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EFFECTS OF DIETS ENRICHED IN CONVENTIONAL AND HIGH-OLEIC ACID CANOLA OILS COMPARED TO A WESTERN DIET ON LIPIDS AND LIPOPROTEINS, GENE EXPRESSION, AND THE GUT ENVIRONMENT IN ADULTS WITH METABOLIC SYNDROME FACTORS

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by

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ABSTRACT

The premise of this dissertation was to investigate the effects of diets that differed only in fatty acid composition on biomarkers for cardiovascular disease (CVD) in individuals with metabolic syndrome risk factors, and to explore the mechanisms underlying the response. In a multi-site, double blind, randomized, controlled, three period crossover, controlled feeding study design, participants were fed an isocaloric, prepared, weight maintenance diet plus a treatment oil for 6 weeks with washouts of ≥ 4 weeks between diet periods. The treatment oils included conventional canola oil, high-oleic acid canola oil (HOCO), and a control oil (a blend of butter oil/ghee, flaxseed oil, safflower oil, and coconut oil). The oils provided approximately 18% of total energy (60 g per 3000 kcal) and were consumed daily in a strawberry orange smoothie. The macronutrient profiles of the three test diets were as follows: canola oil diet [17.5% monounsaturated fatty acid (MUFA), 9.2% polyunsaturated fatty acid (PUFA), 6.6% saturated fatty acid (SFA)], HOCO diet (19.1% MUFA, 7.0% PUFA, 6.4% SFA), and control diet (10.5% MUFA, 10.0% PUFA, 12.3% SFA). The control diet was formulated to emulate contemporary Western fatty acid intakes.

In the first study, we examined the effects the three diets on endpoint lipids, lipoproteins, and apolipoproteins in 119 participants. After 6 weeks, the canola oil and HOCO diets resulted in lower circulating total cholesterol (-4.2% and -3.4%, respectively; $P<0.0001$), low-density lipoprotein-cholesterol (LDL-C; -6.6% and -5.6%; $P<0.0001$), apolipoprotein (apo) B (-3.7% and -3.4%; $P=0.002$), and non-high-density lipoprotein-cholesterol (non-HDL-C; -4.5% and -4.0%; $P=0.001$) compared to the control diet. There were no differences between the two canola oil-based diets in these blood parameters. The total cholesterol: HDL-C and apo B: apo A1 ratios

were lower after the HOCO diet compared to control (-3.7% and -3.4%, respectively). No diet effects on triglyceride, HDL-C, or apo A1 levels were observed.

In the second study, we aimed to investigate the biological mechanisms underlying the lipid and lipoprotein response to the treatments, and assessed the expression of 17 target genes that regulate lipid and lipoprotein metabolism. RNA was extracted from peripheral blood mononuclear cells (PBMC) in a subset of the study participants (n=42) and PCR was utilized to determine the relative mRNA transcript at the endpoint of each diet. Relative to the control diet, the canola oil and HOCO diets decreased expression of the ATP-binding cassette (ABC) transporters A1 (canola: -16%, P=0.0006; HOCO: -11%, P=0.005) and G1 (canola: -9%, P=0.003; HOCO: -15%, P=0.003). No significant treatment effects on expression of the remaining genes were observed. The expression of ABCA1 and ABCG1 was not correlated with endpoint circulating lipids, lipoproteins, or apolipoproteins.

In the third study, we investigated an additional potential mechanism driving the cholesterol-lowering response to the canola oil-based diets versus the Western diet, and assessed the effects on fecal short-chain fatty acid (SCFA) levels. Fecal samples were collected in a subset of participants (n=20), and SCFA were extracted and quantified using gas chromatography-mass spectrometry. After 6 weeks, a trend toward a treatment effect on endpoint propionic acid was observed (P=0.09). Acetic acid was increased from baseline following the control diet (P=0.04). After the control diet only, fecal levels of propionic acid were positively correlated with blood levels of LDL-C, non-HDL-C, and apo B (r_P =0.52 to 0.64, P=0.003 to 0.02), and acetic acid was positively correlated with LDL-C and apo B (r_P =0.48 to 0.49, P=0.03 to 0.04). No significant correlations between fecal SCFA and lipids and lipoproteins were observed after the two canola oil-based diets.

Overall, these data indicate that conventional canola oil and HOCO-based diets elicit comparable, beneficial effects on biomarkers consistent with CVD risk reduction compared to a diet with a Western fatty acid profile. We investigated the underlying cardiovascular health promoting mechanisms in two exploratory analyses, and report evidence of coordinated shifts in genes that regulate cholesterol and phospholipid homeostasis in PBMC, as well as hypothesis-generating data to suggest that the gut environment may mediate the effects of dietary fatty acids on the lipid response. Together, these three studies provide information to add to the existing knowledge of the role of dietary fatty acids in modulating cholesterol homeostasis in humans.

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Abbreviations

ABC	ATP-binding cassette
ACC	American College of Cardiology
AHA	American Heart Association
apo A1	apolipoprotein A1
apo B	apolipoprotein B
BMI	body mass index
CETP	cholesteryl ester transfer protein
CHD	coronary heart disease
COMIT II	canola oil multi-center intervention trial II
C_T	threshold cycle
CVD	cardiovascular disease
CYP7A1	cytochrome P450 family 7 subfamily A member 1
DGA	Dietary Guidelines for Americans
DGAT1	diacylglycerol O-acyltransferase 1
DHA	docosahexaenoic acid
DXA	dual-energy X-ray absorptiometry
EPA	eicosapentaenoic acid
FDA	Food and Drug Administration
GC-MS	gas chromatography-mass spectrometry
HDL-C	high-density lipoprotein-cholesterol
HMG-CoA	3-hydroxy-3-methglutaryl-CoA
HMG-CoAR	3-hydroxy-3-methglutaryl-CoA reductase
HOCO	high-oleic acid canola oil
HPFS	Health Professionals Follow-Up Study
INAF	Institute of Nutrition and Functional Foods
LCAT	lecithin-cholesterol acyltransferase
LDL-C	low-density lipoprotein-cholesterol
LDLR	low-density lipoprotein receptor
LEAR	low-erucic acid rapeseed
LIPC	lipase C hepatic type
LIPE	lipase E hormone-sensitive type
LIPG	lipase G endothelial type
LPL	lipoprotein lipase
LXR	liver X receptor
MetS	metabolic syndrome
MTTP	microsomal triglyceride transfer protein
MUFA	monounsaturated fatty acids
NHANES	National Health and Nutrition Examination Survey
NHS	Nurses’ Health Study
NLA	National Lipid Association
PBMC	peripheral blood mononuclear cell
PCR	polymerase chain reaction
PCSK9	proprotein convertase subtilisin/kexin type 9

PHVO	partially hydrogenated vegetable oil
PLTP	phospholipid transfer protein
PPAR	peroxisome proliferator activated receptor
PPARGC1A	peroxisome proliferator-activated receptor γ, coactivator 1 α
PREDIMED	Prevención con Dieta Mediterránea
PSU	The Pennsylvania State University
PUFA	polyunsaturated fatty acids
RCFFN	Richardson Center for Functional Foods and Nutraceuticals
RCT	randomized controlled trial
RXR	retinoid X receptor
SBRC	St. Boniface Hospital Albrechtsen Research Center
SCFA	short-chain fatty acids
SFA	saturated fatty acids
SREBF	sterol regulatory element binding transcription factor
SREBP	sterol regulatory element binding protein
SR-B1	scavenger receptor class B type 1
TFA	*trans*-unsaturated fatty acids
US	United States
USDA	United States Department of Agriculture
USFA	unsaturated fatty acids
VLDL	very low-density lipoprotein
VLDLR	very low-density lipoprotein receptor

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Chapter 1: Introduction

Heart disease remains the leading cause of death in the United States (US) (1) and globally (2). Approximately 92.1 million (36.6%) US adults had at least one type of cardiovascular disease (CVD) in 2011-2014, and these estimates are projected to increase to over 130 million (45.1%) by 2035 (3). The economic burden of CVD is staggering; annual direct and indirect costs in the US were $329.7 billion in 2013-2014, equating to almost $1 billion per day and accounting for 14% of total health expenditures (3). Trends in total CVD mortality are encouraging with an overall reduction from 2000-2014, attributable to successful implementation of lifestyle and pharmaceutical risk management strategies; however, there is a deflection point in 2011, with a decelerated rate of decline from 2011-2014 (4, 5) and an uptick towards 2015 (3). Continued efforts are vital to reduce CVD incidence and mortality.

Risk for CVD is increased among those with metabolic syndrome (MetS) (6-8). MetS is considered a disease of an unhealthy lifestyle and is defined as a cluster of co-occurring, interrelated conditions (9). Specifically, the presence of at least three of any of the following five criteria constitutes a clinical diagnosis of MetS: elevated waist circumference (ethnicity- and country-specific definitions), triglycerides (≥150 mg/dL), blood pressure (systolic: ≥130 and/or diastolic ≥85 mm Hg), and/or glucose (≥100 mg/dL), and/or reduced high-density lipoprotein-cholesterol (HDL-C; males: <40 mg/dL, females: <50 mg/dL) (10). MetS prevalence among US adults is approximately 35% (11) and, although lower among Canadian adults (21%), 67% and 44% of Canadians have ≥1 and ≥2 MetS factors, respectively (12). According to the latest data from the National Health and Nutrition Examination Survey (NHANES; 2003-2012), MetS prevalence is higher among women than men (35.6% vs. 30.3%, respectively), highest among Hispanics (35.4%), and increases as a function of age (18.3% among those 20-29 years and

46.7% among those 60+ years), underscoring the syndrome relevance as the proportion of the older population grows (11, 12).

Lifestyle practices strongly influence established and novel cardiometabolic disease risk factors and, thus, are the foundation for the prevention and treatment of CVD (13). There is an inverse relationship between CVD and related-mortality and key cardiovascular health metrics defined by the American Heart Association [AHA; smoking status, physical activity, dietary intake, body mass index (BMI), total cholesterol, fasting glucose, and blood pressure (14)] (15). Remarkably, achieving the greatest number of health metrics is associated with an 80% lower risk of total CVD (RR 0.20, 95% CI 0.11–0.37) (15). Dietary habits in particular affect five of the seven health metrics (14). Moreover, 45.4% of all cardiometabolic deaths (i.e., heart disease, stroke, type 2 diabetes) in 2012 were attributable to suboptimal intakes of foods/nutrients, including high intakes of sodium, sugar-sweetened beverages, and processed red meats, and low intakes of n-3 seafood, vegetable, fruits, nuts and seeds (16). Consequently, diet plays an indisputable roll in CVD.

Reducing dietary saturated fatty acids (SFA) and replacing them with unsaturated fatty acids (USFA), as well as eliminating *trans*-unsaturated fatty acids (TFA), in the context of a healthy dietary pattern is a cornerstone of nutrition recommendations for CVD prevention and treatment (17-20). Canola oil is a commonly consumed vegetable oil (21) that has a healthy fatty acid profile and an extensive history of cardiovascular benefit (22). Nevertheless, gaps in knowledge exist as to how canola oil-based diets affect cardiovascular lipid and lipoprotein biomarkers compared to a Western diet with a contemporary fatty acid profile, as well as the mechanisms that account for this. Canola oil is also available as high-oleic acid canola oil (HOCO) that is high in monounsaturated fatty acids (MUFA) at the expense of polyunsaturated

fatty acids (PUFA). High-oleic oils are abundant in the food supply, particularly as a replacement for artificial TFA-containing oils (23); however, existing research on cardiovascular health effects of HOCO are sparse.

The premise of the this dissertation research was to assess the effects of three diets that differed in fatty acid composition (i.e., canola oil diet, HOCO diet, and a control diet with a fatty acid profile emulating contemporary Western fatty acid intakes), on established and novel risk factors for CVD in adults with MetS measures. The specific objectives were to compare the effects of the dietary treatments on: blood lipids, lipoproteins, and apolipoproteins; peripheral blood mononuclear cells (PBMC) expression of lipid- and lipoprotein-related genes; and fecal short-chain fatty acid (SCFA) levels. This study was conducted as part of a larger project termed the Canola Oil Multi-center Intervention Trial II (COMIT II), a multi-site, double-blind, randomized, controlled, three period crossover, controlled feeding, clinical trial.

Chapter 2: Literature review

2.1. Dietary fatty acids: Structure, sources, and intakes

Dietary fatty acids are consumed primarily as triglyceride, a lipid comprised of a three fatty acid molecules attached to a glycerol backbone (24). Each fatty acid consists of a hydrocarbon chain of varied length with a carboxyl group and a methyl group at opposing ends. The number, configuration, and position of double bonds, as well as the amount of carbon atoms in the chain, denote the specific type of fatty acid. The absence of a double bond and, thus, saturation of the carbon chain with hydrogen, indicates a SFA. The presence of at least one double bond indicates an USFA, of which there are two types: MUFA with a single double bond and PUFA with two or more double bonds. The double bonds within dietary fatty acids are found predominantly in the *cis* arrangement, but TFA are also present. The position of the first double bond varies and is described relative to the methyl terminus ("ω" or "n" carbon). MUFA have a single double bond at the n-7 or n-9 position, and PUFA have the first double bond at the n-3 or n-6 position. These structural characteristics define the physiological significance of nutritionally derived fatty acids in human metabolism.

