G, H) at pre- and post-intervention visits. Data were analyzed using a linear mixed model for treatment, time, and visit. * indicates significant treatment by visit interaction. Plasma iAUC at pre- and post-intervention visit for CCK (C), PYY (F), and ghrelin (I) were analyzed using a linear mixed model for treatment and visit. No significant differences were found for iAUC data. Pre- pre-intervention visit, Post- post-intervention visit, iAUC- incremental area under the curve.

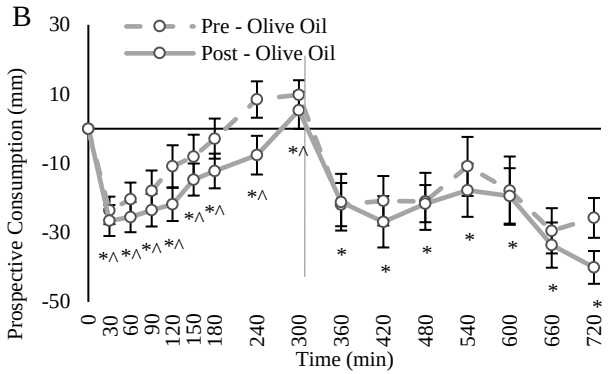


Appetite Score			
P-Values	All Day	LAB	HOME
Treatment	0.37	0.45	0.41
Visit	0.004	<0.001	0.44
Treatment*Visit	0.61	0.45	0.84

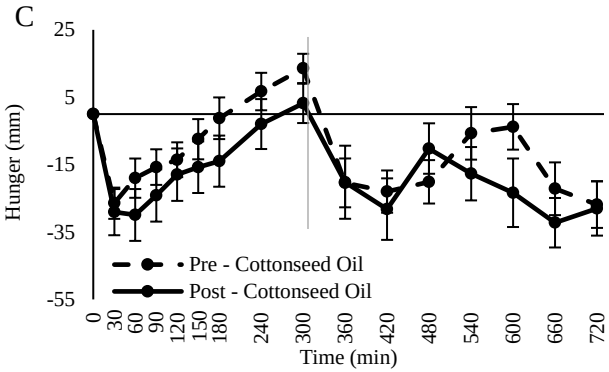
Fullness			
P-Values	All Day	LAB	HOME
Treatment	0.14	0.26	0.15
Visit	0.04	<0.001	0.68
Treatment*Visit	0.04	0.46	0.04

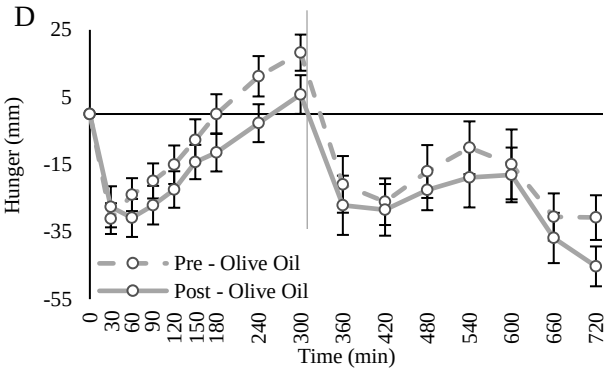
Figure 6.3. Postprandial change from baseline subjective VAS (visual analog scale) appetite score (A, B) and fullness ratings (C, D) at pre- and post-intervention visits. Data were analyzed using a linear mixed model for treatment, time, and visit. * indicates significant treatment by visit interaction for all day measures (time 0-720). # indicates significant treatment by visit interaction for HOME measures. Pre- pre-intervention visit, Post- post-intervention visit, All Day- VAS measures taken across the whole visit day, LAB- VAS measures taken time 0-300, HOME- VAS measures take time 360-720.



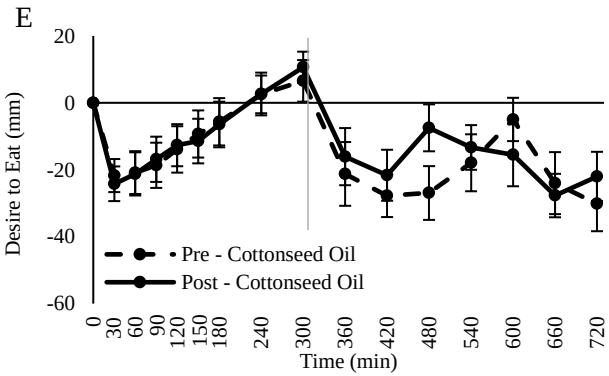


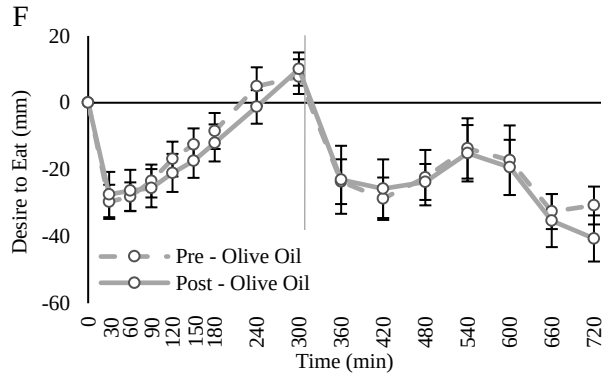
Prospective Consumption			
P-Values	All Day	LAB	HOME
Treatment	0.36	0.36	0.46
Visit	0.003	<0.001	0.32
Treatment*Visit	0.03	0.02	0.23





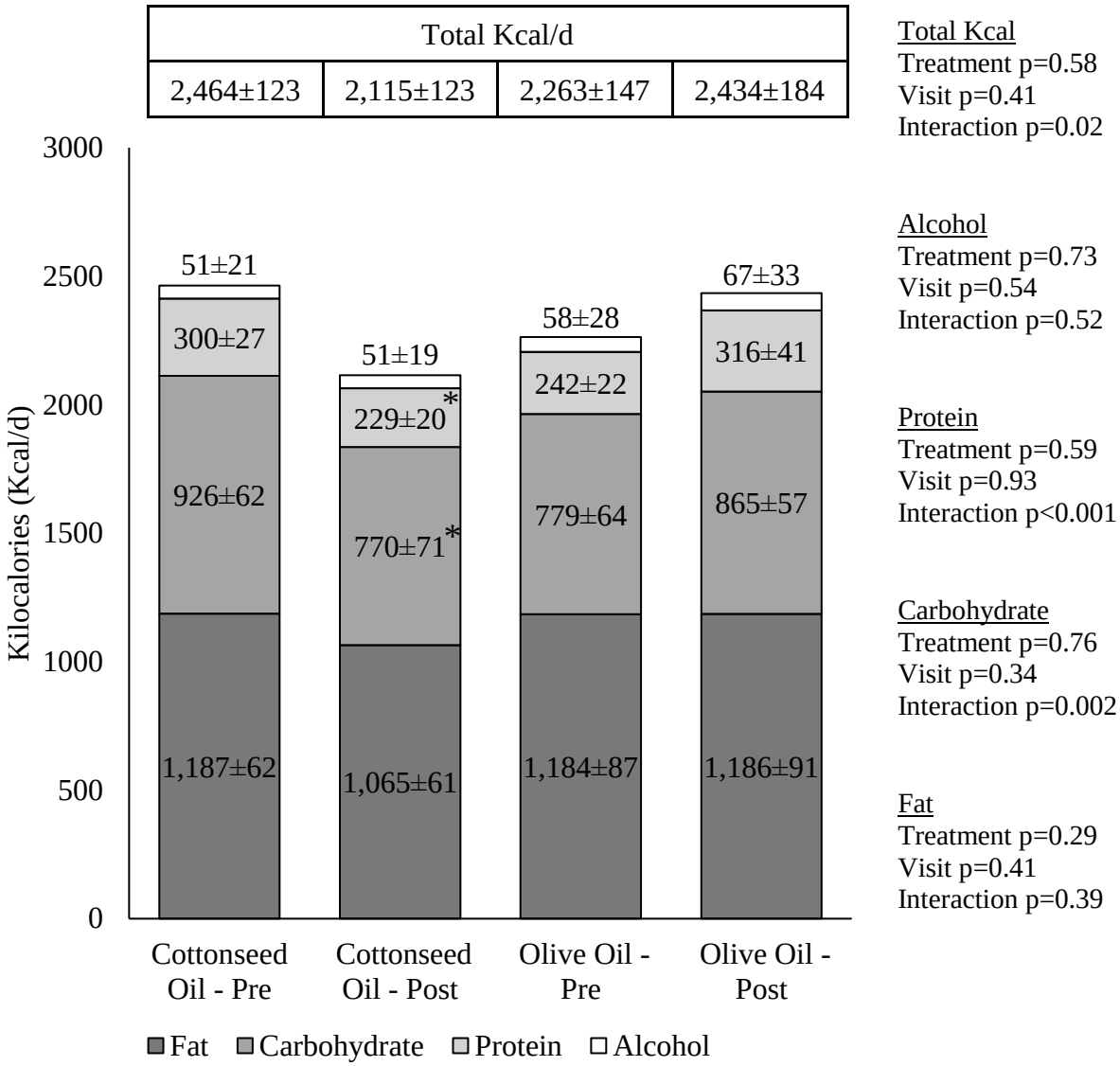
Hunger			
P-Values	All Day	LAB	HOME
Treatment	0.59	0.88	0.66
Visit	<0.001	<0.001	0.009
Treatment*Visit	0.95	0.80	0.79





Desire to Eat			
P-Values	All Day	LAB	HOME
Treatment	0.52	0.49	0.60
Visit	0.92	0.50	0.58
Treatment*Visit	0.13	0.90	0.16

Figure 6.4. Postprandial change from baseline subjective VAS (visual analog scale) ratings of prospective consumption (A,B), desire to eat (C, D), and appetite score (E, F) at pre- and post-intervention visits. Data were analyzed using a linear mixed model for treatment, time, and visit. * indicates significant treatment by visit interaction for all day measures (time 0-720). ^ indicates significant treatment by visit interaction for LAB measures. Pre- pre-intervention visit, Post- post-intervention visit, All day- all VAS measures taken across the whole visit day, LAB- VAS measures taken time 0-300, HOME- VAS measures take time 360-720.



Total Kcal
Treatment p=0.58
Visit p=0.41
Interaction p=0.02

Alcohol
Treatment p=0.73
Visit p=0.54
Interaction p=0.52

Protein
Treatment p=0.59
Visit p=0.93
Interaction p<0.001

Carbohydrate
Treatment p=0.76
Visit p=0.34
Interaction p=0.002

Fat
Treatment p=0.29
Visit p=0.41
Interaction p=0.39

Figure 6.5. Total and macronutrient intake consumed on the days of the pre- vs. post-intervention visits. Data were analyzed using a linear mixed model for treatment and visit and are expressed in kcals (kilocalories). * indicates significant treatment by visit interaction. Pre- pre-intervention visit, Post- post-intervention visit.

Kilojoule (KJ) equivalents of energy intake are as follows.

CSO pre: total EI 10,309±515 KJ/d; alcohol 213±87 KJ/d; protein 1,256±112 KJ/d; carbohydrate 3,874±261 KJ/d; fat 4,964±258 KJ/d.

CSO post: total EI 8,849±515 KJ/d, alcohol 212±81 KJ/d; protein 958±82 KJ/d; protein 3,223±297 KJ/d; fat 4,456±256 KJ/d.

OO pre: total EI 9,468±615; alcohol 241±118 KJ/d; protein 1,013±92 KJ/d; carbohydrate 3,259±267 KJ/d; fat 4,956±365 KJ/d.

OO post: total EI 1,184±770 KJ/d; alcohol 280±138 KJ/d; protein 1,322±172 KJ/d; carbohydrate 3,620±240 KJ/d; fat 4,962±382 KJ/d.

CHAPTER 7

NO OBSERVED DIFFERENCE IN INFLAMMATORY AND COAGULATION MARKERS FOLLOWING DIETS RICH IN N-6 POLYUNSATURATED FAT VS MONOUNSATURATED FAT IN ADULTS WITH UNTREATED HYPERCHOLESTEROLEMIA: A RANDOMIZED TRIAL[5]

[5] Prater M.C., Scheurell A. R., Paton C. M., Cooper J. A. 2024.
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Abstract

Background: Inflammatory and prothrombotic responses are hallmark to the progression of cardiovascular disease (CVD) and may be influenced by the type of dietary fat. Cottonseed oil (CSO) is rich in omega-6 polyunsaturated fats (n-6 PUFA) and improves traditional CVD risk factors such as cholesterol profiles. However, some clinicians are still hesitant to promote n-6 PUFA consumption despite growing evidence suggesting they may not be independently pro-inflammatory.

Objective: To investigate the inflammatory and coagulation marker responses to an 8-week diet intervention rich in either CSO or olive oil (OO; monounsaturated fat rich) in adults with untreated hypercholesterolemia.

Design: This was a secondary analysis of a parallel-arm randomized clinical trial with the main outcome of cholesterol measures.

Participants/setting: Participants included in this analysis were 42 sedentary adults age 30-75 (62% female) in the Athens, GA area, between May 2018 and June 2021, with untreated hypercholesterolemia or elevated blood lipids and BMIs >18.5. Hypercholesterolemia was defined as at least two blood lipids in a "borderline undesirable/at risk" range (total cholesterol (TC) ≥ 180 mg/dL, Low-density lipoprotein cholesterol (LDL-c) ≥ 110 mg/dl, high-density lipoprotein cholesterol (HDL-c) < 50 mg/dL, or triglycerides (TG) ≥ 130), or at least one in an "undesirable" range (TC ≥ 240 mg/dL, LDL-c ≥ 160 mg/dl, HDL-c< 40 mg/dL, or TG ≥ 200).

Intervention: Participants were randomly assigned to either the CSO or OO group in a partial outpatient feeding trial. Meals from the study provided approximately 60% of their energy needs with 30% of energy needs from either CSO or OO for eight weeks. Participants fulfilled their remaining energy needs with meals of their choosing.

Main outcome measures: Fasting plasma concentrations of inflammatory markers including C-reactive protein (CRP), tumor necrosis factor-α (TNF-α), interleukin-6 (IL-6), and interleukin-1β (IL-1β) were measured at baseline and 8-weeks. Markers of coagulation potential including plasminogen activator inhibitor-1 (PAI-1), and tissue factor (TF) were measured at the same time points.

Statistical analyses performed: Repeated measures linear mixed models were used with treatment and visit in the model for analyses of all biochemical markers.

Results: There were no significant differences in fasting CRP (p=0.70), TNF-α (p=0.98), IL-6 (p=0.21), IL-1β (p=0.13), PAI-1 (p=0.29), or TF (p=0.29) between groups across the intervention.

Conclusion: Inflammation and coagulation marker responses to diets rich in CSO vs. OO were not significantly different between groups, and neither group showed changes in these markers in adults with untreated hypercholesterolemia. This provides additional evidence suggesting that dietary n-6 PUFAs may not promote inflammation compared to MUFAs, even in adults at increased risk for CVD.

Introduction

The cholesterol lowering effects of omega-6 poly-unsaturated fatty acids (n-6 PUFAs), such as linoleic acid (LA), are well documented (1-3). However, hesitation still surrounds the promotion of the health benefits of n-6 PUFAs (4), likely from the prior belief that n-6 PUFAs increase inflammation, exacerbate coagulation potential, and/or promote cardiovascular disease (CVD) (5). While it is true that inflammation and coagulation pathways, when dysregulated, can drive CVD and other chronic disease risk (6, 7), understanding of the effects of n-6 PUFAs on these markers has changed in recent years based on a new growing body of evidence.

Reexamination of LA metabolism has revealed that the physiological effects are not as detrimental as once believed (5, 8). The conversion of dietary LA to arachidonic acid (AA) drove health concerns because tissue AA is a substrate for the production of a wide variety of eicosanoids, some of which are pro-inflammatory and increase coagulation potential (9, 10). It is now known that only ~0.2% of dietary LA is converted to AA (11), variations in LA consumption do not correspond to similar variation in tissue AA (12), and some LA metabolites including prostaglandin E_1 (PGE_1) and thromboxane A_1 (TxA_1) are anti-inflammatory and reduce markers of coagulation potential (13-15). Furthermore, animal models have shown that diets rich in LA do not independently induce inflammation (16, 17).

In addition to animal model and cell culture data, it is important to consider changes in biomarkers of inflammation and coagulation after people consume LA. Relatively few clinical trials have assessed dietary interventions rich in LA for markers of inflammation and coagulation. However, systematic review and meta-analysis of available data has shown little to no evidence of a relationship between dietary LA and increases in markers of inflammation or coagulation (18, 19). While promising, these results suffer from heterogeneity due to variations

in study design, use of different inflammation and coagulation markers, and a low number of trials. Thus, more research is needed to inform the inflammation and coagulation potentials of n-6 PUFA-rich diets.

Cottonseed oil (CSO) is a n-6 PUFA-rich oil (LA 56.97%) (20) that is commonly used in commercial food products in the United States. Despite containing a relatively high amount of saturated fat (~22%) (20), CSO diet enrichment has been shown to improve multiple markers of CVD risk (20-23), including large reductions in fasting (20, 22, 24) and postprandial (20, 22) blood lipids. However, its effect on markers of inflammation and coagulation has never been assessed. Olive oil (OO), on the contrary, is familiar to consumers and considered a healthy oil that is rich in mono-unsaturated fats (MUFA), specifically oleic acid (OA)(25, 26). It has been shown to reduce blood lipids when replacing or compared to some saturated fats (27, 28) and impart anti-inflammatory effects in the context of the Mediterranean diet (29); however, OA is not known to influence markers of inflammation or coagulation potential alone (30-32). Only high phenolic extra virgin OO independently provides improvement in these markers (32-34). This lends to commercially available OO being a good comparator to CSO. The purpose of this study was to compare the changes in circulating markers of inflammation and coagulation potential in response to 8-week diets enriched with CSO (n-6 PUFA rich) or OO (MUFA rich) in adults with dyslipidemia. The null hypothesis was that there would be no difference between CSO and OO diets with respect to changes in circulating markers of inflammation and coagulation potential. It was predicted that there would not be enough evidence to reject the null hypothesis.

Methods

Study Design

This was a secondary analysis of an 8-week single-blind, randomized, parallel-arm trial (clinicaltrials.gov: NCT04397055) utilizing a partial outpatient feeding intervention with the primary outcome assessing cholesterol responses. Recruitment began in May 2018 at the University of Georgia, Athens campus via listservs, Facebook ads and flyers. Final testing addressing all primary outcomes took place in June 2021. Participants attended four visits including a screening visit and three testing visits (pre-, mid- and post-intervention). A balanced blocks random assignment method was used to assign participants to either an OO or CSO intervention arm as previous described (20). This study was conducted according to the guidelines laid down by the Declaration of Helsinki and the Institutional Review Board for human participants at the University of Georgia approved all procedures involving human participants (STUDY00005869). Prior to any testing procedures, informed written consent was obtained from each participant.