Both plants and animals are sources of dietary fatty acids, with most foods containing a combination of fatty acid types (18). SFA are highest in butter, beef fat, lard, and chicken fat, as well as coconut, palm kernel, and palm oils. The major source of SFA in the US among individuals aged 2+ is mixed dishes (i.e., burgers/sandwiches, pizza, rice/pasta/grain dishes, meat/poultry/seafood dishes, and soups), followed by snacks and sweets, protein foods, and dairy. TFA in the diet are of both natural origin (i.e., ruminant fat) and industrially produced [i.e., partially hydrogenated vegetable oils (PHVO) and refined vegetable oils], PHVO being the primary dietary source. Animal-based sources of MUFA include beef fat, lard, chicken fat, and

wild game, while plant-based MUFA include vegetable oils (i.e., canola, olive, and peanut oils), avocados, and nuts. PUFA, particularly the n-6 PUFA linoleic acid (18:2), are found in vegetable oils (i.e., sunflower, corn, cottonseed, and soybean oils), as well as nuts and seeds. Flaxseed, walnuts, and canola oil are significant sources of the n-3 PUFA α-linolenic acid (18:3). The longer-chain n-3 PUFA eicosapentaenoic acid (EPA; 20:5) and docosahexaenoic acid (DHA; 22:6) are marine-based and obtained through intake of fish and seafood, such as salmon, herring, mackerel, tuna, and anchovies (25).

According to the latest NHANES food consumption data (2015-2016), the average intake of SFA, MUFA, and PUFA among the US population is 12%, 12%, and 8% of total energy, respectively (26). The most recent TFA analysis (2003-2006) estimated mean intake from both natural and artificial sources as 1.1% of total energy (27).

2.2. USFA as a replacement for SFA

2.2.1. Dietary recommendations for SFA

Current diet and lifestyle guidelines from authoritative sources recommend healthy dietary patterns that are low in SFA to reduce CVD risk (28). For example, the 2015-2020 Dietary Guidelines for Americans (DGA) recommend a healthy dietary pattern for the general population that includes: a variety of vegetables from all subgroups (i.e., dark green, red and orange, legumes, starchy, etc.), fruits (especially whole), grains (half of which are whole), fat-free or low-fat dairy (i.e., milk, yogurt, cheese, and/or fortified soy beverages), a variety of proteins (i.e., seafood, lean meats and poultry, eggs, legumes, nuts, seeds, and soy products), and oils; and limits: saturated fats, *trans*-fats, added sugars, and sodium (18). The DGA nutrient-

based recommendation for SFA is <10% of calories, a threshold met by only 29% of individuals in the US (18). Leading cardiovascular-centric organizations, including the National Lipid Association (NLA) and AHA/American College of Cardiology (ACC), recommended lower intakes of <7% and 5-6%, respectively, specifically to decrease low-density lipoprotein-cholesterol (LDL-C) (17, 19). The Institute of Medicine, National Academy of Sciences has not established an Adequate Intake, Recommended Dietary Allowance, or Tolerable Upper Intake Level for SFA (29).

2.2.2. Macronutrient substitution [1]

Achieving the recommended reduction in SFA results in a corresponding shift in a different macronutrient (i.e., USFA, carbohydrate, or protein) to maintain the total energy of the diet. The 2015–2020 DGA recommend replacing SFA with USFA, preferably PUFA given its stronger evidence base for CVD benefit compared to MUFA (18). Similarly, the NLA recommends substitution with USFA or protein for those in need of atherogenic cholesterol lowering, and acknowledges that n-6 PUFA causes greater reductions in atherogenic cholesterol levels than MUFA (19). The AHA/ACC recommendation for adults who would benefit from LDL-C lowering does not specify optimal macronutrient substitution, but notes lipid benefit is greatest when SFA is replaced with PUFA, then MUFA, followed by carbohydrate (preferably whole grains) (17). A recent AHA Presidential Advisory on Dietary Fats and CVD reaffirmed

[1] Sentences in this section (2.2.2.) were extracted, with written permission, from a published article in *Current Atherosclerosis Reports* [Bowen KJ, Sullivan VK, Kris-Etherton PM, Petersen KS. Nutrition and cardiovascular disease – an update. Curr Atheroscler Rep 2018;20(20):8]. Kate Bowen wrote the sentences selected from the publication in their entirety. The final publication is available at https://link.springer.com/article/10.1007%2Fs11883-018-0704-3.

the organization's stance of replacing SFA with USFA in the context of a healthy diet to lower CVD incidence, and emphasized the n-6 PUFA linoleic acid (20).

The available epidemiological evidence from observational cohort studies of cardiovascular hard outcomes (i.e., events and mortality) supports these SFA replacement recommendations. A 2015 analysis of repeated dietary assessments from the Nurses' Health Study (NHS) and Health Professionals Follow-Up Study (HPFS) reported replacing 5% of energy from SFA with 5% of energy from PUFA, MUFA, MUFA+PUFA, or whole grains reduced risk of coronary heart disease (CHD) by 25% (HR 0.75, 95% CI 0.67 to 0.84), 15% (HR 0.85, 95% CI 0.74 to 0.97), 17% (HR 0.83, 95% CI 0.75 to 0.91), and 9% (HR 0.91, 95% CI 0.85 to 0.98), respectively (30). Conversely, isocaloric substitution of SFA with refined starches and added sugars was not associated with change in CVD risk. An earlier pooled analysis of 11 prospective cohort studies from 1997-1993 also found isocaloric replacement of 5% of energy from SFA with PUFA was associated with a reduced risk of coronary events (HR 0.87, 95% CI 0.77 to 0.97), although replacement with carbohydrates or MUFA did not reduce risk (carbohydrates: HR 1.07, 95% CI 1.01 to 1.14; MUFA: HR 1.19, 95% CI 1.00 to 1.42) (31). Consideration of differences in carbohydrate quality (i.e., refined grains and added sugars vs. whole grains) and confounding nutrients from animal sources of MUFA (i.e., SFA and TFA) resolves the discordance between the latter findings from the pooled cohorts (31) and the more recent observational report (30).

In a 2016 analysis of the NHS and HPFS data, Wang et al. reported that during follow-up there was a decrease in the correlation between MUFA and SFA, and a shift in the major food sources of MUFA from animal- to plant-based (32). For example, in the NHS, the three major contributors to MUFA intake in 1986 were beef/lamb as a main dish, margarine, and regular

hamburger, whereas in 2010 the top contributors were olive oil, other nuts, and salad dressing. Similarly, in the HPFS, the primary contributors shifted from beef/lamb as a main dish, regular hamburger, and beef/pork/lamb as a sandwich or mixed dish in 1986, to olive oil, other nuts, and peanuts in 2010. A 2018 analysis of theses prospective cohorts separated MUFA from plant and animal sources to delineate their independent associations with CHD risk (33). SFA intake was highly correlated with animal-MUFA ($r \geq 0.81$, $P<0.001$), but not plant-MUFA ($r \leq 0.21$, $P<0.001$), intake, which reflects the overlapping food sources of SFA and animal MUFA (i.e., red meat and dairy). In multivariable models, including adjustment for SFA, highest compared to lowest plant-MUFA intake was associated with a reduced risk of CHD (HR 0.93, 95% CI 0.85-1.03, *P* trend=0.04), whereas highest animal-MUFA intake was associated with increased risk (HR 1.23, 95% CI 1.06-1.42, *P* trend=0.002). Isocaloric replacement of SFA (5% of energy) with plant-MUFA, but not animal-MUFA, reduced CHD risk (plant: HR 0.83, 95% CI 0.68-1.00, $P=0.05$; animal: HR 1.04, 95% CI 0.79-1.38, $P=0.76$). Moreover, CHD risk was 24% lower when 5% of energy from plant-MUFA was substituted for animal-MUFA (HR 0.76, 95% CI 0.65-0.88, $P<0.001$), and 19% lower when substituted for the sum of SFA plus animal-MUFA (HR 0.81, 95% CI 0.73-0.90, $P<0.001$), respectively. The authors concluded a beneficial role of MUFA in CHD prevention when consumed as plant-based foods (i.e., vegetable oils, nuts) and food products containing them.

In addition to the observational data, a meta-analysis of eight randomized controlled trials (RCT) reported lowering SFA and replacing with PUFA reduced CHD risk by 19% (RR 0.81, 95% CI 0.70 to 0.95), corresponding to a 10% lower risk of CHD for every 5% energy replacement of SFA with PUFA (RR 0.90, 95% CI 0.83 to 0.97) (34). The 2017 AHA Presidential Science Advisory on Dietary Fats and CVD applied stringent inclusion criteria to

perform a meta-analysis of a subgroup of these studies (n = 4), and reported that lowering SFA by replacing with PUFA lowered CHD by 29% (RR 0.71, 95% CI 0.62 to 0.81) (20). Similarly, a 2015 Cochrane Review of RCT reported a 27% reduction in cardiovascular events when PUFA replaced SFA (RR 0.73, 95% CI 0.58 to 0.92), whereas there was no significant effect of carbohydrate or protein replacement, and MUFA was inconclusive due to the paucity of RCT on cardiovascular outcomes (35).

Although there is a dearth of RCT evidence assessing substitution of plant-based MUFA for SFA on cardiovascular event outcomes, the macronutrient substitution recommendations are supported by existing RCT evidence that demonstrates a beneficial effect on intermediate cardiovascular biomarkers. Mensink performed a comprehensive systematic review and multiple regression analysis to evaluate the effects of isocalorically exchanging SFA for PUFA (primarily linoleic acid and α-linolenic acid), MUFA (primarily oleic acid), or carbohydrate (mixture) on serum lipids and lipoproteins (**Figure 2-1**) (24). This analysis included 84 clinical trials that were strictly controlled, weight maintenance, randomized, dietary studies, and totaled 2,353 apparently healthy adults. Decreasing 1% of total energy from SFA and increasing 1% from PUFA significantly lowered total cholesterol by 0.064 mmol/L (95% CI: –0.070, –0.058), LDL-C by 0.055 mmol/L (95% CI: –0.061, –0.050), triglycerides by 0.010 mmol/L (95% CI: –0.014, –0.007), and HDL-C by 0.005 mmol/L (95% CI: –0.006, –0.003), while increasing 1% from MUFA significantly decreased total cholesterol by 0.046 mmol/L (95% CI: –0.051, –0.040), LDL-C by 0.042 mmol/L (95% CI: –0.047, –0.037), triglycerides by 0.004 mmol/L (95% CI: –0.007, –0.001), and HDL-C by 0.002 mmol/L (95% CI: –0.004, 0.000). Isocaloric PUFA or MUFA substitution was superior to carbohydrates [total cholesterol: –0.041 mmol/L (95% CI: –

0.047, –0.035); LDL-C: -0.033 mmol/L (95% CI: –0.039, –0.027); triglycerides: + 0.011 mmol/L (95% CI: 0.007, 0.014), HDL-C: -0.010 mmol/L (95% CI: –0.012, –0.008)].

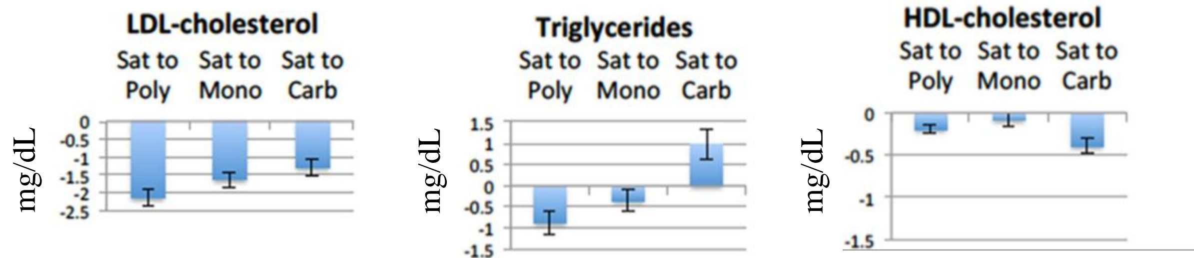


Figure 2-1. Effects of isocaloric substitution (1%) of SFA with PUFA, MUFA, or carbohydrate on circulating LDL-C, triglycerides, and HDL-C levels (20, 24). The data from a meta-regression analysis performed by Mensink (24) was converted from mmol/L to mg/dL and presented in a Presidential Advisory from the AHA: Dietary Fats and CVD (20). The figure has been modified for this dissertation.

In summary, the nature of the macronutrient replacement for SFA has profound effects on the degree of cardiovascular benefit. Substitution analyses of epidemiological and interventional data provide robust evidence for isocaloric replacement of dietary SFA with USFA for improvement in both cardiovascular outcomes and intermediate biomarkers for CVD. Although PUFA confer the greatest benefit, MUFA from plant origins are also favorable. Future RCT designed to assess the effects of plant-MUFA as a replacement for sources of SFA on coronary outcomes are warranted.

2.2.3. Vegetable oils to increase USFA intake

This reduction is SFA and simultaneous replacement with USFA can be achieved by exchanging sources of SFA, such as animal fats or tropical oils, for sources of USFA, such as

liquid vegetable oils (non-tropical and non-hydrogenated). Data presented in the 2015-2020 DGA show that the average intake of dietary oils (both plant- and marine-based) are marginally below guidelines for nearly all age and sex subgroups (18). Vegetable oils rich in PUFA include soybean oil, corn oil, safflower oil, cottonseed oil, and nut- and seed-derived oils (i.e., walnut and flaxseed oils) (18). Appreciable amounts of both n-6 linoleic acid and n-3 α-linolenic acid are also found in canola oil (36). Vegetable oils rich in MUFA include canola oil, olive oil, avocado oil, high-oleic acid oil varieties (i.e., high-oleic safflower, sunflower, and canola), and nut-derived oils (i.e., almond, hazelnut, and peanut oils) (18, 37).