Participants

One hundred and twelve sedentary adults between the ages of 30 and 75y were assessed for further eligibility (**Figure 7.1**). Inclusion and exclusion criteria were measured at the screening visit and has been described previously (20). Briefly, exclusion criteria included ages outside 30-75y, excessive alcohol use, tobacco use, exercise (>3h/week), weight instability (gain or loss >5% of body weight in the past 3mo), lipid-lowering medications, diabetes medications, steroid/hormone therapies, ADD/ADHD medications, or chronic/metabolic diseases. Hypercholesterolemia was defined as at least two blood lipids in a “borderline undesirable/at

risk" range, or at least one in an "undesirable" range. The "borderline undesirable/at risk" ranges were as follows: total cholesterol (TC) ≥ 180 mg/dL, Low-density lipoprotein cholesterol (LDL-c) ≥ 110 mg/dl, high-density lipoprotein cholesterol (HDL-c) < 50 mg/dL, or triglycerides (TG) ≥ 130 (to convert TC, LDL-c, and HDL-c from mg/dL to mmol/L multiply by 0.0259, to convert TG from mg/dL to mmol/L multiply by 0.0113). The "undesirable" measures were defined as TC ≥ 240 mg/dL, LDL-c ≥ 160 mg/dl, HDL-c < 40 mg/dL, or TG ≥ 200.

Protocol

Screening visit

Prior to testing procedures at all visits, participants were instructed to fast overnight (8-12h) and abstain from alcohol and exercise for 24h. At the screening visit, participants arrived at the University of Georgia (lab) where height, weight, waist/hip circumference, and blood pressure were measured after written informed consent was provided. Height, weight and waist/hip circumferences were measured without shoes, in light clothing using a stadiometer, a clinical scale, and an anthropometric tape respectively. Further, circumferences were taken in triplicate and averaged. Blood pressure was taken using an automated cuff, in triplicate, and averaged. Additionally, self-reported sex, age, and race/ethnicity were collected. Next, a 30-min indirect calorimetry measurement (TrueOne 2400; Parvo Medics, Sandy, Utah, USA) was taken under standard conditions (35). The Weir equation was applied to the final 20min of respiratory gasses to calculate resting metabolic rate (RMR) (36). The resultant RMRs were multiplied by an average U.S. physical activity factor of 1.65 (37), to estimate total daily energy needs for assignment to diet intervention energy tiers. After the screening visit, a researcher not involved in data collection or analysis randomly assigned participants to either the OO or CSO

interventions using a random number generator and stratified balanced blocks with an allocation ratio of 1:1 (20).

Pre-intervention visit (V1)

Prior to the pre-intervention visit, participants completed a two-day food record consisting of one week day and one weekend day recording everything they ate and drank on their selected days (38). Participants were not required to record consecutive days but did need to record two days in the same week. At v1, participants arrived to the lab in the fasted state after consuming a standardized dinner meal and snack (energy: 50% carbohydrate, 15% protein, 35% fat) the evening before the visit (provided by research personnel). In addition to the anthropometrics described for the screening visit, body composition was measured by Bod Pod (Cosmed USA, Inc.), and a fasting blood sample was collected. At the end of v1, participants were instructed on how to use the compliance logs to record meals consumed or missed and were told how much energy was being provided by the study foods. Lastly, they were sent home with a one-week supply of foods made with their assigned intervention oil.

Intervention protocol

Participants began the 8-week diet intervention the day after v1. They were blinded to their assignment of either CSO or OO intervention groups. Via the partial outpatient feeding design, ~60% of energy needs were provided, permitting control of most of the diet, while allowing participants to maintain some of their usual diet. Each week, participants were provided with a one-week supply of daily meals and snacks made with the oil of their assigned group. They were given information including their estimated total daily energy needs, how many kilocalories were provided by the study foods, and the instruction to fill their remaining

estimated energy requirement with their usual foods. Additionally, context was provided to communicate that participants typically only needed one meal of their choosing in addition to the provided study foods.

The two intervention diets provided identical foods, with the exception of the type of oil being used (CSO or OO). A 7-day rotating menu provided two meals plus snacks daily. Research personnel prepared all study foods, including soups, sautéed vegetables with rice, turkey meatloaf with mashed potatoes and baked goods, and ingredients were weighted to the 0.01g. Participants were provided breakfast shakes 5d per week as one of their two meals as described previously (20). The rest of the meals were pre-portioned and frozen by research personnel for participants to take home.

Kilocalorie tiers, as described previously (20), were used to provide ~60% of the estimated energy needs as foods from the study, leading to about 30% of their total energy needs being provided by the assigned oil (CSO or OO). The non-random allocation to kilocalorie tiers was dependent on their estimated energy requirement, derived from their RMR at screening. Participants were instructed to consume foods of their choosing for the remaining ~40% of their energy needs daily.

During the intervention, participants completed weekly compliance checklists as a self-reported measure of compliance. The checklists displayed the 7-day rotating menu and allowed participants to check off the meals consumed while also marking missed meals. Participants also kept four two-day food records including a weekday and a weekend day during weeks 2, 4, 6, and 8. Participants returned to the lab on a weekly basis to pick up meals and turn in study materials. Food records and compliance checklists were reviewed by study staff weekly and participants clarified questions before leaving the weekly pick-up visits.

Mid- and post- intervention visits (V2 and V3)

Participants returned to the lab for their mid-intervention visit (V2) after the first 4 weeks, and the post-intervention visit (V3) after completing all eight weeks of the partial outpatient feeding. All procedures from the pre-intervention visit were repeated at the mid- and post-intervention visits with one exception. Body fat percentage was not measured at the mid-intervention visit.

Plasma sample analyses

All blood samples were drawn into EDTA vacutainers (Becton, Dickinson and Company, Franklin Lakes, NJ), immediately placed on ice, and then centrifuged for 15 min at 4°C. The plasma was aliquoted and stored at -80°C until analysis. Outcomes of plasma concentrations of inflammatory markers (C-reactive protein (CRP) tumor necrosis factor-α (TNFα) interleukin-6 (IL-6) and interleukin-1β (IL-1β)) and markers of coagulation potential (plasminogen activator inhibitor-1 (PAI-1) and tissue factor (TF)) were measured using ELISA kits according to manufacturer directions. Specifically, plasma concentrations of CRP and PAI-1 were assayed using DuoSet ELISAs, TF using Quantikine ELISAs, and TNF-α, IL-6, and IL-1β using Quantikine HS ELISAs (R&D systems Minneapolis, MN, USA). Each participant's total number of samples were run within the same assay.

Dietary intake analysis

ESHA Food Processor SQL software (version 10.12.0) (39) was used to assess all food record data. For analysis of self-reported dietary intake, two averages were calculated based on food records and has been previously described (20). Briefly, the first was an average of the two-day food record completed before V1 and describes the baseline diet. The second is an average

of the four two-day food records taken during the intervention and describes the intake during the intervention.

Statistical analysis

SAS version 9.2 (SAS Institute Inc., Cary, NC, USA) (40) was used for statistical analyses. All values are reported as mean ± SEM unless otherwise noted. Statistical significance was set at $p \leq 0.05$. A sample size of 40 (20 per group) was estimated using a Cohen's F of 0.225 to detect a difference in C reactive protein (CRP) based on the results from Bumrungpert et al (41). Sample size was calculated using G*power 3.19.7 (42) assuming at least 80% power and an α of 0.05. The decision to use per-protocol analyses was made a priori and acceptable compliance rates were set at 75% of provided foods reported as consumed via the compliance checklists. A repeated measures linear mixed model for treatment and visit was used to determine between and within group differences for fasting inflammatory and coagulation markers, as well as differences in self-reported intake between baseline and intervention averages. In all linear mixed models, participants were modeled as random effects. When significance was found, post hoc analyses were done using Tukey's test. Finally, unpaired t-tests were used to assess differences between groups in self-reported compliance. As an exploratory analysis, mixed models were adjusted for TC and LDL-c, which has been published previously (20).

Results

Participants

Fifty-three participants were randomized to either the CSO or OO group; however, eleven did not start or complete the intervention and/or testing visits (**Figure 7.1**). The forty-two

individuals (12 women and 9 men in CSO and 14 women and 7 men in OO) who completed the study have been included in the final analysis. Both groups primarily reported identifying as white with one individual in the CSO group reporting as white and Hispanic/Latino and one individual in the OO group reporting as Asian. Baseline characteristics are presented in **Table 7.1**. There were no differences observed between groups at baseline for any demographic or clinical characteristics measured. All participants that completed the final testing visit were deemed compliant by consuming at least 75% of their provided foods. Participant compliance (self-reported from the compliance checklists) averaged at 91±2% and 92±1% for CSO and OO groups, respectively, with no differences between groups.

Markers of Inflammation

Markers of inflammation are presented in **Figure 7.2**. There were no significant main effects or interactions for CRP, IL-6, or IL-1β. There was a visit effect for TNF-α ($p=0.034$). Post-hoc tests showed a slight increase at V2 compared to V1 ($p=0.026$) with no differences between groups, and no differences between V1 vs. V3. No treatment effects or interactions for TNF- α were detected. However, the visit effect did not remain after adjusting for TC and LDL-c.

Markers of Coagulation Potential

Markers of coagulation potential are presented in **Figure 7.3**. There were no significant main or interaction effects for PAI-1. There was a visit effect for TF ($p=0.014$) that revealed higher TF at V2 compared to both V1 ($p=0.034$) and V3 ($p=0.025$) with no differences between groups. No treatment effects or interactions were TF were detected. The visit effect did not

remain after adjusting for TC and LDL-c. **Figure 7.4** displays individual variations in responses to all markers of inflammation and coagulation potential.

Discussion

This study has shown that enriching diets with CSO for eight weeks did not change select inflammatory markers or markers of coagulation potential and were not different when compared to similar diets enriched with OO. This was observed in a population at risk for CVD. There were visit effects revealing slight increases at V2 (4 weeks), but not V3 (8 weeks), in TNF-α and TF. These increases were transient, not different between groups, and returned to baseline values by the conclusion of the intervention. It is of additional importance to note that the lack of observed increases in inflammatory markers and coagulation potential were in the context of high-fat diets (rich in unsaturated fats), and slight weight gain (20).

Previous clinical trials providing diets rich in LA have found similar results. In hypercholesterolemic populations, circulating levels of TNFα, CRP, serum amyloid A, and IL-6 have shown no significant changes with LA-rich diets, and even showed some tendencies for reductions (43, 44). In the same trials, alpha-linolenic acid (ALA; n-3 PUFA) rich diets were able to reduce circulating levels of these markers; however, the LA-rich diets reduced circulating blood lipids more than the ALA-rich diets (43, 44). This combination of greater reductions in blood lipids, and a lack of inflammatory response, shows the potential value of LA-rich foods for reducing CVD risk. The results of the current analysis are also in line with a systematic review of trials providing LA-rich diets suggesting there is little to no clinical trial evidence of LA-rich diets resulting in increases in inflammatory markers in healthy adults (18).

Due to the increasing incidence of CVD (45), it is important to characterize the inflammatory and pro-thrombotic responses, or lack thereof, of LA-rich diets in order to properly inform dietary guidelines. Rich sources of n-6 PUFA are well documented for their cholesterol and lipid lowering effects (1-3); however, due to concerns regarding inflammation and n-6 PUFA-rich sources, some groups recommend their reduction in the diet (46-48). The main concern with n-6 PUFA-rich foods is that consuming them frequently would drive chronic low-grade inflammation, which is believed to promote many other chronic and metabolic diseases (49, 50). While clinical trials are often not long enough to characterize the chronic effect of LA consumption, prospective cohort studies have shown associations of modestly lower risk of mortality from all causes, CVD, and cancer with increased consumption of LA (51), as well as a lower risk of type 2 diabetes (52). This information is valuable in demonstrating that the effects observed in randomized controlled trials with higher LA intake are translatable into the general population and may elicit lower risk of chronic diseases that have inflammatory pathologies.

Major contributors to the development and progression of thromboembolisms and atherosclerotic plaques are the pro-coagulant proteins PAI-1 and TF. Both of these factors are regulators of separate coagulation pathways (53, 54). PAI-1 inhibits tissue-type plasminogen activator (t-PA) resulting in the inhibition of the fibrinolytic system which stops the degradation of fibrin (54). TF, on the other hand, is the primary initiator of the extrinsic blood coagulation cascade when complexed with factor VIIa (53). When these systems are appropriately regulated, they are critically important for maintaining hemostatic regulation (6). However, inflammation has been shown to influence the production of these pro-coagulant proteins making it additionally important to include markers of coagulation potential when studying inflammation and CVD risk (55). Specifically, CRP, IL-1, and TNF-α have all been shown to stimulate the

expression of PAI-1 and TF in animal models and cultured endothelial cells (56-60). This is problematic since, as mentioned above, both PAI-1 and TF increase clotting potential (61, 62), and higher levels of these coagulation markers increase risk of ischemic CVD and atherosclerosis (63-67). There is also a reciprocal relationship where TF has been shown to induce the expression of inflammatory markers including TNFα, IL-1 and IL-6 (60, 68, 69). Therefore, the lack of change in markers of both inflammation and coagulation potential is clinically relevant for each system independently, but together provides a picture of the lack of increased risk with LA-rich CSO consumption.

It is important to remember that other nutrients of both CSO and OO in addition to their fatty acid profiles could be contributing to the observed protection against changes in inflammation and/or coagulation potential given that the participants of this trial had a slight positive energy balance (significant weight gain) (20) and were following a high-fat diet. CSO is a rich source of the antioxidant vitamin E, which could contribute to the lack of a pro-inflammatory response by protecting its PUFA content from reactive oxygen species and potentially improving oxidative stress in the body (70). Additionally, CSO contains a bioactive lipid called dihydrosterulic acid that has been shown to contribute to its benefits for blood lipid profiles beyond that of other n-6 PUFA rich oils (20, 22, 23). Aside from being a good source of vitamin E, OO is known to contain polyphenols, which could have offered protection against changes in inflammation or coagulation potential due to its potential role in reducing oxidative stress (32, 33, 71). This emphasizes the importance of taking a whole foods approach for clinical interventions to fully understand how the foods we eat can impact overall health.

This study is not without limitations. First, this trial provided foods that differed based on the type of oil, not individual fatty acids. This was intentional and allowed for the analysis of

responses to the whole oils as sources of fats, making the conclusions more applicable to clinical practice as people consume whole fat sources rather than isolated fatty acids. The potential drawback is not being able to identify what specific nutrients or bio-actives are executing what function related to the study outcomes. Another limitation is the lack of a placebo group. This study compared CSO, a relatively unfamiliar oil to American consumers that is showing promise for cardiovascular benefits (20-23), to the recognizably "healthy" OO (72, 73), which may limit the results but better matches previous literature. The use of a partial outpatient feeding trial design did allow participants to consume one meal of their choosing each day which may have introduced higher variability between participants; however, this was done to increase external validity as the results are in the context of some individual dietary choices. Furthermore, overall differences in the nutrient composition of the diets were previously shown to be driven by the provided oils (20). Other limitations include the use self-reported measures of dietary intake and compliance, and high-fat diets. Self-reported data is known to introduce some under- and over-reporting (74). A high-fat diet was chosen to better align with the previous literature on CSO diets (21, 22), but it may limit the translatability of the results to low-fat dietary patterns. While high-fat dietary patterns like the ketogenic diet have gained popularity (75), further research is needed to determine the effects of lower doses of CSO. Lastly, this study was not designed to evaluate the effect of BMI, race, or sex on the outcome variables; thus, future trials are needed to further explore these interactions.

Conclusions

In conclusion, this study assessed inflammatory markers and coagulation potential responses to diets enriched with CSO in adults with untreated hypercholesterolemia. The current study found that eight weeks of CSO vs. OO diet enrichment did not produce observed

differences in the markers of inflammation and coagulation potential measured. However, further research is needed to completely elucidate CSO's effect in the diet particularly in the context of lower doses, different populations, and longer intervention durations.

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Table 7.1. Baseline demographics and clinical characteristics of adults with hypercholesterolemia in cottonseed oil or olive oil diet groups. [a]

	Cottonseed Oil (n=21)	Olive Oil (n=21)	p-values [d]
	V1	V1	Treatment
Age (years) [b]	53±2	54±2	0.73
Sex (% female) [c]	57.1%	66.7%	0.53
Ethnicity (% Hispanic or Latino) [c]	4.8%	0.0%	0.33
Race (% white) [c]	100%	95.2%	0.33
Height (cm) [b]	169.1±2.2	168.0±1.9	0.73
Weight (kg) [b]	78.6±3.6	77.9±3.6	0.88
Body Mass Index (kg/m^2) [b]	27.3±0.9	27.6±1.2	0.87
Waist to Hip ratio [b]	0.85±0.02	0.82±0.02	0.33
Body Fat (%) [c]	31.0±2.1	32.9±2.6	0.65
Total Cholesterol (mg/dL) [b, c]	230±6.57	228±5.81	0.73
LDL cholesterol (mg/dL) [b, c]	162±5.41	162±4.90	0.99
HDL cholesterol (mg/dL) [b, c]	56.5±2.79	59.7±2.63	0.52
Triglyceride (mg/dL) [b, f]	169±22.9	131±10.8	0.13
ApoB (mg/dL) [b, g]	112±4.48	111±3.86	0.83

[a] Participants were recruited from the Athens, GA area between May 2018 and June 2021.
[b] Values are presented as mean±SEM.
[c] Values are presented as percentage of total.
[d] Analyzed using a two-sample t-test.
[e] To convert Total Cholesterol, LDL cholesterol, and HDL cholesterol from mg/dL to mmol/L multiply by 0.0259.
[f] To convert TG from mg/dL to mmol/L multiply by 0.0113.
[g] To convert ApoB from mg/dL to g/L multiply by 0.01.