Globally, the most commonly consumed vegetable oils are soybean oil, canola oil, and palm oil (21), the latter of which is a tropical oil and is not recommended due to the high-SFA content compared to other edible oils (18). Canola oil is a vegetable oil of particular interest given its suitable fit within a healthy dietary pattern as a source of both MUFA and PUFA to substitute for SFA-containing foods, as well as its substantial global consumption.

2.3. Dietary canola oils

2.3.1. Conventional canola oil

Canola oil is derived from the seeds of the canola crop, a yellow-flowering plant that belongs to the *Brassica* genus of the Brassicaceae (or mustard) family (22, 38). Commercially consumed canola oil, also know as low-erucic acid rapeseed (LEAR) oil, has been developed through traditional plant breeding of rapeseed plant varieties to yield oilseed crops low in erucic acid (22:1 n-9), a naturally occurring toxic compound present in rapeseed cultivars (38). LEAR oil, containing 2% or less of erucic acid, was granted Generally Recognized As Safe status by the US Food and Drug Administration (FDA) in 1985 (39). The term “canola,” originally derived

from “Canadian” and “ola” (meaning oil), was coined to distinguish this improved crop from the rapeseed, with reduced characteristics of concern and suitable for human consumption, but is no longer strictly a Canadian crop (38). According to the Codex Alimentarius International Food Standards, canola oil or LEAR oil can only be applied if the oil contains no more than 2% erucic acid as a percentage of total fatty acids (40). Presently, canola oil is third most commonly consumed vegetable oil in the world (21), and is consumed in the following ways: in spreads, salad dressings, margarines, vinaigrettes, and marinades; as cooking oil or pan release spray for sautéing, baking, stir-frying, and deep-frying; and in processed and packaged foods.

Classic, or commodity, canola oil has a fatty acid profile that is rich in MUFA (63% of total energy), moderate in PUFA (27%; 19% n-6 linoleic acid, 9% n-3 α-linolenic acid), and low in SFA (7%) **(Table 2-1)** (36). Compared to other edible fats and oils, canola oil has the lowest SFA content, among the highest MUFA content, and the second highest α-linolenic acid content, behind flaxseed oil (18). There are trace amounts of TFA in canola oil (<0.5%) due to deodorization, a high-heat process in the refining of all vegetable oils that causes thermal reactions (41); however, this does not reach the 0.5 g per serving threshold for Nutrition Facts labeling required by the US FDA and, thus, is labeled as 0 g (42). Canola oil is a source of Vitamin E (36), providing approximately 1/3 of the recommended dietary allowance [15 mg (43)] of α-tocopherol per 2 tablespoons or 28 g. Additionally canola oil contains phytosterols and phytostanols, such as campesterol, β-sitosterol, β-sitostanol, campestanol, and Δ-5 avenasterol (36), plant compounds that have serum cholesterol-lowering properties (19).

Table 2-1. Nutrient composition of canola and high-oleic acid canola oils. [1]

Nutrient	Canola Oil	HOCO
Total Energy (kcal)	124	126
Fat (g)	14	14
SFA	1.03	0.95
MUFA	8.86	10.18
Oleic acid	8.64	10.05
PUFA	3.94	2.21
Linoleic acid	2.66	1.88
α-linolenic acid	1.28	0.31
TFA	0.06	0.13
Micronutrients		
Vitamin E (α-tocopherol) (mg)	2.44	2.44
Vitamin K (phylloquinone) (ug)	10	10

[1] Nutrient data from the US Department of Agriculture (USDA) National Nutrient Database for Standard Reference Legacy, April 2018 Software v.3.9.4.1 (36, 44). Rather than specific brands, a generic food description was selected from the database (04582, Oil, Canola; 04698, Oil, industrial, canola, high oleic). Values are per 1 tablespoon (14 g) of oil.

Abbreviations: HOCO, high-oleic acid canola oil; MUFA, monounsaturated fatty acid; PUFA, polyunsaturated fatty acid; SFA, saturated fatty acid; TFA, *trans*-unsaturated fatty acids

2.3.2. HOCO

Canola oil is also available in a higher-MUFA variety termed HOCO, or high-stability canola oil. This specialty canola cultivar was developed through traditional plant breeding (45) to selectively reduce the total PUFA content, namely linoleic acid and α-linolenic acid. Compared to its conventional counterpart, HOCO is equivalent in SFA, higher in MUFA, and proportionally lower in PUFA (**Table 2-1**) (44). HOCO is available in varieties ranging from 65-80% oleic acid (45) and is predominately utilized in commercial production of processed and packaged foods and in food service.

The incorporation of HOCO within the food supply was spurred, in part, by the recognition of the adverse health effects of industrially produced TFA from the partial hydrogenation processing of vegetable oils (46). The scientific evidence indicated that artificial TFA consumed in PHVO were a detriment to cardiovascular health, particularly through causal elevation in circulating LDL-C (46). The DGA first recommended reducing TFA intake in the 2005-2010 edition (47) and, in 2003, the US FDA mandated declaration of the TFA content on the Nutrition Facts labels by 2006 to improve transparency of consumption and to assist in maintaining healthy diets (42). Finally, in 2015, the US FDA made a final determination that PHVO were no longer Generally Recognized As Safe with compliance of removal by June 18, 2018 (48). Increased consumer health awareness combined with ultimate required elimination from the food supply drove the food industry to reformulate oil-containing products and to seek alternatives to PHVO high in TFA. Independent from the now known health effects, PHVO were originally a desirable ingredient in a wide range of oil-containing food products due to the increased flavor stability, extended shelf life, oxidative resistance, low cost, higher melting point, and consistent availability compared to non-hydrogenated oils, butter, and lard (23, 49). In addition, substituting PHVO for sources of SFA was considered a responsible approach by the food industry to address the public health recommendations to decrease intake of SFA and cholesterol (50). PHVO in margarines, shortenings, and the oil itself was used in a wide variety of food applications, including baking and frying of fast foods, frozen foods, packaged snacks, and bakery products (46). As such, the edible oils industry sought a fat-based replacement that simultaneously satisfied or exceeded the functional characteristics of PHVO, preserved the structure and palatability of foods, complied with US FDA regulations, and met consumer health demands.

High-MUFA oils, including high-oleic canola, safflower, sunflower, and soybean, are a reasonable substitute given their favorable fatty acid profiles consistent with dietary guidance (i.e. low in SFA, high in USFA, and negligible TFA) and ability to achieve the same functional characteristics provided by PHVO. Vegetable oils rich in PUFA are less stable and prone to oxidation due to the degree of unsaturation, and, consequently, are not optimal for prolonged packaging periods or high-temperature applications. High-MUFA oils have a higher oxidative stability index compared to both PHVO and their conventional counterparts, primarily attributed to the reduced PUFA composition, although endogenous antioxidant content is also a factor (45). A shelf-life study of packaged breakfast cereals compared high-oleic soybean cereal to conventional soybean cereal aged at 140°F for 12 days (equivalent to 1 year shelf-life) in high- or low-oxygen barrier bags and reported significantly lower peroxide values in the high-oleic cereal in both packaging types (45). In addition to the improved shelf-life for ambient temperature food applications regardless of packaging material, high-MUFA oils are also of high-fry quality with thermo-oxidative stability to withstand high-heat temperatures, an extended fry-life, and a neutral flavor with less susceptibility to producing off flavors (23). The extended fry-life has environmental implications; improved oil stability requires less product, transport, equipment washing, and waste generation compared to conventional oils (51).

Current food product applications of high-MUFA oils include: within oil blends as a replacement for margarine and shortening in commercial baked goods, such as cookies and cakes; as frying oils to replace frying fats and shortenings for restaurant deep frying, such as French fries; and as frying oils to replace frying fats and shortenings for commercial frying of packaged and processed foods, such as chips (23). These foods are substantial sources of energy in the diet. For example, according to a 2012 analysis of NHANES (2003-2006) data, the

category of cake/cookies/quick bread/pastry/pie ranks second in principal food sources of energy among Americans aged 19+ years (52). Despite the incorporation of high-oleic oils into the food supply as the "new standard" to replace SFA- and TFA-containing products within a range of foods, there is very limited knowledge of their effects on cardiovascular health. Widespread consumption of high-oleic oils may affect the intake of other fatty acids, particularly PUFA; these are the preferentially recommended class of USFA to replace SFA for cardioprotection and also include the essential fatty acids α-linolenic acid and linoleic acid (49).

A 2018 dietary modeling exercise by Raatz et al. estimated the impact of replacing conventional soybean oil and canola oil with their high-oleic counterparts on fatty acid intakes in the US (53). Using the What We Eat In America dietary component of NHANES (2007-2014) from individuals aged 20+ years (n=21,029), foods reported from 24-hour recalls were disaggregated into ingredients and the Food and Commodity Intake Database was utilized to estimate the amount of soybean oil and canola oil consumed in each food. Modeling scenarios of 10%, 25%, or 50% replacement of the conventional oil with the high-oleic variety were calculated using the nutrient composition in the USDA Nutrient Database or de novo laboratory analysis of the oils. Changes in fatty acid intakes for the total population and across age- and sex-specific groups showed increases in total MUFA intake and reductions in total SFA and PUFA intakes across all replacement levels; notably, linoleic acid and α-linolenic acid were significantly reduced. Numerous adult age and sex subgroups were at risk of not meeting the Dietary Reference Intake Adequate Intake for linoleic acid and α-linolenic acid [linoleic acid 12-17 g/d; α-linolenic acid 1.1-1.6 g/d (29)] at the 25% and/or 50% replacement scenarios (53). In addition to potential essential fatty acid deficiency, this modeling suggested that, while SFA

intake will decrease, adults will continue to consume more than the amounts recommended by the AHA and DGA 2015-2020.

High-oleic oil consumption is likely to become pervasive, although the exact proportion of substitution of commodity oils is unknown. HOCO had over 90% of the high-oleic market share in 2012, whereas sunflower and safflower oleic varieties moved into niche markets that utilize non-genetically modified oils (45). High-oleic soybeans recently received global regulatory approval and are projected to become the fourth-largest grain and oilseed crop in the US within the next decade (54). Thus, further investigation of the cardiovascular health impacts of high-oleic oils, such as HOCO, is necessary.

2.4. Cardiovascular health benefits of canola oils

2.4.1. Cardiovascular outcomes

No RCT have been designed to specifically investigate the effects of canola oil on cardiac events and cardiac mortality. However, the results of Mediterranean diet intervention trials that incorporate high-MUFA oils (i.e., canola oil, olive oil) into the experimental dietary pattern can be utilized to speculate on the potential benefits of canola oil in primary and secondary prevention. These studies include the Lyon Diet Heart Study and Prevención con Dieta Mediterránea (PREDIMED).

The Lyon Diet Heart Study was a parallel, randomized, single blind, multi-center, free-living, clinical trial conducted in France aimed at using diet modification to reduce the risk of cardiovascular death and recurrent myocardial infarction in survivors of a first myocardial infarction (55). Males and females aged <70 years who experienced a myocardial infarction up

to 6 months prior were randomly assigned to a Mediterranean-type diet (n=302) or a prudent post-myocardial infarction diet (n=303). The experimental group was counseled by a research cardiologist and dietician during a 1-hour session, with additional counseling at subsequent visits, and advised to consume: more bread, root vegetables, green vegetables, and fish; less meat (red meat replaced with poultry); fruit daily; butter and cream replaced with provided margarine; and olive oil and rapeseed oil (canola oil) for salads and food preparation. Because participants were not amenable to using olive oil, rapeseed oil-based margarine was provided, which was higher in linoleic acid (16.4% vs. 8.6%) and α-linolenic acid (4.8% vs. 0.6%) compared to olive oil. The control group that followed a prudent diet did not receive dietary advice apart from that of the dieticians and physicians at the hospital post-initial cardiac event. An intermediate analysis (mean 27 months of follow-up) showed a reduced risk of combined cardiac deaths and non-fatal myocardial infarction (adjusted RR 0.27, 95% CI 0.12-0.59, *P*=0.001), as well as total mortality (adjusted RR 0.30, 95% CI 0.11-0.82, *P*=0.02), in the experimental group. This cardioprotective effect was confirmed in an extended follow-up (mean 46 months), with a reduction in the composite measure of cardiac death and recurrent nonfatal myocardial infarction (adjusted RR 0.28, 95% CI 0.15-0.53, *P*=0.0001) (56). The authors concluded that there was an impressive cardioprotective effect of a Mediterranean-type dietary pattern on secondary prevention. However, there are methodological limitations in this trial, including the lack of assessment of the baseline diet in the control group and assumption of comparable baseline intake between arms, and ascertainment of nutrient intake data at the study conclusion from only 30% of the control and 50% of the experimental groups (57). Although rapeseed oil-based margarine was the sole food provided, it is difficult to infer which dietary component, or combination thereof, precisely drove the observed effects. Moreover, intakes of numerous nutrients were different

between groups (i.e., decreases in SFA, linoleic acid, and cholesterol, and increases in oleic acid, α-linolenic acid, and fiber in the Mediterranean arm) (56), confirmed by plasma fatty acid analyses (55).

The PREDIMED trial was a parallel, free-living, primary prevention trial conducted at multiple sites in Spain designed to compare the effects of a Mediterranean diet supplemented with extra-virgin olive oil or mixed nuts to a control reduced-fat diet on major cardiovascular events (i.e., myocardial infarction, stroke, or death from cardiovascular causes) (58). This study was designed to be a randomized trial, but protocol deviations occurred with 425 participants assigned to the same intervention as a household member, rather than individual randomization. The PREDIMED primary outcome reported herein was analyzed using statistical models that do not rely on the assumption of random assignment. Participants (n=7,447) included men (aged 55-80 years) and women (aged 60-80 years) who were at high CVD risk, defined as type 2 diabetes or having three major cardiovascular risk factors. Participants in the Mediterranean diet plus olive oil arm were provided with extra-virgin olive oil (1 L per household per week) and recommended to consume ≥4 tablespoons (56 g) per day, while participants in the Mediterranean diet plus nuts arm were provided with 30 g of mixed nuts per day (walnuts, hazelnuts, almonds); dieticians counseled the Mediterranean groups at the baseline visit and quarterly thereafter. The control group was provided with small, nonfood gifts and was counseled at baseline, followed by annual leaflets for 3 years. A protocol change was implemented to provide control subjects with dietary sessions with the same intensity and frequency as the Mediterranean diet groups. After a median follow-up of 4.8 years, both Mediterranean diets reduced the risk of major cardiovascular events compared with the control (extra-virgin olive oil: adjusted HR 0.69, 95% CI 0.53 to 0.91; mixed nuts: HR 0.72, 95% CI, 0.54 to 0.95). Interestingly, although the interventions were

intended to change overall dietary patterns, the primary between group differences diet reflected the fat profile of the provided food items.

In summary, the Lyon Diet Heart study and PREDIMED results indicate beneficial effects of the Mediterranean diet on both secondary and primary prevention, respectively. We cannot assume currently available canola oil would have identical effects as the Lyon rapeseed oil-based margarine or the PREDIMED extra-virgin olive oil, nor can we tease out the effects of the dietary pattern versus the provided foods; however, these studies support incorporating high-MUFA plant-based sources into overall healthy dietary patterns to improve cardiovascular incidence and recurrence.

While no trials to date directly assessed contemporary canola oil on cardiovascular outcomes, the beneficial relationship between canola oil and CVD intermediate biomarkers has been studied for over three decades (22). In 2006, the US FDA concluded that there was sufficient scientific evidence to support a canola oil health claim, and issued the following: "Limited and not conclusive scientific evidence suggests that eating about 1 ½ tablespoons (19 grams) of canola oil daily may reduce the risk of CHD due to the USFA content in canola oil (59). To achieve this possible benefit, canola oil is to replace a similar amount of saturated fat and not increase the total number of calories you eat in a day. One serving of this product contains [x] grams of canola oil." The following section (*2.4.2*) will describe the evidence of canola oil on intermediate lipid and lipoprotein biomarkers for CVD.

2.4.2. Atherogenic lipid and lipoprotein biomarkers

The primary therapeutic target for the prevention of atherosclerotic CVD and its clinical indicators (i.e., myocardial infarction, ischemic stroke) is atherogenic cholesterol, defined as the cholesterol carried by circulating apolipoprotein (apo) B-containing lipoproteins [i.e., LDL-C and non-HDL-C (total cholesterol – HDL-C)] (60). Lipoproteins are transport particles composed of a hydrophobic lipid core containing differing ratios of esterified cholesterol and triglycerides, a hydrophilic plasma membrane, and surface apolipoproteins. LDL, intermediate-density lipoprotein, very low-density lipoprotein (VLDL), chylomicrons, and lipoprotein (a) are a class of lipoproteins containing a single apo B particle (chylomicrons: apo B48; all others: apo B100); LDL carries ~75% of the cholesterol contributing to non-HDL-C, whereas the other apo B-containing lipoproteins and their remnants are largely triglyceride-rich and carry lesser amounts of cholesterol. The NLA categorizes circulating non-HDL-C and LDL-C levels at the following clinical cut points (in mg/dL): non-HDL-C <130 desirable, 130-159 above desirable, 160-189 borderline high, 190-219 high, ≥220 very high; LDL-C <100 desirable, 100-129 above desirable, 130-159 borderline high, 160-189 high, ≥190 very high (60).

There is strong and consistent evidence to support that LDL-C is not only a biomarker of atherosclerotic CVD, but is a causal factor. A 2017 panel from the European Atherosclerosis Society reviewed the existing research from meta-analyses of observational prospective cohort studies, RCT of pharmacological therapies, and Mendelian randomization studies of LDL-lowering, including >200 studies (n>2 million) with >20 million person-years of follow-up and >150,000 cardiovascular events, and concluded that the evidence "unequivocally establishes that LDL causes atherosclerotic CVD" (61). An earlier meta-analysis of 22 clinical trials (n>170,000) of statin regimens, including more intense versus less intense statin programs and statin versus

control, reported that a 1.0 mmol/L reduction in LDL-C at 1 year reduced major vascular events by 22% (RR 0.78, 95% CI 0.76-0.80) and CHD-related mortality by 20% (RR 0.80, 99% CI 0.74-0.87) (62). Accordingly, interventions that lower LDL-C are vital for atherosclerotic cardiovascular risk reduction, including not only pharmacologic but also dietary strategies.

There is a substantial evidence base to support that conventional canola oil improves atherogenic blood lipids and lipoproteins. Lin et al. published a review of 31 clinical trials that assessed the effects of canola oil-based diets on the lipid/lipoprotein profile in heterogeneous populations, and concluded that canola oil-based diets reduce total cholesterol and LDL-C, with inconclusive effects on triglycerides and HDL-C (22). Of particular interest are studies in which a diet with canola oil is compared to a diet with the fatty acid profile of Western intakes. This approach evaluates the dietary recommendations of replacing SFA with USFA using a food-based substitution strategy. Study design details and pertinent results of trials that compared canola oil diets to diets reflecting Western intakes are described in **Table 2-2**. In general, the canola oil diets reduced circulating total cholesterol, LDL-C, and apo B compared to the Western diets, whereas triglyceride, HDL-C, and apo AI results are inconsistent.

Table 2-2. Clinical trials that compared conventional canola oil to a Western diet on endpoint lipids, lipoproteins, and apolipoproteins.

Ref., location	**Study design**	**Subjects**	**Duration**	**Treatment**[2]	**Diet composition (% of total energy)**				**Results** [1]							
					Total F	**MUFA**	**PUFA**	**SFA**	**TC**	**LDL-C**	**Non-HDL-C**	**HLD-C**	**TG**	**apo A1**	**apo B**	**Canola vs. Western**
McDonald et al. (1989), Canada (63) [3]	Randomized, crossover, controlled feeding 4 treatments within a Western-type diet	Healthy men n=8 age=19-32 years	6 days	**Pre-experimental mixture of fats**	36	15	7	14	4.4	2.8	-	1.9	1.3	-	-	-Canola-sunflower randomization: TC, LDL-C, HDL-C reduced after canola compared to pre-experimental baseline diet. No effect on TG. -Sunflower-canola randomization: TC reduced after canola compared to pre-experimental baseline diet. No diet effect on LDL-C, HDL-C, or TG.
			18 days	**Canola oil**	36	20	10	5	3.7	2.3	-	1.3	0.8	-	-	
			18 days	Sunflower oil	36	7	22	7	3.7	2.2	-	1.2	0.8	-	-	
			6 days	Post-experimental mixture of fats	36	15	7	14	4.1	2.6	-	1.3	0.8	-	-	
Wardlaw et al. (1991), US (64)	Randomized, parallel, blind, controlled feeding Typical US diet followed by one vegetable oil	Healthy men n=16 Mean age=33	21 days	**Typical US diet before canola oil**	38	14	9	15	5.4	3.6		1.1	1.5	1.5	1.2	-TC, LDL-C, and apo B-100 reduced after canola compared to typical US diet. -No diet effect on HDL-C, TG, or apo AI.
			56 days	**Canola oil**	40	22	11	7	4.9	3.2		1.1	1.4	1.5	0.9	
			21 days	Typical US diet before safflower oil	38	14	9	15	5.4	3.8		1.0	1.4	1.4	1.1	
			56 days	Safflower oil	39	9	22	7	4.6	3.0		1.0	1.3	1.4	0.9	
Lichtenstein et al. (1993), US (65) [4]	Randomized, crossover, double-blind, controlled feeding	LDL-C >130 mg/dL n=15 (7 males, 8 females) Mean age=61	32 days	**Typical US diet**	35	12	8	13	221	152	-	48	107	134	99	-TC, LDL-C, apo B, and HDL-C reduced after canola oil compared to typical US baseline diet. -No diet effect of apo AI or TG.
				Canola oil	30	15	7	5	194	126	-	44	109	131	80	
				Corn oil	29	9.0	11	7	194	125	-	44	108	133	79	
				Olive oil	30	17	4	7	205	132	-	46	112	133	83	

	Typical US diet followed by 3 vegetable oils within NCEP ATP Step 2 diet	years														
Gustafsson et al. (1994), Sweden (66)	Randomized, parallel, controlled feeding Typical Swedish diet followed by 1 vegetable oil within lipid-lowering diet	Moderate hypercholesterolemia with or without hypertriglyceridemia 73 males, 22 females Rapeseed oil n=46 Sunflower oil=49	7 days	**Baseline Swedish diet before rapeseed** [5]	37	13	6	16	6.8	4.9	-	1.1	2.1	1.2	1.4	-TC, TG, LDL-C, HDL-C, apo B, and apo AI reduced after rapeseed oil compared to Swedish baseline diet.
			21	**Rapeseed oil**	30	14	7	7	5.7	4.1	-	1.0	1.8	1.1	1.2	
			7 days	Baseline Swedish diet before sunflower [5]	37	13	6	16	7.3	5.2	-	1.1	2.4	1.2	1.4	
			21	Sunflower oil	30	10	11	8	6.1	4.5	-	1.0	1.7	1.0	1.3	

[1] Endpoint values for total cholesterol, LDL-C, HDL-C, triglycerides, apo A1, and apo B expressed in in mmol/L and non-HDL-C expressed as a ratio, unless otherwise indicated.
[2] Treatments in bold are of emphasis for this dissertation.
[3] McDonald et al. reported separate values and significance tests for treatment order (i.e., canola-sunflower randomization, sunflower-canola randomization). Values presented here are the combined mean endpoints of the two treatment orders.
[4] Results expressed as mg/dL. apo B specific to LDL apo B.
[5] Baseline diet from 7-day food record, not controlled feeding.

Abbreviations: apo, apolipoprotein; HDL-C, high-density lipoprotein-cholesterol; HOCO, high-oleic acid canola oil; DHA, docosahexaenoic acid; LDL-C, low-density lipoprotein-cholesterol; MUFA, monounsaturated fatty acid; PUFA, polyunsaturated fatty acid; SFA, saturated fatty acid; TC, total cholesterol; Total F, total fat; TG, triglyceride

There are numerous limitations of previous trials specifically comparing canola oil diets to Western diets on lipid and lipoprotein response that warrant future research. For example, all described trials were published > 20 years ago and investigated healthy participants (63, 64) or those with elevated lipids (66) or LDL-C (65) at baseline, with small sample sizes (n=8-16) (63-65). No studies have assessed these interventions in individuals with MetS, a CVD risk factor and disease of an unhealthy lifestyle with prevalence rates of approximately 35% and 21% among US and Canadian adults, respectively (11, 12). In addition, three trials included control diets with 14-16% of energy from SFA (63, 64, 66), considerably higher than the current average intake of 12% of total energy among US adults (26), and only one trial matched the percentage of energy from total fat across treatments (63). Comparator diets higher in saturated fat versus actual intake and/or higher in total fat confound the interpretation of canola oil's treatment effect relative to a typical Western dietary pattern. To address these limitations, future research should investigate canola oil compared to the current SFA intake, maintain total fat composition across diets, and investigate a fairly contemporary, yet widespread, disease state.

Although conventional canola oil has beneficial effects on lipid and lipoprotein biomarkers, namely total cholesterol and LDL-C (22), we cannot assume that HOCO will elicit identical effects because the increased MUFA is at the expense of decreased PUFA. Two trials to date assessed the effects of HOCO on lipid and lipoprotein endpoints; details of the study design and endpoint values are described in **Table 2-3**. Gillingham et al. was the first to assess the effects of HOCO on blood lipids and lipoproteins in humans and compared controlled feeding of a high-oleic acid rapeseed oil diet, a high-oleic acid rapeseed oil and flax oil blend diet, and a Western control diet (67). After 28 days, endpoint total cholesterol, LDL-C, and non-HDL-C were lower following the high-oleic acid rapeseed oil diet compared to control. There was no

treatment effect on endpoint triglycerides, and HDL-C was higher after the high-oleic acid rapeseed oil compared to the high-oleic acid rapeseed oil and flax oil blend. Apolipoproteins were not reported. The authors concluded a cardioprotective benefit of high-oleic acid rapeseed oil compared to a Western diet control. A more recent study investigated the effects of consumption of five dietary oils that varied in USFA compositions, including HOCO and regular canola oil, in individuals at risk for or with MetS in the COMIT I study, the trial preceding the dissertation project described herein (68). After 4 weeks of feeding, the canola oil and HOCO diets did not significantly differ in endpoint lipids, lipoproteins, or apolipoproteins, suggesting the oils exert comparable effects. Although these two studies provide important insights into HOCO effects on cardiovascular biomarkers compared to a Western diet (67) and to conventional canola oil (68), no trials have simultaneously examined conventional canola oil, HOCO, and a Western diet for direct comparisons.