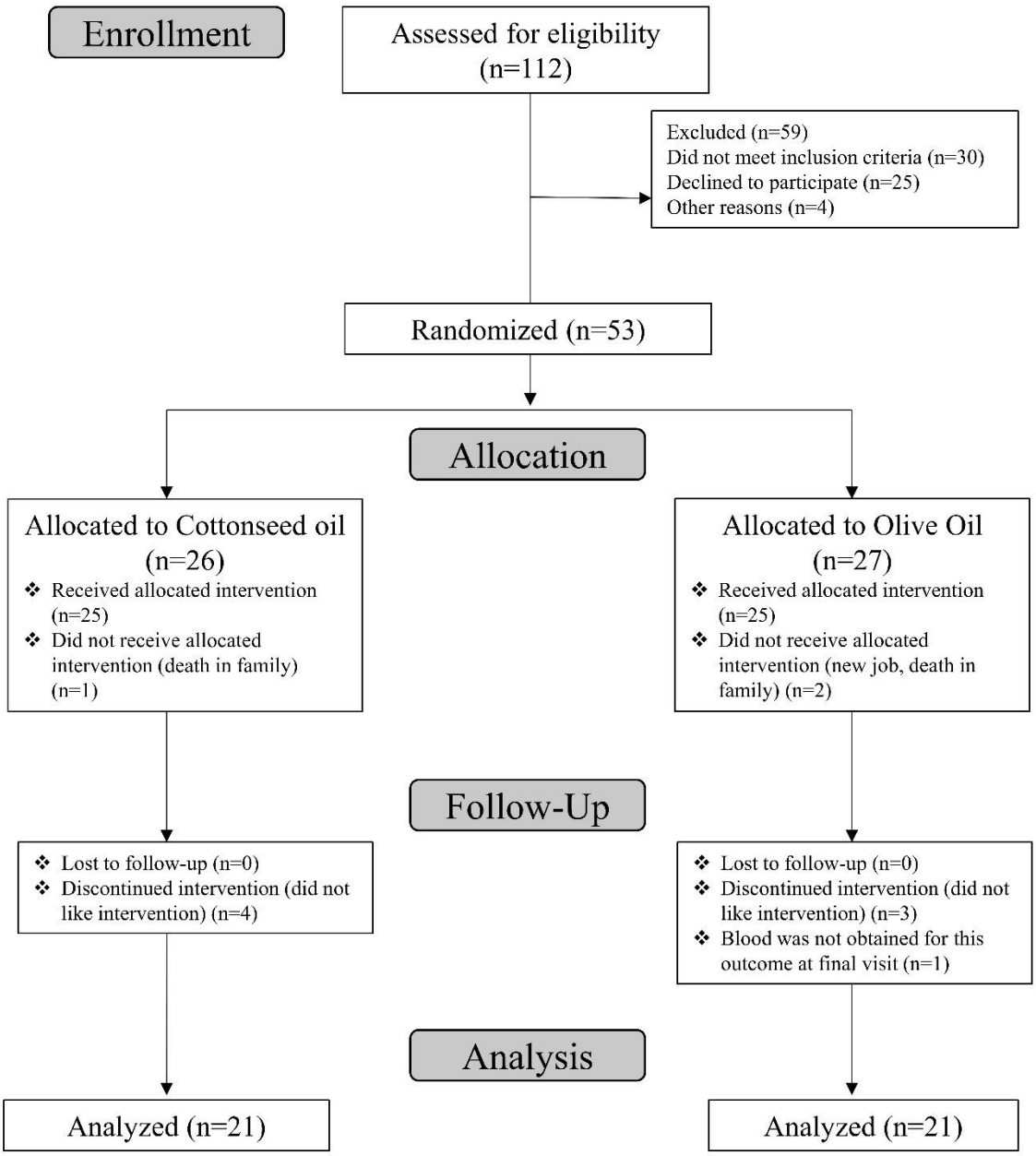

Figure 7.1. CONSORT flow diagram determining the eligibility of participants for a partial outpatient feeding trail comparing cottonseed oil to olive oil enriched diets in adults with untreated hypercholesterolemia conducted from May of 2018 to June of 2021at the University of Georgia in Athens, GA.

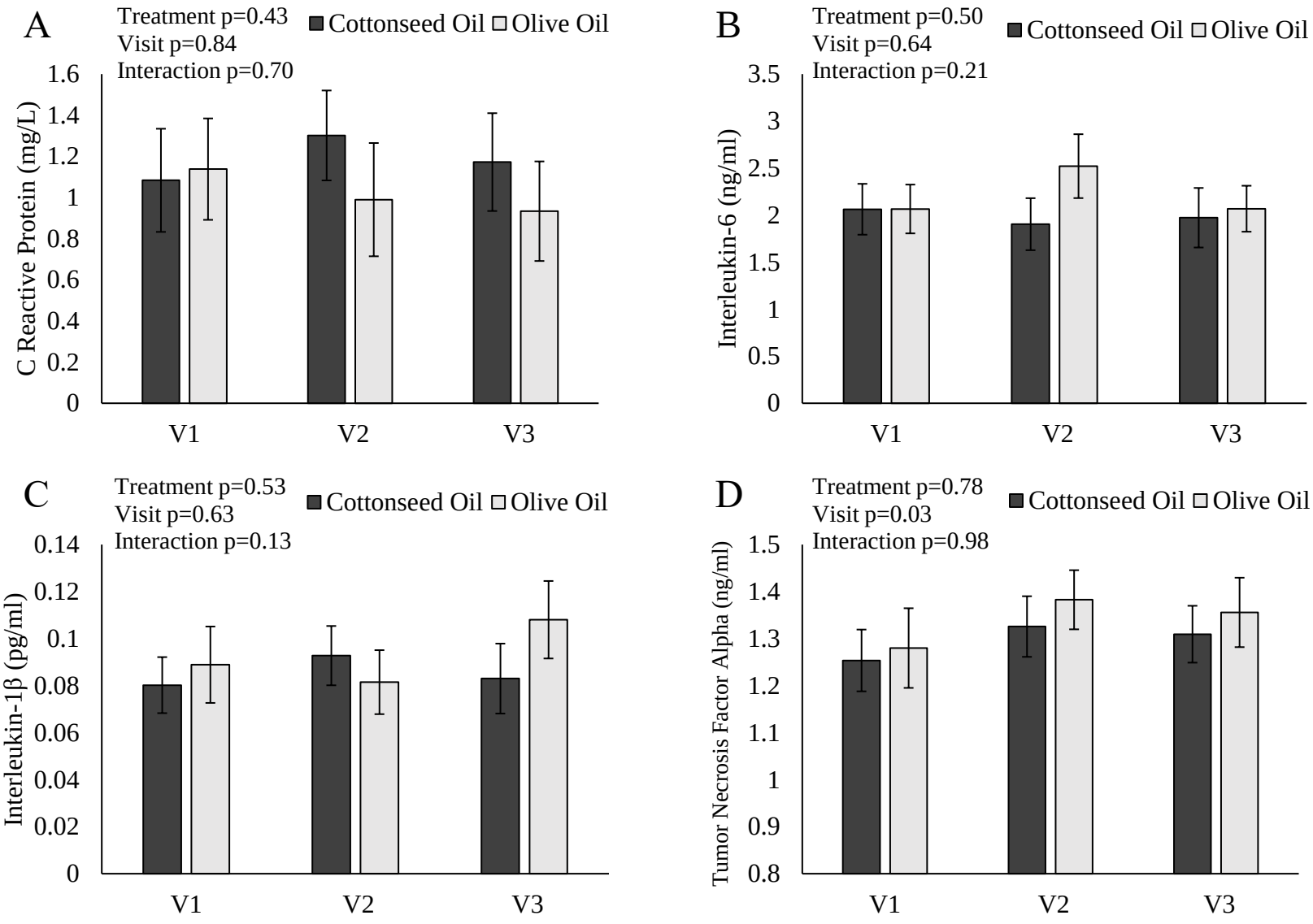
A
Treatment p=0.43
Visit p=0.84
Interaction p=0.70
Cottonseed Oil
Olive Oil
C Reactive Protein (mg/L)
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
V1
V2
V3
B
Treatment p=0.50
Visit p=0.64
Interaction p=0.21
Cottonseed Oil
Olive Oil
Interleukin-6 (ng/ml)
3.5
3
2.5
2
1.5
1
0.5
0
V1
V2
V3
C
Treatment p=0.53
Visit p=0.63
Interaction p=0.13
Cottonseed Oil
Olive Oil
Interleukin-1β (pg/ml)
0.14
0.12
0.1
0.08
0.06
0.04
0.02
0
V1
V2
V3
D
Treatment p=0.78
Visit p=0.03
Interaction p=0.98
Cottonseed Oil
Olive Oil
Tumor Necrosis Factor Alpha (ng/ml)
1.5
1.4
1.3
1.2
1.1
1
0.9
0.8
V1
V2
V3

Figure 7.2. Plasma concentrations of C reactive protein (CRP) (A), interleukin-6 (IL-6) (B), interleukin 1-β (IL-1β) (C) and tumor necrosis factor-α (TNF-α) (D) at V1, V2, and V3 in adults with untreated hypercholesterolemia for cottonseed oil vs. olive oil enriched diets conducted from May of 2018 to June of 2021at the University of Georgia in Athens, GA. Data were analyzed using linear mixed model for treatment and visit. A visit effect was found for greater TNF-α at V2 compared to V1. No other significant differences were observed. V1 – baseline visit, V2 – week-4 visit, V3 – week-8 visit. To convert CRP from mg/dL to mg/L multiply by 10.