Table 2-3. Clinical trials that assessed the effects of HOCO on lipids, lipoproteins and apolipoproteins.

Reference, location	Study Design	Subjects	Diet composition (% of total energy)					Results [1]						
			Diet	Total Fat	MUFA	PUFA	SFA	TC	LDL-C	Non-HDL-C	HLD-C	TG	apo A1	apo B
Gillingham et al. (2011), Canada (67)	Randomized, crossover, single-blind, controlled feeding design Experimental period: 28 days	Hypercholesterolemic adults n=36 (13 males, 23 females) Mean age=47.5	High-oleic rapeseed oil	36.8	22.9	5.7	5.6	5.3	3.1	3.9	1.33	1.8	-	-
			High-oleic rapeseed oil and flax oil blend	36.9	15.9	12.3	6.1	5.1	3.1	3.8	1.28	1.7	-	-
			Western diet (butter, extra-virgin olive oil, vegetable lard, sunflower oil)	36.8	16.1	6.5	11.2	5.7	3.5	4.3	1.4	1.6	-	-
Jones et al. (2014), Canada (68)	Randomized, crossover, double-blind, controlled feeding design Experimental period: 28 days	Increased waist circumference plus at least one additional MetS risk factor n=130 (60 males, 70 females) Mean age= 46.5	Canola oil	35	17.6	9.1	6.6	4.81	2.9	-	1.20	1.60	1.42	0.9
			HOCO with DHA	35	17.8	8.0	6.9	4.87	3.0	-	1.30	1.25	1.46	1.0
			Corn oil and safflower oil blend	35	9.5	16.3	6.7	4.74	2.9	-	1.20	1.56	1.41	0.9
			Flax oil and safflower oil blend	35	9.6	16.3	6.8	4.69	2.8	-	1.17	1.56	1.38	0.9
			HOCO	35	19.3	6.9	6.5	4.77	2.9	-	1.18	1.64	1.43	0.9

[1] Endpoint values for TC, LDL-C, HDL-C, TG, apo A1, and apo B expressed in in mmol/L and non-HDL-C expressed as a ratio.

Abbreviations: Apo, apolipoprotein; HDL-C, high-density lipoprotein-cholesterol; HOCO, high-oleic acid canola oil; DHA, docosahexaenoic acid;

LDL-C, low-density lipoprotein-cholesterol; MetS, metabolic syndrome; MUFA, monounsaturated fatty acid; PUFA, polyunsaturated fatty acid; SFA, saturated fatty acid; TC, total cholesterol; TG, triglyceride

As noted above, the COMIT I trial assessed canola oil and HOCO in individuals with or at risk for MetS. Two additional trials to date also assessed the effects of canola oil on lipids and lipoproteins in this population (69, 70). Palomaki et al. compared 35 mL/day cold-pressed turnip rapeseed oil to 37.5g/day butter in a free-living, crossover, randomized study of Finnish men diagnosed with MetS (n=37), and reported lower mean LDL-C (butter: 3.35 ± 0.21 mmol/L, oil: 3.00 ± 0.18 mmol/L; reduction by 11%; $p < 0.001$) and total cholesterol (butter: 5.43 ± 0.23 mmol/L, oil: 4.98 ± 0.23 mmol/L; reduction by 8%; $p < 0.001$) after 6-8 weeks of oil compared to butter (69). No differences between HDL-C and triglycerides were observed. This study exhibited numerous design limitations, including lack of blinding as evident by treatment delivery (i.e., spread butter on bread or drink oil), and included smokers and individuals with current metabolic disease (diabetes, CVD), and allowed lipid-lowering therapies. Cold-pressed turnip rapeseed oil was selected as the intervention because it is the primary source of plant-based MUFA among the Finnish; however, commercially available canola oil is not commonly cold-pressed in the US and, thus, the demonstrated lipid lowering effects in this sample cannot necessarily be generalized to conventional canola oils (22). Baxheinriech et al. compared low energy density diets (nutritionist-prescribed diets for subject preparation) enriched in rapeseed oil or olive oil in participants with MetS (n=81) in a randomized, parallel weight reduction trial (70). After 6 months, there were no between treatment differences, but total cholesterol (5.43 ± 0.88 to 5.13 ± 0.90 mmol/l), LDL-C (3.42 ± 0.82 to 3.20 ± 0.81 mmol/l), apo B (0.92 ± 0.21 to 0.85 ± 0.19 g/l) and triglycerides (1.94 ± 1.13 to 1.49 ± 0.79 mmol/l) were reduced from the baseline to final study visit in the rapeseed arm. Together, these studies demonstrate that canola oil has beneficial effects on lipids and lipoproteins in the MetS population.

2.5. Potential mechanisms by which canola oil improves lipids/lipoproteins

As described above, there is a substantial evidence-base to support that canola oil benefits total cholesterol and LDL-C, in particular when replacing a Western diet fat profile higher in SFA. However, the precise biological mechanisms by which canola oils confer benefit have yet to be elucidated. Putative modes of action are explored in the following sections and include: intrinsic effects of dietary fatty acids on expression of genes implicated in lipid- and lipoprotein-related pathways; phytosterol composition and modulation of cholesterol absorption and synthesis; and indirect effects of diet on gut microbial-produced metabolites and their role in modulating lipid metabolism.

2.5.1. Gene expression

Circulating fatty acids are of physiological significance and can exert their biological effects through regulation of gene transcription. Numerous cellular systems function as fatty acid sensors [i.e., peroxisome proliferator activated receptors (PPAR), liver X receptor (LXR), sterol regulatory element binding proteins (SREBP), hepatic nuclear factors, farnesoid X receptor, among others] and respond to the fatty acid environment by altering gene transcription of gene targets in metabolic (i.e., lipid and glucose) and inflammatory pathways (**Figure 2-2**) (71). For example, PPAR are well-characterized nuclear transcription factors that sense fatty acids through direct fatty acid ligand activation and promote transcription of genes involved in fatty acid catabolism (71). Because circulating fatty acids are derived mainly from diet (72), the functional impact of dietary fat interventions, such as canola oil, may be through modulation of the fatty acid pool for subsequent gene regulation. Plasma fatty acids are typically used as a biomarker of

compliance in dietary fat quality trials (73), supporting the indication that circulating fatty acids reflect the diet. For example, in the COMIT I trial, five oils with different USFA profiles were provided and, following 4 weeks of intake, the circulating fatty acids represented the major fatty acids consumed in the respective oil intervention; canola oil and HOCO had the highest circulating total MUFA, a flaxseed oil and safflower oil blend as well as a corn oil and safflower oil blend had the highest total PUFA, a canola oil and DHA blend had the highest total n-3 PUFA, the flaxseed and safflower oil blend had the highest α-linolenic acid, and HOCO had the highest oleic acid (74).

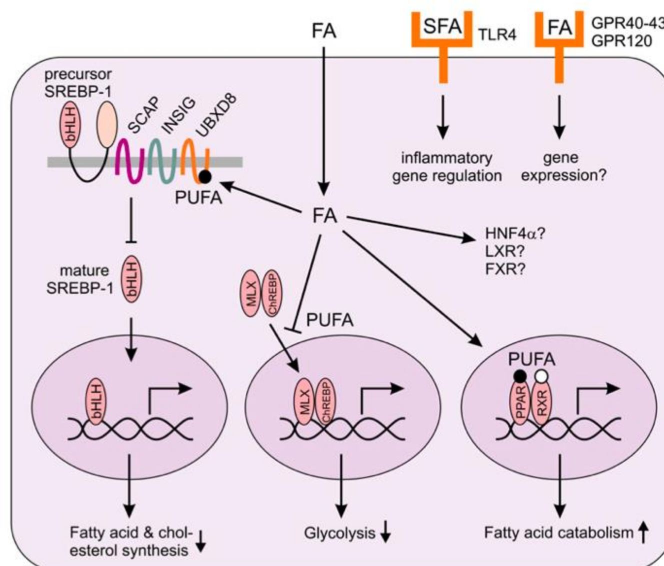


Figure 2-2. General mechanisms by which fatty acids regulate gene expression to modulate metabolism and inflammation, as shown in a hepatocyte (71).

Results from clinical trials of various dietary fat interventions indicate that fatty acids can mediate expression of genes related to lipid and lipoprotein metabolism in human blood cells, including PBMC. These feeding trials are limited to two supplemental studies (75, 76) and two post-prandial high-fat challenges (77, 78). Schmidt et al. assessed the effects of fish oil capsules (1.14 g DHA and 1.56 g DPA/day) in healthy (n=10) and dyslipidemic (n=10) men in a parallel

design on expression of lipid/lipoprotein-related genes to explore the mechanisms by which n-3 PUFA lower triglycerides and increase HDL-C (75). Gene expression was assessed in whole blood cells by microarray analysis and real time-polymerase chain reaction (rt-PCR) (79) and, after 12 weeks of supplementation, numerous genes were altered from baseline that coincided with the triglyceride and HDL-C response, including transcription factors [i.e., PPARα, retinoid X receptors (RXR), HNFs] and genes related to lipid and lipoprotein metabolism [i.e., low-density lipoprotein receptor (LDLR), diacylglycerol O-acyltransferase 1 (DGAT1), 2-acylglycerol O-acyltransferase 2 and 3, phospholipid transfer protein (PLTP), apo CII, ATP-binding cassette (ABC) subfamily G member 5, and others]. These findings were particularly apparent in the dyslipidemic men, suggesting differences in health status affect the diet-gene response. In contrast, Tsunoda et al. supplemented participants with DHA (1.8 g/day; n=18), EPA plus olive oil (1.8 g/day EPA and 3.0 g/day olive oil; n=16), or olive oil (6.0 g/day; n=16) for 6 weeks in a randomized, double blind, parallel design, and reported no treatment effect on lipid-related genes (76). The authors attributed this to the assessment of PBMC rather than hepatocytes (76), although there is substantial evidence to support using PBMC as a surrogate for the liver environment (80). Esser et al. assessed fatty acid-specific gene expression following a high-MUFA (high-oleic acid sunflower oil) or high-SFA (palm oil) post-prandial challenge in a crossover study of 32 men aged 50-70 years (77). PBMC gene expression was assessed at baseline and 4 hours post-prandial and the change was compared between the challenges. Compared to MUFA, the SFA challenge decreased expression of genes involved in cholesterol biosynthesis [i.e., 3-hydroxy-3-methyl-glutaryl-coenzyme A reductase (HMG-CoAR) and others], uptake (i.e., LDLR and others), and transcription factors [i.e., sterol regulatory element binding transcription factor (SREBF) 2], and increased cholesterol efflux genes (i.e., ABCA1

and ABCG1). These coordinated changes suggest an increased cellular cholesterol environment after SFA, resulting in cholesterol deactivation of SREBP2 to decrease sterol biosynthesis and uptake, as well as increased cellular cholesterol removal to reduce the lipid load. Additional results include increased inflammatory genes and PPARα target genes involved in fatty acid β-oxidation after MUFA compared to SFA. A limitation of this study was the fat challenge was arguably not a physiological relevant dose (95 g fat, or 855 kcal). In another post-prandial study, Bouwens et al. compared high-fat shakes (55 g fat) with MUFA (high-oleic acid sunflower oil), PUFA (high-DHA fish oil), or SFA (butter) on lipid/lipoprotein gene expression in healthy males (n=21) aged 19-27 in a crossover design. Whole-genome expression profiles in PBMC at baseline and after 6 hours in the PUFA and SFA arms (MUFA not assessed) showed opposite effects between shakes in genes within the LXR signaling pathway: ABCG1, ABCA1, and SREBF1 were down-regulated following PUFA compared to the SFA shake. The three shakes were compared using rt-PCR and, at 6 hours post-shake, the MUFA and PUFA arms had lower expression of SREBF1, LXR, and ABCA1 compared with the SFA arm. These studies provide evidence that dietary fat interventions of various qualities can alter lipid- and lipoprotein-related gene expression in humans.

In summary, these clinical trials indicate that dietary fat interventions can alter the expression profile of lipid and lipoprotein-related genes, particularly in blood cells as the target biological compartment. However, the effects of dietary fatty acid interventions following prolonged consumption in controlled feeding in humans have not been investigated. In addition, few trials included women in the sample. Such studies will provide mechanistic insight into the biological pathways by which higher-MUFA, lower-SFA oils, such as canola oil, improve clinical lipid and lipoprotein parameters compared to higher-SFA, lower-MUFA treatments .

2.5.2. Phytosterols

From a macronutrient perspective, canola oil is strictly composed of dietary fatty acids; however, canola oil also contains other nutritional factors that may contribute to improving the atherogenic lipid and lipoprotein profile, chiefly phytosterols (36). It is well established that phytosterols, including sterols and stanols, have a dose response LDL-C-lowering effect, with intakes of 3 g/day reducing LDL-C by approximately 12% (81). Phytosterols exert their cholesterol modifying effects through multiple mechanisms, including: lowering intestinal cholesterol absorption by competing with cholesterol for incorporation into mixed micelles and through modulation of Niemann-Pick C1-Like 1 transporters; increasing sterol efflux from the enterocyte to the intestinal lumen for excretion through up-regulation of ABCG5 and G8; and reducing hepatic sterol content to cause up-regulation of hepatic LDLR to remove apo B-containing particles from the bloodstream (19, 82). The record of evidence of benefit is such that the NLA recommends the use of supplemental plant phytosterols (~2 g/day) to enhance atherogenic cholesterol lowering (19).