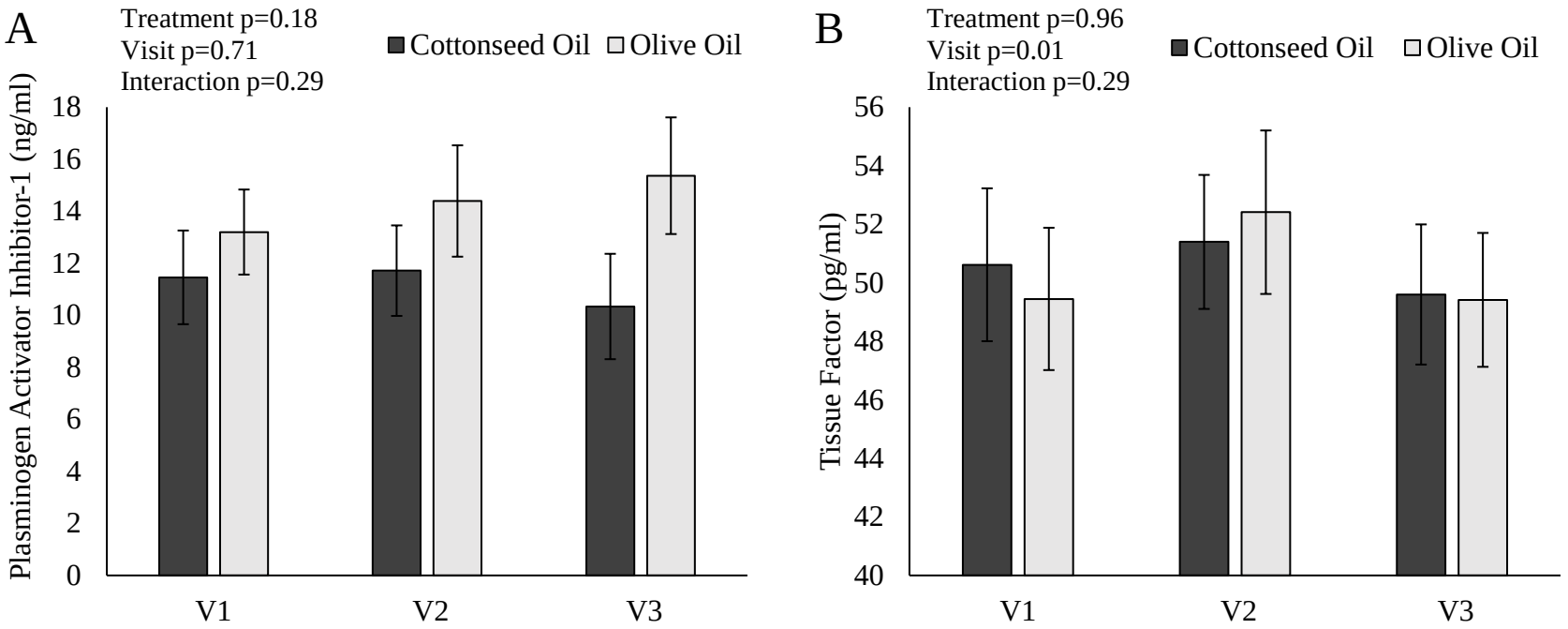

Figure 7.3. Plasma concentrations of plasminogen activator inhibitor-1 (PAI-1) (A) and tissue factor (TF) (B) at V1, V2, and V3 of adults with untreated hypercholesterolemia for cottonseed oil vs. olive oil enriched diets conducted from May of 2018 to June of 2021at the University of Georgia in Athens, GA. Data were analyzed using linear mixed model for treatment and visit. A visit effect was found for greater TF at V2 compared to V1 and V3. No other significant differences were observed. V1 – baseline visit, V2 – week-4 visit, V3 – week-8 visit. To convert PAI-1 from ng/mL to pmol/L multiply by 22.19.

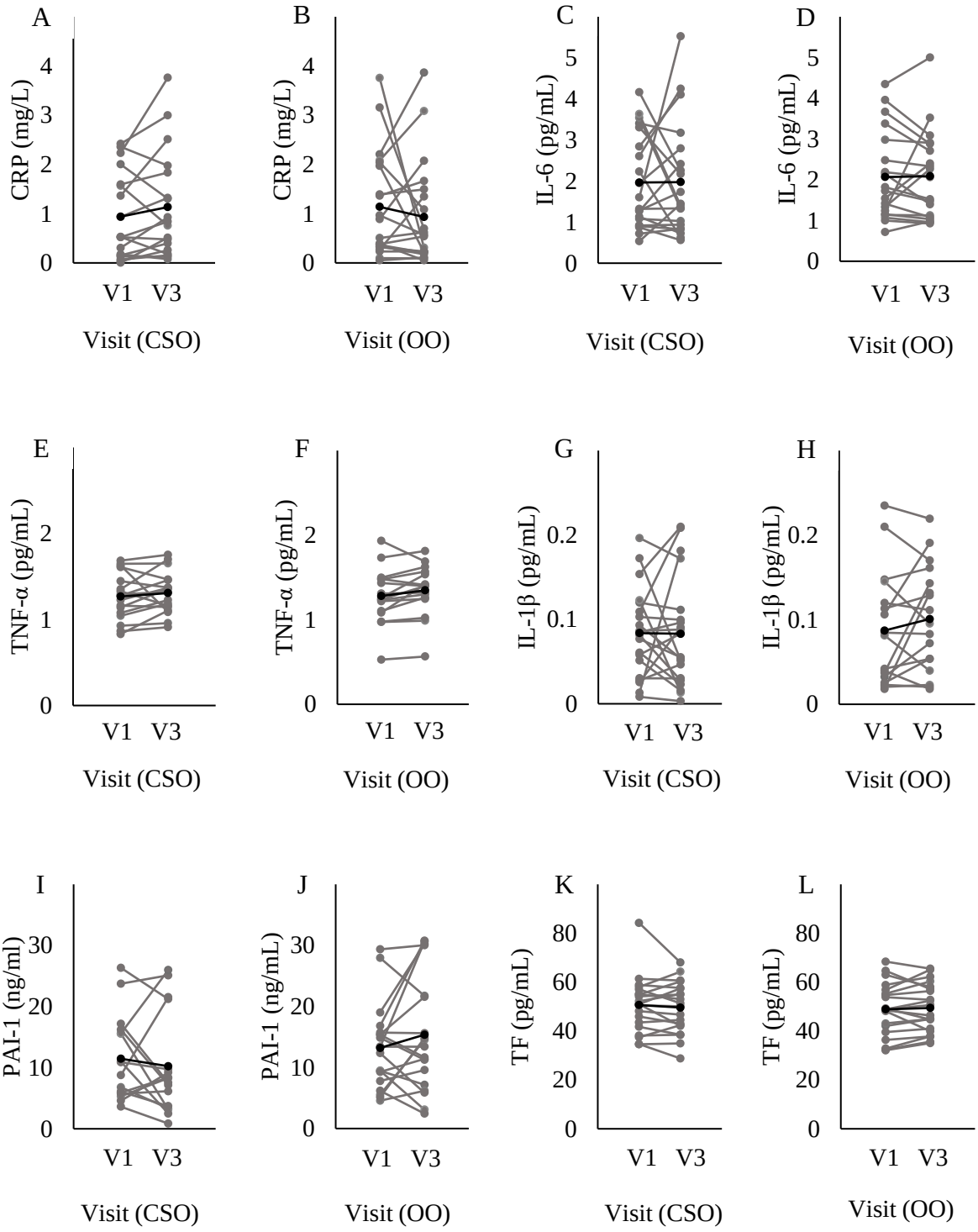
A
CRP (mg/L)
0
1
2
3
4
V1
V3
Visit (CSO)
B
CRP (mg/L)
0
1
2
3
4
V1
V3
Visit (OO)
C
IL-6 (pg/mL)
0
1
2
3
4
5
V1
V3
Visit (CSO)
D
IL-6 (pg/mL)
0
1
2
3
4
5
V1
V3
Visit (OO)
E
TNF-α (pg/mL)
0
1
2
V1
V3
Visit (CSO)
F
TNF-α (pg/mL)
0
1
2
V1
V3
Visit (OO)
G
IL-1β (pg/mL)
0
0.1
0.2
V1
V3
Visit (CSO)
H
IL-1β (pg/mL)
0
0.1
0.2
V1
V3
Visit (OO)
I
PAI-1 (ng/ml)
0
10
20
30
V1
V3
Visit (CSO)
J
PAI-1 (ng/mL)
0
10
20
30
V1
V3
Visit (OO)
K
TF (pg/mL)
0
20
40
60
80
V1
V3
Visit (CSO)
L
TF (pg/mL)
0
20
40
60
80
V1
V3
Visit (OO)

Figure 7.4. Plasma concentrations of individual participants of C reactive protein (CRP) (A, B), Interlukin-6 (IL-6) (C, D), tumor necrosis factor-α (TNF-α) (E, F), interleukin 1-β (IL-1β) (G, H), plasminogen activator inhibitor-1 (PAI-1) (I, J), and tissue factor (TF) (K, L) at V1, and V3 in adults with untreated hypercholesterolemia for cottonseed oil vs. olive oil enriched diets conducted from May of 2018 to June of 2021at the University of Georgia in Athens, GA. Gray dots are individual participants with their values connected by a gray line. Black dots with black lines represent the group mean. CSO – Cottonseed Oil, OO – Olive Oil, V1 – baseline visit, V3 – week-8 visit. To convert CRP from mg/dL to mg/L multiply by 10. To convert PAI-1 from ng/mL to pmol/L multiply by 22.19.