Previous studies designed to assess the effects of replacement of SFA with plant-based sources of USFA on lipid and lipoprotein parameters have been confounded by differences in other non-fat plant-derived compounds. For example, Maki et al. compared the effects of 4 tablespoons/day of corn oil or extra-virgin olive oil on lipid and lipoprotein outcomes in adults with hypercholesterolemia (n=54) in a randomized, double blind, crossover, controlled feeding trial consistent in macronutrient composition (82). Compared to extra-virgin olive oil, 21 days of corn oil resulted in a greater reduction in percent change from baseline (P<0.001 for all) in LDL-C (-7.4%), total cholesterol (-6.4%), non-HDL-C (-7.7%), and the total cholesterol: HDL-C ratio (-3.9%). The higher PUFA (+6.9% energy) and lower SFA (-0.2% energy) content in the corn oil

was hypothesized to cause a greater reduction in the lipid and lipoprotein response compared to the olive oil; however, the observed differences were substantially greater than predicted from dietary fatty acid replacement equations (LDL-C prediction: -2.4%; actual: -7.4%). The additional benefit was attributed to differences in phytosterol content (corn oil: 989 mg/100 g, extra-virgin olive oil: 189 mg/100 g). Thus, it is possible that the phytosterols within canola oil may contribute, at least in part, to the lipid modifying effects of canola oil. Although this dissertation will not systematically assess the phytosterol composition and biological effects, it is important to consider that this is a potential mode of action of canola oil lipid-lowering.

2.5.3. Gut microbial metabolites

A novel potential mechanism by which canola oil may confer lipid and lipoprotein benefit is through its efffects on the gut environment. Foods, individual nutrients, and their combinations within dietary patterns have a direct effect on the composition and activity of resident gut microbes (83). The microorganisms within the gut are able to influence host physiology through the production of a variety of microbial metabolites; among those are the SCFA. These volatile fatty acids are <6 carbon structures that are the end products of anaerobic microbial fermentation of carbohydrate (and less protein), and include acetic acid, propionic acid, butyric acid, isobutyric acid, valeric acid, and isovaleric acid (84). Acetic, propionic, and butyric acid are the most abundant SCFA and, although clinical research is ongoing concerning the effects, these fatty acids are suggested to influence host health through their ability to: alter the luminal pH for prevention of overgrowth of pathogenic bacteria and increase absorption of select nutrients, provide a source of fuel to intestinal epithelial cells, reduce appetite, modulate

hepatic glucose and lipid metabolism, among others (84). The role in hepatic lipid metabolism is of particular relevance to this dissertation.

Den Besten et al. published an in-depth review of the current state of knowledge of the role of SCFA in host energy metabolism and underlying mechanisms, summarized herein (85). SCFA are produced in the large intestine, mainly the cecum and colon, and access the host circulation and extra-gastric tissues through absorption across the intestinal epithelium. Once transported across the apical membrane of colonocytes, these cells preferentially use butyrate as an energy source and oxidize the SCFA to ketone bodies and CO_2; the remaining, non-oxidized butyrate and other SCFA are transported across the basolateral membrane to the hepatic portal vein. The liver clears a large fraction of the SCFA from the hepatic portal circulation, where they can function as substrates for biosynthetic processes (acetate: cholesterol and long-chain fatty acid synthesis; propionate: gluconeogenesis) and regulate fatty acid and cholesterol metabolism. SCFA regulate fatty acid metabolism to increase fatty acid β-oxidation and decrease de novo synthesis and lipolysis in target tissues (i.e., liver, adipose, and muscle) (**Figure 2-3**). SCFA can exert biological effects through binding to G-protein coupled receptors 41 and 43 [also known as free fatty acid receptors 3 and 2, respectively], as well as through activation of AMP-protein activated kinase. An example of the latter, metabolism of acetate by acetyl-CoA synthetase in hepatocytes results in the production of AMP that can subsequently activate AMP-activated protein kinase to increase fatty acid oxidation and decrease synthesis (86), as shown in Figure 2-3. Thus, SCFA can regulate fatty acid metabolism through numerous pathways.

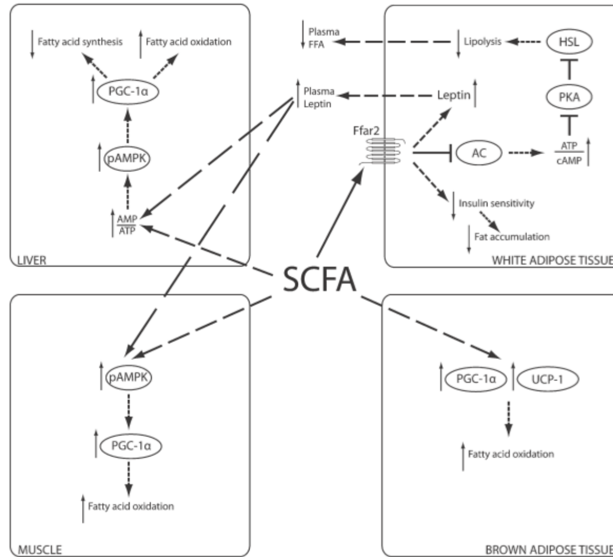


Figure 2-3. Mechanisms by which SCFA regulate fatty acid metabolism to promote utilization of fatty acids as an energy source and decrease de novo lipogenesis (85).

SCFA have been shown to regulate cholesterol metabolism through suppression of cholesterol biosynthesis both *in vivo* and *in vitro*. ^3H_2O tracer studies show that intake of SCFA reduces hepatic cholesterol synthesis rates in rat models (87), with supportive *in vitro* evidence of inhibition of cholesterol synthesis in rat hepatocytes exposed to various concentrations of propionate (88). Further, concentration of 3-hydroxy-3-methyl-glutaryl-coenzyme A (HMG-CoA), an intermediate in the cholesterol synthesis pathway, as well as ATP-citrate lyase activity, an enzyme that plays a role in cholesterol biosynthesis, were reduced in the liver of rats fed acetic acid (86). Moreover, incubation of human enterocytes with butyrate or propionate downregulated numerous genes within the cholesterol biosynthesis pathway, including HMG-CoA synthase and HMG-CoAR(89). These findings indicate SCFA function in the regulation of cholesterol, and are supported by the cholesterol-lowering effects of oral SCFA in studies described below.

Research on supplementation of dietary SCFA and cholesterol response provides insight into the potential effects of microbial-derived SCFA absorbed in the lower intestine. However, interpretation of the results should consider that the entirety of oral SCFA will not be delivered to the large intestine and that SCFA are absorbed and metabolized in combination. Numerous studies in animal models have demonstrated that intake of SCFA lowers total cholesterol. Hara et al. fed rats the cecal fermentation products of sugar beet fiber, a highly fermentable fiber, and found they lowered plasma cholesterol when added to a basal fiber-free diet compared to a fiber-free diet alone (90). The major fermentation products were identified as acetic, propionic, and butyric acid, and feeding of these SCFA in equal amounts lowered plasma cholesterol compared to a fiber-free diet, with a similar effect compared to a high-fiber diet. Feeding of acetate in isolation, but not propionate or butyrate, lowered cholesterol compared to the low-fiber diet, suggesting that acetate was the SCFA from the mix responsible for the sterol-lowering response (90). However, the effects of propionate administration on circulating cholesterol in animal models are conflicting (91). In a subsequent study from Hara et al., rats were fed the sodium salts of acetic, propionic, and butyric acid in a ratio reflecting cecal composition (i.e., acetic > propionic > butyric) in a fiber-free diet and confirmed the cholesterol-lowering effect of SCFA compared to a fiber-free control diet, with a reduction comparable to a high-fiber sugar-beet diet (87). Moreover, rats fed a cholesterol-containing diet (1% w/w) plus acetic acid had lower serum total cholesterol compared to a diet equivalent in cholesterol content (86).

Human trials that assess dietary SCFA interventions and report cholesterol levels provided SCFA in isolation, rather than in combination. The effects of acetic acid were assessed through consumption of 500 mL/day of vinegar (low: 750 mg acetic acid; high: 1,500 mg acetic acid) for 12 weeks in a randomized, double blind, parallel (n=~50 per group) study of Japanese

adults with a BMI 25-30 kg/m^2 (obese by Japanese definitions) (92). Total cholesterol was reduced from baseline at both low and high dosages, with no effect of placebo. In another study supplementation of propionate consumed in a bread matrix for 1 week did not alter lipid and lipoproteins compared to standard bread in healthy adults (n=6) (93). In contrast, supplementation of propionate in ester form (>80% delivery of propionate to the colon) reduced total cholesterol, LDL-C, and HDL-C from baseline after 24-weeks of supplementation in overweight and obese adults (n=25) (94)

The studies above provide evidence to suggest that SCFA may modulate circulating cholesterol, with mechanisms delineated in animal models. However, there is a dearth of human research assessing the effects of diet on microbial-produced SCFA and their relationship with lipid parameters. Assessing microbial metabolites within fecal samples provides a snapshot of the gut environment and few studies have assessed the effects of fat interventions on fecal SCFA in humans (95, 96). Interestingly, two trials have reported significant negative correlations between fecal SCFA composition and lipid parameters (97, 98), although more research is needed to corroborate these exploratory findings.

In summary, SCFA are regulators of fatty acid and cholesterol metabolism through a variety of pathways. Evidence from supplementation in animal and human studies suggests a potential lipid-lowering effect of SCFA. Because diet can alter the gut microbial composition, which in turn modulates presence and concentration of microbial metabolites, it is reasonable to hypothesize that microbial metabolites may mediate the effects of canola oil of lipid outcomes. However, no studies to date have investigated the effects of dietary oils, such as canola oil, on fecal SCFA and their associations with lipid parameters.

2.6. Rationale for current research

High-oleic acid oils are arguably the new standard to replace SFA- and TFA-containing fats and oils, particularly in frying and baking applications, and are currently consumed in a range of foods products. There is very limited knowledge of the effects of these oils on cardiovascular health; of particular concern is that widespread consumption of higher-MUFA, lower-PUFA oils will decrease the intake of total PUFA, which are the preferred class of USFA to replace SFA in the context of a healthy diet for cardioprotection. The health impacts of HOCO are of particular interest because this oilseed crop currently has the majority of the high-oleic global market share, and its conventional counterpart is one of the top three globally consumed oils. Direct comparison of HOCO to conventional canola oil will allow identification of the impact of the fatty acid intake shifts of increased MUFA at the expense of decreased PUFA on the cardiovascular risk profile.

It is well established that conventional canola oil elicits beneficial effects on lipid and lipoprotein biomarkers, namely total cholesterol and LDL-C, when replacing a Western-like diet. However, the clinical trials contributing to this conclusion were conducted >20 years ago and dietary fat intake recommendations have since changed; thus, it is unknown how diets with canola oil fare relative to a Western diet with contemporary fatty acid intakes. Further, MetS is a highly prevalent condition in the US with strong predictive power of CVD, and few trials investigated canola oil in this population. Simultaneous examination of a conventional canola oil diet, a HOCO diet, and a Western diet reflecting current fatty acid intakes in individuals with MetS factors is pertinent for primary prevention of CVD.

The precise biological mechanisms by which canola oil benefits atherogenic total cholesterol and LDL-C have yet to be elucidated. Nutritional factors can influence gene

expression in human tissues. It is possible that the fatty acids within canola oil exert physiological effects through regulation of expression of genes that encode for proteins involved in lipid metabolism pathways. Alternatively, SCFA are regulators of hepatic cholesterol metabolism and are end products of gut microbial metabolism. Because dietary components can alter the gut microbial composition, which in turn modulates presence and concentration of microbial-derived SCFA, it is possible that SCFA may mediate the lipid-lowering effects of canola oil. Exploration of such candidate biological mechanisms is necessary to determine how canola oil-based diets confer benefit compared to Western-fatty acid diets.

In summary, this dissertation research was conducted to address the knowledge gaps in the existing canola oil, HOCO, and CVD prevention literature, with the overarching goal of identifying the cardiovascular health impacts of commonly consumed oils and exploring underlying mechanisms.

2.7. Objectives and hypotheses

The purpose of COMIT II is to investigate the effects of three diets containing oils that vary in fatty acid composition on established and novel risk factors for CVD in adults with MetS factors. COMIT II was a multi-site, double-blind, randomized, controlled, three period crossover, controlled feeding clinical trial in which participants were provided with an isocaloric, weight maintenance diet plus conventional canola oil, HOCO, or a control oil blend for 6 weeks followed by washout periods of at least 4 weeks. The diet containing the control oil blend was formulated to represent a Western diet fatty acid profile. The specific objectives and hypotheses of my research within the COMIT II study are:

1) Objective: Examine the effects of diets containing conventional and high-oleic acid canola oils on lipids (total cholesterol, triglycerides), lipoproteins (LDL-C, HDL-C, non-HDL-C), and apolipoproteins (apo A1, apo B) compared to a control diet with a Western fatty acid profile.