CHAPTER 8

SUMMARY AND CONCLUSIONS

The main purpose of this dissertation project was to compare cardiometabolic responses (blood lipids, ANGPTL, appetite, metabolism, inflammation and coagulation potential) of diets rich in cottonseed oil vs. olive oil in adults with dyslipidemia. To address our research question, we conducted an 8-week partial outpatient feeding trial providing ~60% of participant's daily EE. This allowed us to deliver ~30% of daily EE as either CSO (n=21), or OO (n=22).

The results presented in Chapter 3 demonstrate that 8 weeks of CSO diet enrichment improved TC, LDL-c, non – HDL-c, and apo B, to a greater extent than similar OO diet enrichment. These findings are clinically meaningful because the magnitude of suppression of fasting TC, LDL-c, and apo B correspond to an estimated 14.6% to 21.4% reduction in coronary artery disease risk (1, 2), and a 7% to 10% reduction in CVD risk (3). The impact of these results is further emphasized by the finding that 57% of the participants in the CSO group experienced a reduction in TC and/or LDL-c by at least 1 diagnostic category (e.g., "high" to "borderline high"). It is important to note that the improvements with CSO diet enrichment were observed despite a slight energy surplus in both groups.

In addition to the important results in the fasted state, we show in chapter 3 that the protection from increased postprandial TG and glucose after a saturated fat-rich test meal with CSO vs. OO diet enrichment. Postprandial TG and glucose are gaining attention for their predictive value for CVD (4, 5). Thus, any protection from increases in these postprandial

markers may be beneficial in CVD prevention. In Chapter 4, we reported that postprandial ANGPTLs 3 and 4 increased in the OO group compared to the CSO group. These isoforms of ANGPTLs have been implicated in the regulation of TG and glucose metabolism such that an increase in ANGPTLs can lead to increases in circulating TG levels (6). Together, the observed postprandial differences in TG, glucose, and ANGPTLs between CSO and OO 1) suggest that ANGPTLs may be involved in the mechanism influencing TG and glucose responses to these oils and 2) suggest a postprandial protective effect of CSO dietary enrichment compared to OO enrichment, even in a state of energy surplus.

In Chapter 5, we show no changes in substrate utilization at fasting or postprandially, but an increase in postprandial EE and diet induced thermogenesis (DIT) with OO but not CSO. The increase in DIT in the OO group corresponds to only a 10-kcal increase over the 3.5-h period of postprandial metabolic measurements, which is small compared to total daily EE. Despite this seemingly small change in DIT, it's clinical relevance could be important long-term. On average, it is estimated that adults gain and average of 0.5-1 kg annually, which corresponds to only a 10-20 kcal daily energy surplus (7). In this regard, OO dietary enrichment may be useful in curbing the typical lower DIT response to high saturated fat meals (8) promoting long term weight maintenance. Importantly this did not correspond to weight loss over the short 8-week duration of the intervention. Conversely, Chapter 6 displays the direct reduction in energy intake after the CSO diet (-349 ± 34 kcal) compared to a slight increase in the OO diet (+171 ± 39 kcal). The observed reduced intake in CSO was supported by physiologic changes such as increased fasting cholecystokinin and suppressed postprandial ghrelin responses, as well as enhanced feelings of fullness overall compared to the OO group. Both energy intake driven by appetite, and energy expenditure are important in the consideration weight management. Despite the small increase in

energy expenditure in the OO group, the superior reduction in energy intake and appetite would suggest that CSO may be more useful than OO in terms of weight management.

The last important cardiometabolic outcome assessed in this study was inflammation and coagulation potential (discussed in Chapter 7). CSO is a rich source of omega-6 polyunsaturated fatty acids (n-6 PUFAs) which are commonly associated with increased inflammation and exacerbated coagulation potential (9). Despite mechanistic studies contradicting these associations (10), this is one of very few clinical trials having compared markers of inflammation and coagulation potential in response to n-6 PUFA vs. monounsaturated fat-rich diets. These results showed no differences between diets in these markers and emphasizes the potential of CSO to serve as a nutrition intervention for cardiometabolic disease risk reduction.

To further explore the dietary intake data from this trial, a theoretical average EI required to produced the observed changes in body weight were calculated using the NIH bodyweight planner (https://www.niddk.nih.gov/bwp). The difference between the theoretical EI and self-reported EI during the intervention period was also calculated and serves as a potential indication of under or over reporting of EI on the self-reported food records. This difference in reported and theoretical EI was compared with the change in weight (kg) for each participant and is displayed in **Figure 8.1**. There were no clear relationships between over or under reporting EI and change in bodyweight in either group. There was also no clear relationship between weight gain and inaccuracy of EI reporting. To further explore this relationship, the percent change in weight and the percent difference in EI reporting were calculated and showed a similar result in **Figure 8.2**. There were no clear relationships with the magnitude of over or under reporting of EI with the magnitude of weight change.

Beyond health effects of foods, consumers also consider cost and convenience when making purchasing decisions. Despite the data presented in this dissertation showing the potential of CSO to have superior CVD risk reduction compared to OO, CSO is not typically present on grocery store shelves. With the exception of specific Sam's Club stores in Texas, pure CSO is typically sold online by restaurant suppliers or on Amazon.com. While CSO may be more limited in convenience, it is a cost-effective oil. On Amazon, CSO cost per fluid ounce (fl.oz.) ranged from $0.27 to $0.48. In contrast, OO at a local grocery store (Athens, GA) range in price from $0.35 to $0.86/fl.oz. Further, CSO is also more cost effective than other trendy cooking oils (Avocado oil ~$0.71/fl.oz. or liquid coconut oil ~$0.62/fl.oz.). Therefore, CSO may be less convenient to purchase for the average American household, but it is a cost-effective cooking oil that may offer health benefits over the more common OO.

The data presented in Chapters 3 through 7 are a robust comparison of the cardiometabolic responses to diets enriched with CSO vs OO. This is the first trial to assess CSO consumption in adults with hypercholesterolemia, and displays the treatment potential of CSO in adults at cardiometabolic risk. Namely, this trial showed CSO to improve blood lipids and appetite control without detriment to inflammation. Despite the focus on improvements with CSO in this trial, these results do not demonize OO. Generally regarded as healthy dietary fat source, there is a robust body of research demonstrating the health qualities of OO (11) often in the context of the Mediterranean diet (12). The presented results showed multiple comparable results between OO and CSO, and even superior EE and DIT responses. OO was chosen as the active comparator because it is well established as a healthy fat source. Within this study, any results that showed CSO diets produce similar or superior results to the already established as healthy OO, would further support our hypotheses of CSO being beneficial dietary approach for improvements in

cardiometabolic function. Further differences between these oils include the fatty acid profiles and differences in microconstituents that may have differing bioactive properties. The multiple differences are important in the interpretation of this data. True conclusions can only be drawn from this work based on the comparison of the two oils as a whole, and can only suggest evidence towards hypotheses based solely on individual differences such as fatty acid profiles or bioactive components. The final conclusion of this body of work suggests that CSO may be a more favorable than OO as a dietary fat source for the improvement of cardiometabolic health.