   Hypothesis: I hypothesize the lipid, lipoprotein, and apolipoprotein response will be similar between the canola oil and HOCO-based diets, with benefit relative to the Western diet. Specifically, I hypothesize the total cholesterol, LDL-C, non-HDL-C, and apo B levels will be reduced after the canola oil diets compared to the Western diet, and with no treatment effect on HDL-C, triglycerides, and apo A1.

2) Objective: Assess the expression of candidate genes related to lipid and lipoprotein metabolism using a PCR-based approach to investigate the mechanisms underlying the lipid and lipoprotein response to the dietary oils.

   Hypothesis: I hypothesize the two canola oil diets will cause differential expression in genes regulating lipid and lipoprotein metabolism compared to the Western diet control oil, and that the gene expression response following the dietary oils will correspond with the lipid and lipoprotein shifts determined in *Objective 1*.

3) Objective: Determine the effects of the three diets that differ in fatty acid composition on fecal SCFA levels, and assess their associations with established CVD lipid biomarkers.

   Hypotheses: I hypothesize that dietary fat quality will differentially affect fecal SCFA and there will be significant associations between fecal SCFA levels and circulating endpoint total cholesterol, LDL-C, non-HDL-C, and apo B.

Chapter 3: Diets enriched with conventional or high-oleic acid canola oils decrease atherogenic lipids and lipoproteins compared to a diet with a Western fatty acid profile in individuals at risk for MetS

Abstract

Background: Novel vegetable oils high in MUFA and low in SFA are a desirable alternative to partially hydrogenated fats and oils high in TFA. There is widespread use of high-MUFA oils across the food industry; however, limited knowledge of their CVD impact exists compared to conventional counterparts.

Objective: We investigated the effects of diets containing conventional canola oil, HOCO, and a control oil (blend of ghee, safflower, coconut, and flaxseed oils; diet formulated to emulate a Western diet fatty acid profile) on lipids, lipoproteins, and apolipoproteins in adults with MetS risk factors.

Methods: In a multi-center, double blind, randomized, three-period crossover, controlled feeding clinical trial, 119 participants with an increased waist circumference plus ≥1 additional MetS risk factor consumed prepared, isocaloric, macronutrient matched, weight maintenance diets containing conventional canola oil (17.5% of total energy from MUFA, 9.2% PUFA, 6.6% SFA), HOCO (19.1% MUFA, 7.0% PUFA, 6.4% SFA), or control oil (10.5% MUFA, 10.0% PUFA, 12.3% SFA) for 6 weeks separated by ≥4-week washouts.

Results: Compared to the control diet, the canola oil and HOCO diets resulted in lower circulating total cholesterol (-4.2% and -3.4%, respectively; *P*<0.0001), LDL-C (-6.6% and -5.6%; *P*<0.0001), apo B (-3.7% and -3.4%; *P*=0.002), and non-HDL-C (-4.5% and -4.0%; *P*=0.001), with no differences between the canola diets for these parameters. The total

cholesterol: HDL-C and apo B: apo A1 ratios were significantly lower after the HOCO diet than the control diet (-3.7% and -3.4%, respectively). No diet effects were seen on triglyceride, HDL-C, or apo A1 level.

Conclusions: HOCO, with increased MUFA at the expense of decreased PUFA, elicited beneficial effects on lipids and lipoproteins comparable to conventional canola oil and greater than the Western diet control in individuals at risk for or with MetS. These findings are consistent with atherogenic CVD risk reduction. This trial was registered at www.clinicaltrials.gov as NCT02029833.

Introduction

Reduction of SFA intake and replacement with USFA in the context of a healthy dietary pattern represents a cornerstone of nutritional recommendations for CVD prevention and treatment (17-20). Canola oil is a commonly consumed vegetable oil that is low in SFA, moderate in PUFA, and rich in MUFA (>60% oleic acid) (36), with numerous cardioprotective benefits (22, 59). Canola oil is also available in a high-oleic acid variety, HOCO (>70% oleic acid), that is equivalent in SFA and proportionally lower in PUFA (44).

The development of HOCO and incorporation into the food supply was spurred, in part, by the recognition of the adverse cardiovascular health effects of industrially produced TFA from partial hydrogenation of vegetable oils (46). High-MUFA oils are a reasonable substitute for TFA-containing fats and oils given their favorable fatty acid profiles consistent with dietary guidance and ability to achieve or exceed the functional characteristics of PHVO, (i.e., oxidative stability, shelf-life, fry-life, neutral flavor) (23, 45, 49). Food applications for HOCO include as a replacement of margarine and shortening in commercial baked goods and as frying oil for restaurant deep-frying and commercial frying of packaged snacks and chips (23, 49). These foods are primary energy sources among US adults (52). Despite use as the “new standard” of oil across the food supply, which is likely to become pervasive given the US FDA required removal of added TFA (99), there is very limited knowledge of HOCO health effects. Research on the cardiovascular impact of HOCO is scarce (67, 68) and, although conventional canola oil has beneficial effects on CVD lipid and lipoprotein biomarkers (22), we cannot assume that increased MUFA at the expense of decreased PUFA in HOCO will elicit identical impacts.

MetS is defined as a cluster of three or more co-occurring interrelated conditions, including abdominal obesity, dysglycemia, dyslipidemia, and/or hypertension, and is associated

with increased cardiometabolic disease risk (10). MetS prevalence is approximately 35% and 21% among US and Canadian adults, respectively, with substantially higher estimates of individuals having components of the syndrome (i.e., 67% have ≥1 and 44% have ≥ 2 MetS criteria) (11, 12). Assessing dietary interventions in a sample of metabolically compromised adults is relevant to a considerable portion of the population.

The objective of the present study was to examine the effects of diets containing conventional or high-oleic acid canola oils on lipids, lipoproteins, and apolipoproteins compared to a control diet with a fatty acid composition characteristic of a Western diet in individuals with MetS risk factors. We hypothesized the lipid, lipoprotein, and apolipoprotein response would be similar between the canola diets, with greater benefit relative to the Western diet. This paper presents the first systematic assessment of the shift in dietary fatty acids in reformulated canola oil simultaneously compared to conventional canola oil and a Western diet.

Methods

Participants

Males and females (ages 20-65 years) with MetS risk factors were eligible for the study. Risk for MetS was defined as an increased waist circumference (International Diabetes Federation cut points: men ≥94 cm, women ≥80 cm) plus at least one of the following secondary inclusion criteria: Elevated fasting blood glucose (≥5.6 mmol/L), triglycerides (≥1.7 mmol/L), systolic blood pressure (≥130 mmHg), diastolic blood pressure (≥85 mmHg); and/or decreased HDL-C (men <1 mmol/L, women <1.3 mmol/L). Exclusion criteria included: smokers; consumption of >14 alcoholic beverages per week; use of prescription lipid-modifying

medications in the last three months or chronic anti-inflammatory medications; kidney disease, liver disease, diabetes, or uncontrolled thyroid disease; and pregnant or lactating women.

Study design

COMIT II was a double blind, randomized, controlled feeding, crossover, clinical trial that consisted of three, 6-week feeding periods separated by ≥4-week washout periods. The trial was conducted from 2014- 2016 at four research centers in North America [Richardson Center for Functional Foods and Nutraceuticals, University of Manitoba (RCFFN); Institute of Nutrition and Functional Foods, Laval University (INAF); Canadian Center for Agri-Food Research in Health and Medicine, St. Boniface Hospital Albrechtsen Research Center (SBRC); Department of Nutritional Sciences, The Pennsylvania State University (PSU)]. The respective centers' ethics review boards approved the COMIT II protocol and related documents, and the procedures followed were in accordance with the Declaration of Helsinki. All participants provided written informed consent (**Appendix A**) at screening prior to enrollment. Randomization.com was used to generate the random allocation sequence, with six possible sequences and an allocation ratio of 1:1:1:1:1:1. The sequences were assigned to each participant in the pre-specified order as he or she was enrolled in the trial by the study coordinators. This trial was registered at clinicaltrials.gov as NCT02029833.

Controlled diet and oil interventions

During the feeding periods, participants were provided with an isocaloric, healthy, weight maintenance base diet (**Appendix B**) with one of the following treatment oils: canola oil (Canola Harvest Canola Oil, Richardson International), HOCO (Canola Harvest Canola Oil, Richardson International, Canada), or control oil [blend of butter oil/ghee (Verka), safflower oil (eSutras), coconut oil (eSutras), and flaxseed oil (Shape Foods)]. The oil blend in the control diet was approximately 49% ghee, 29% safflower oil, 14% flaxseed oil, and 8% coconut oil. The fatty acid profile of the oils is presented in **Table 3-1** and the macronutrient profile of the full diets (i.e., base diet plus treatment oil) is presented in **Table 3-2**. The three experimental diets were identical in percentage of energy from macronutrients, but differed in fatty acid composition due to the presence of the treatment oils. The canola diets were higher in MUFA and lower in SFA compared to the control diet. The control diet was designed to approximate the average fatty acid profile of a contemporary Western-style diet; the most recent estimate of average intake among US adults (NHANES 2015-2016) for SFA, MUFA, and PUFA is 12% 12%, 8% of total energy, respectively (26).

The oils were incorporated into a smoothie containing frozen strawberries, orange sherbet, and milk, which was divided into two portions and consumed daily in the morning and evening to avoid gastrointestinal distress from the fat load. The amount of oil consumed was adjusted to participant's caloric needs and provided approximately 18% of total energy (e.g., 60 g of oil for a 3000 kcal/day diet). The kitchen staff at each site prepared breakfast, lunch, dinner, and snacks for the participants, adhering to a 7-day rotating menu (**Appendix B**). Participants were fed a calorie controlled diet for weight maintenance, calculated using the Harris Benedict Formula, and monitored by daily weighing at each participating center prior to food pick-up.

Calorie levels were adjusted appropriately during the first 2 weeks of diet period 1. Participants were instructed to consume all foods provided and to avoid consumption of extraneous food items and calorie-containing beverages. Compliance was optimized by consumption of at least one meal and smoothie per weekday at the participating center; assessment of remaining food in returned coolers; documentation of additional or omitted foods on daily compliance sheets; and daily weight measurement. All study personnel and participants were blinded to the treatments, with the exception of the kitchen staff responsible for smoothie preparation.

Table 3-1. Fatty acid profiles of the three oils. [1]

	Canola Oil	HOCO	Control Oil Blend
MUFA	66.1	75.7	26.7
PUFA			
α-linolenic acid	7.9	2.6	8.7
n-6	19.5	15.3	27.8
SFA	6.7	6.5	36.8

[1] Average GC values from oil samples analyzed at numerous time points throughout the COMIT II study. Values are expressed as percent of total energy.

Abbreviations: HOCO, high-oleic acid canola oil; MUFA, monounsaturated fatty acid; PUFA, polyunsaturated fatty acid; SFA, saturated fatty acid.

Table 3-2. Macronutrient composition of the three experimental diets containing the oils. [1]

	Canola Oil Diet	HOCO Diet	Control Oil Diet
Protein [2]	15.87	15.87	15.71
Carbohydrate	50.79	50.79	50.75
Fat	35.26	35.26	35.21
MUFA	17.45	19.11	10.50
Oleic acid	15.55	17.86	5.92
PUFA	9.21	7.02	9.96
α-linolenic acid	2.10	0.76	1.73
Linoleic acid	6.42	5.56	7.28
SFA	6.56	6.43	12.26

[1] The average macronutrient composition from the 7-day rotating menu, estimated at the 3000 kcal level, using Food Processor Nutrition Analysis Software (ESHA Research; Salem, OR).
[2] Nutrients presented as percentage of total energy.

Abbreviations: HOCO, high-oleic acid canola oil; MUFA, monounsaturated fatty acid; PUFA, polyunsaturated fatty acid; SFA, saturated fatty acid.

Sample collection and analyses

Participants underwent various clinical tests on two consecutive days at baseline (days 1 and 2) and endpoint (days 41 and 42) of each diet period, and the mean values were calculated for all parameters. Anthropometric measures included weight, height, and waist circumference, and clinical procedures included seated blood pressure, dual-energy X-ray absorptiometry (DXA) scans, and fasting blood draw. All blood draws were following 12-hours without food or drink except water and 48-hours without alcohol. Blood was allowed to clot, separated by centrifugation, aliquoted into microtubes, and stored at -80°C. Frozen serum samples were

shipped on dry ice to St. Michael's Hospital (Toronto, ON, Canada), the central laboratory for multi-site analyses of lipids, lipoproteins and apolipoproteins.

The endpoints of interest were total cholesterol, triglycerides, LDL-C, HDL-C, non-HDL-C, apo A1, apo B, and the total cholesterol: HDL-C and apo B: apo A1 ratios. Total cholesterol, triglyceride, and HDL-C were quantitatively determined by an enzymatic, colorimetric method on a Roche/Hitachi cobas c 501 analyzer (Roche Diagnostics). LDL-C was estimated according to the Friedewald equation [LDL-C (mmol/L) = total cholesterol – HDL-C – (triglyceride/2.2)] (100). However, for 4 time point samples, due to high serum triglyceride concentrations (>4.52 mmol/L), LDL-C was not calculated and recorded as a missing value. Non-HDL-C was calculated as total cholesterol – HDL-C. Apo A1 and apo B were quantitatively determined by end-point nephelometry on a BN ProSpec (Siemens) nephelometer. The total cholesterol: HDL-C and apo B: apo A1 ratios were calculated from original values.