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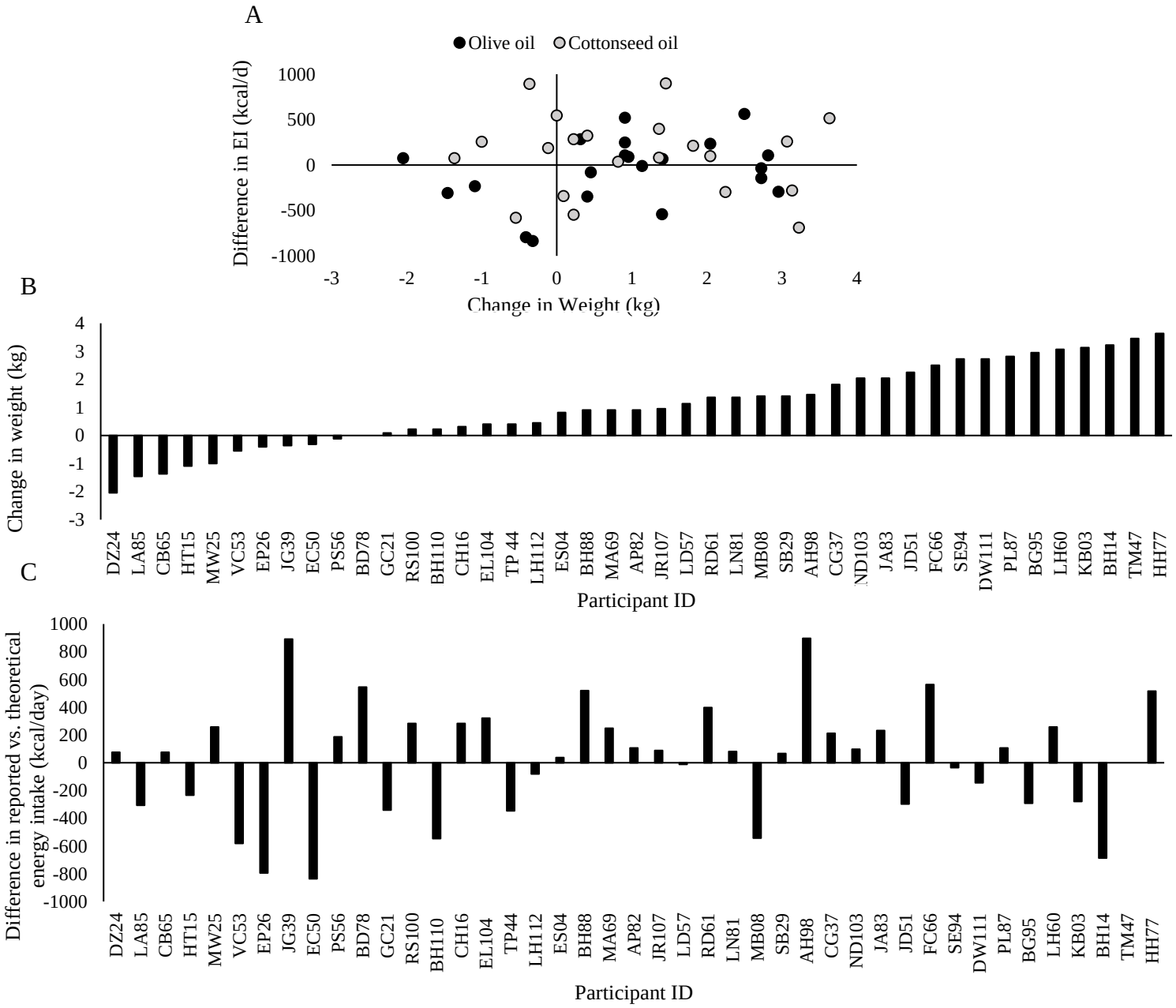
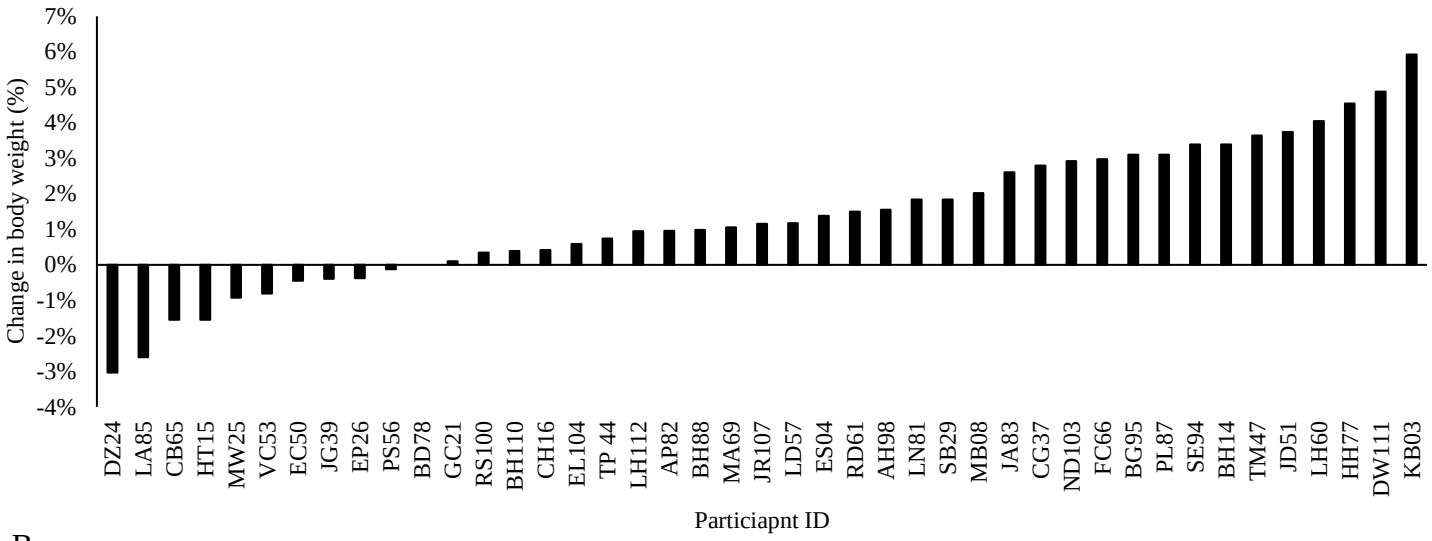

A
Olive oil
Cottonseed oil
Difference in EI (kcal/d)
1000
500
0
-500
-1000
-3
-2
-1
0
1
2
3
4
Change in Weight (kg)
B
Change in weight (kg)
4
3
2
1
0
-1
-2
-3
DZ24 LA85 CB65 HT15 MW25 VC53 EP26 JG39 EC50 PS56 BD78 GC21 RS100 BH110 CH16 EL104 TP 44 LH112 ES04 BH88 MA69 AP82 JR107 LD57 RD61 LN81 MB08 SB29 AH98 CG37 ND103 JA83 JD51 FC66 SE94 DW111 PL87 BG95 LH60 KB03 BH14 TM47 HH77
Participant ID
C
Difference in reported vs. theoretical energy intake (kcal/day)
1000
800
600
400
200
0
-200
-400
-600
-800
-1000
DZ24 LA85 CB65 HT15 MW25 VC53 EP26 JG39 EC50 PS56 BD78 GC21 RS100 BH110 CH16 EL104 TP44 LH112 ES04 BH88 MA69 AP82 JR107 LD57 RD61 LN81 MB08 SB29 AH98 CG37 ND103 JA83 JD51 FC66 SE94 DW111 PL87 BG95 LH60 KB03 BH14 TM47 HH77
Participant ID

Figure 8.1. Comparison of the change in weight (kg) with the difference between self-reported energy intake and estimated energy intake (kcal/d) in both cottonseed oil and olive oil groups (A). Individual variation in change in weight (kg) (B) and differences in self-reported vs. theoretical energy intake (kcal/d) (C). kcal/d – kilocalories per day, kg – kilograms, participant ID – participant identification code.

A


7%
6%
5%
4%
3%
2%
1%
0%
-1%
-2%
-3%
-4%
Change in body weight (%)
DZ24 LA85 CB65 HT15 MW25 VC53 EC50 JG39 EP26 PS56 BD78 GC21 RS100 BH110 CH16 EL104 TP 44 LH112 AP82 BH88 MA69 JR107 LD57 ES04 RD61 AH98 LN81 SB29 MB08 JA83 CG37 ND103 FC66 BG95 PL87 SE94 BH14 TM47 JD51 LH60 HH77 DW111 KB03
Particiapnt ID

B

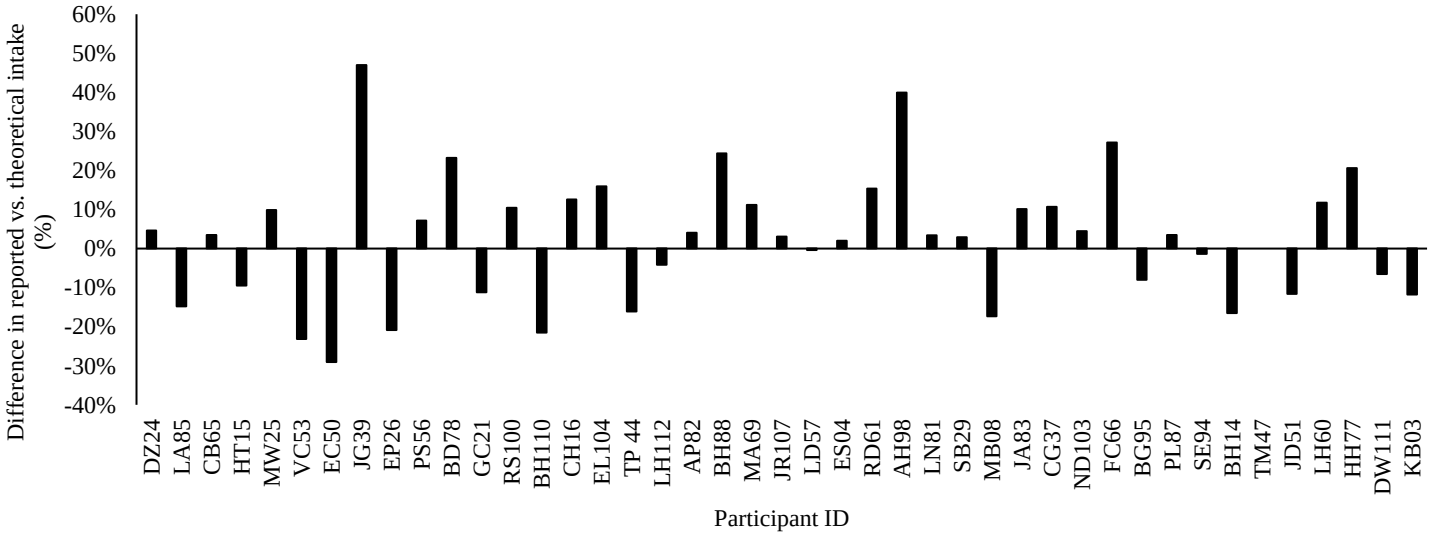

60%
50%
40%
30%
20%
10%
0%
-10%
-20%
-30%
-40%
Difference in reported vs. theoretical intake (%)
DZ24 LA85 CB65 HT15 MW25 VC53 EC50 JG39 EP26 PS56 BD78 GC21 RS100 BH110 CH16 EL104 TP 44 LH112 AP82 BH88 MA69 JR107 LD57 ES04 RD61 AH98 LN81 SB29 MB08 JA83 CG37 ND103 FC66 BG95 PL87 SE94 BH14 TM47 JD51 LH60 HH77 DW111 KB03
Participant ID

Figure 8.2. Individual variation of the percent change in body weight (%) (A) and the percent difference in self-reported vs. theoretical energy intake (%) (B). participant ID – participant identification code.