Statistical methods

Statistical analyses were performed using SAS 9.4 (SAS Institute Inc., Cary, NC, US). The primary analysis was endpoint-to-endpoint comparison (mean of days 41 and 42) of lipids, lipoproteins, and apolipoproteins across the three treatment oils. A secondary analysis was performed to assess absolute change from baseline within each diet. Data were analyzed per-protocol and missing data were not imputed. All values for the primary and secondary analyses are presented as least squares mean ± standard errors of the means and $P \leq 0.05$ was considered significant.

The effects of the treatment oils on the lipid, lipoprotein, and apolipoprotein outcomes

were assessed using a mixed model, with subject, treatment sequence, and study center as random effects and visit as the repeated effect. Factors assessed in the model include treatment oil (canola oil, HOCO, control oil), visit (diet period 1, 2, 3), sex (male, female), center (RCFFN, INAF, SBRC, PSU), and treatment sequence, and the following interactions: treatment-by-visit, treatment-by-sex, treatment-by-center, and treatment-by-sequence. The diet period specific baseline value of the dependent variable was used as a covariate. Final models included treatment oil and only significant terms. Tukey-Kramer adjusted *P*-values were used for multiple pairwise comparisons between treatments, only when there was a significant main effect of treatment. Normality of the residuals from the final models was assessed and non-normal dependent variables were log transformed. Within treatment changes from baseline were assessed by the least squares means *P*-values from the final mixed model output. The effect of treatment on DXA-measured weight (both endpoint and change from baseline) was assessed as described above.

The COMIT II sample size was calculated according to the pre-specified trial primary outcome, defined as body composition with supplementary measurement of visceral adiposity measured by DXA (clinicaltrials.gov NCT02029833). A sample size of 140 was determined to detect a 55 gram change in android fat mass using the variance parameter in android fat mass from the COMIT I trial (101), and assuming a 20% drop-out rate. Post-hoc calculations indicate a sample size of 119 offered 97% power to detect a difference of 10% in LDL-C between treatments, with α=0.017.

Results

Baseline characteristics

The flow of participants through COMIT II and inclusion for the lipid and lipoprotein analyses are depicted in **Figure 3-1**. One hundred and twenty five participants completed the study, with a dropout rate of approximately 28%. Participants who had a weight change of >5% during any treatment period were removed from the analyses to eliminate the confounding effects of weight change on the outcomes (n=6). **Table 3-3** presents the baseline characteristics (period 1, days 1 and 2) of the remaining COMIT II participants included in the analyses (n=119). Participants were predominately female (male: n=44, 36.97%) and middle-aged with class I obesity (BMI 30.0-34.9 kg/m^2). Approximately 38% of the participants met the clinical criteria for a MetS diagnosis at baseline (i.e. at least three risk factors).

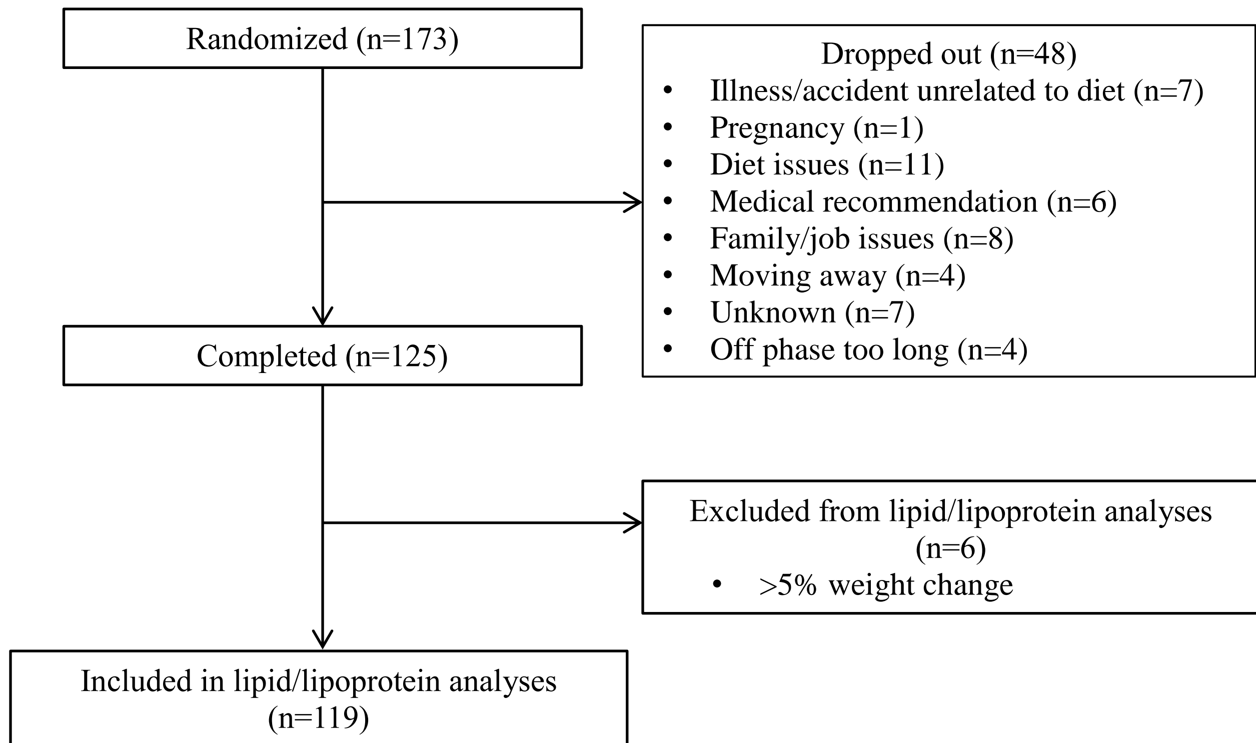


Figure 3-1. Flow diagram of the COMIT II participants for inclusion in the lipid and lipoprotein outcome analyses.

Table 3-3. Baseline characteristics of the COMIT II participants (n=119). [1]

Variable	Value [2]
Sex (female)	75, 63%
Anthropometric measures	
Age (years)	44.24 ± 13.11 (22.00 – 65.00)
Weight (kg) [3]	91.25 ± 18.68 (60.40 – 146.35)
BMI (kg/m^2)	31.71 ± 5.33 (22.55 – 52.58)
MetS criteria	
Waist circumference (cm)	104.97 ± 12.57 (80.00 – 150.90)
Female	102.89 ± 12.08 (80.00-130.75)
Male	108.52 ± 12.72 (93.85-150.90)
Triglycerides (mmol/L) [4]	1.60 ± 0.73 (0.33 – 3.67)
HDL-C (mmol/L) [4]	1.33 ± 0.35 (0.67 – 2.49)
Female	1.41 ± 0.35 (0.87 – 2.49)
Male	1.20 ± 0.31 (0.67 – 1.97)
Glucose (mmol/L) [4]	5.30 ± 0.59 (4.16 – 8.00)
Blood pressure (mmHg)	
Systolic blood pressure	120.01 ± 13.93 (87.67 – 163.67)
Diastolic blood pressure [4]	78.90 ± 10.71 (53.67 – 100.33)
Number of MetS Criteria [5]	
0	1, 0.85%
1	29, 24.79%
2	43, 36.75%
3	27, 23.08%
4	12, 10.26%
5	5, 4.27%
Additional cardiovascular risk biomarkers	
Total cholesterol (mmol/L) [4]	5.17 ± 0.90 (3.38 – 7.36)
LDL-C (mmol/L) [4]	3.11 ± 0.75 (1.04 – 5.33)

[1] Data collected on days 1 and 2 of diet period 1.
[2] Categorical variables are expressed as frequency, percent and continuous variables expressed as mean ± standard deviation (minimum – maximum).
[3] Weight was measured using a scale at each participating center (i.e., not DXA weight).
[4] n=118 due to missing values.
[5] n=117 due to missing values. Enrolled participants met the requirements of an increased waist circumference plus one additional factor at the screening visit; values present here are from the baseline visits of diet period 1.

Abbreviations: BMI, body mass index; HDL-C, high-density lipoprotein-cholesterol; LDL-C, low-density lipoprotein-cholesterol; metabolic syndrome, MetS.

Dietary compliance

Weight stability over the course of six weeks was utilized as a quantitative index of compliance. **Table 3-4** shows mean DXA-measured body weight at baseline (day 1 or 2) and endpoint (day 41 or 42), and the absolute weight change for the COMIT II participants used in the lipid and lipoprotein analyses (n=119). All diets modestly reduced body weight from baseline (<1 kg; *P*<0.0001 for all). No treatment effects on endpoint weight or absolute weight change from baseline were observed (*P* for treatment=0.19).

Table 3-4. DXA-measured weights (kg) for each diet period (n=119). [1]

Treatment	Baseline	Endpoint	Change
Canola Oil	90.03 ± 1.71	89.12 ± 1.68	-0.65 ± 0.16 *
HOCO	90.37 ± 1.69	89.46 ± 1.68	-0.92 ± 0.15 *
Control Oil	90.28 ± 1.69	89.41 ± 1.69	-0.87 ± 0.16 *

[1] Values are expressed as mean ± standard error of the mean.
* indicates significant change from 0, *P*≤0.05.

Abbreviations: DXA, dual energy X-ray absorptiometry; HOCO, high-oleic acid canola oil.

Endpoint-to-endpoint mean comparisons

The primary analysis of endpoint-to-endpoint comparisons (mean of days 41 and 42) among the three treatments is presented in **Table 3-5**. Compared to the control oil, consumption of both regular canola oil and HOCO resulted in lower endpoint means for total cholesterol (canola vs. control: *P*=<0.0001; HOCO vs. control: *P*=0.002), LDL-C (canola vs. control: *P*=<0.0001; HOCO vs. control: *P*=0.0002), apo B (canola vs. control: *P*=0.005; HOCO vs.

control: *P*=0.01), and non-HDL-C (canola vs. control: *P*=0.002; HOCO vs. control: *P*=0.008). There were no significant differences between canola oil and HOCO treatments for these parameters. The total cholesterol: HDL-C ratio was lower following HOCO compared to control (HOCO vs. control: *P*=0.01), as well as the apo B: apo A1 ratio (HOCO vs. control: *P*=0.02; canola vs. control: *P*=0.06). No treatment effects on triglycerides, HDL-C, and apo A1 were observed. There was a significant treatment-by-center interaction for apo B, with a higher endpoint value after HOCO at RCFFN vs. SBRC (differences of LSM estimate=0.09 g/L; *P* for interaction=0.04).

Table 3-5. Endpoint-to-endpoint comparisons of lipids, lipoproteins, and apolipoproteins following the dietary treatments (n=119). [1]

	Canola Oil	HOCO	Control Oil	*P* for treatment
Total cholesterol (mmol/L)	4.54 ± 0.04 [a]	4.58 ± 0.04 [a]	4.74 ± 0.04 [b]	<0.0001
Triglycerides (mmol/L)	1.45 ± 0.04	1.44 ± 0.04	1.40 ± 0.04	0.66
HDL-C (mmol/L)	1.25 ± 0.01	1.28 ± 0.01	1.26 ± 0.01	0.09
LDL-C (mmol/L)	2.64 ± 0.04 [a]	2.67 ± 0.04 [a]	2.83 ± 0.04 [b]	<0.0001
TC: HDL-C ratio	3.82 ± 0.04 [a b]	3.77 ± 0.04 [a]	3.92 ± 0.04 [b]	0.02
apo A1 (g/L)	1.44 ± 0.01	1.46 ± 0.01	1.45 ± 0.01	0.15
apo B (g/L)	0.87 ± 0.01 [a]	0.88 ± 0.01 [a]	0.91 ± 0.01 [b]	0.002
apo B: apo A1 ratio [2]	0.62 ± 0.01 [a b]	0.62 ± 0.01 [a]	0.64 ± 0.01 [b]	0.01
Non-HDL-C	3.30 ± 0.05 [a]	3.31 ± 0.05 [a]	3.45 ± 0.05 [b]	0.001

[1]Data are expressed as least squares means ± standard errors of the means. Different superscript letters within a row indicate statistically significant differences between treatments, *P*≤0.05. A mixed model was used to assess the effects of treatment oil, visit, sex, center, and treatment sequence, and the interactions treatment-by-visit, treatment-by-sex, treatment-by-center, and treatment-by-sequence. The diet period specific baseline value used as a covariate. Final models included treatment oil and only significant terms. . Pairwise comparisons were adjusted using the Tukey-Kramer method when there was a significant effect of treatment.
[2] Canola LSM=0.619 and HOCO LSM=0.616.

Abbreviations: apo A1, apolipoprotein A1; apo B, apolipoprotein B; HDL-C, high-density lipoprotein-cholesterol; high-oleic acid canola oil, HOCO; LDL-C, low-density lipoprotein-cholesterol; TC, total cholesterol.

Absolute change from baseline

The secondary analysis of change from baseline for all lipid and lipoprotein parameters within each treatment is shown in **Figure 3-2**. All diet conditions reduced total cholesterol, LDL-C, non-HDL-C, HDL-C, apo B, and apo A1 from baseline ($P<0.0001$ for all). Triglyceride (canola: $P=0.0182$, HOCO: $P=0.0053$, control: $P=0.0002$), the total cholesterol: HDL-C ratio (canola and HOCO: $P<0.0001$, control: $P=0.0002$), and the apo B: Apo A1 ratio (canola and HOCO: $P<0.0001$, control: $P=0.006$) were also reduced from baseline. Treatment effects were identical to those reported in endpoint analyses.

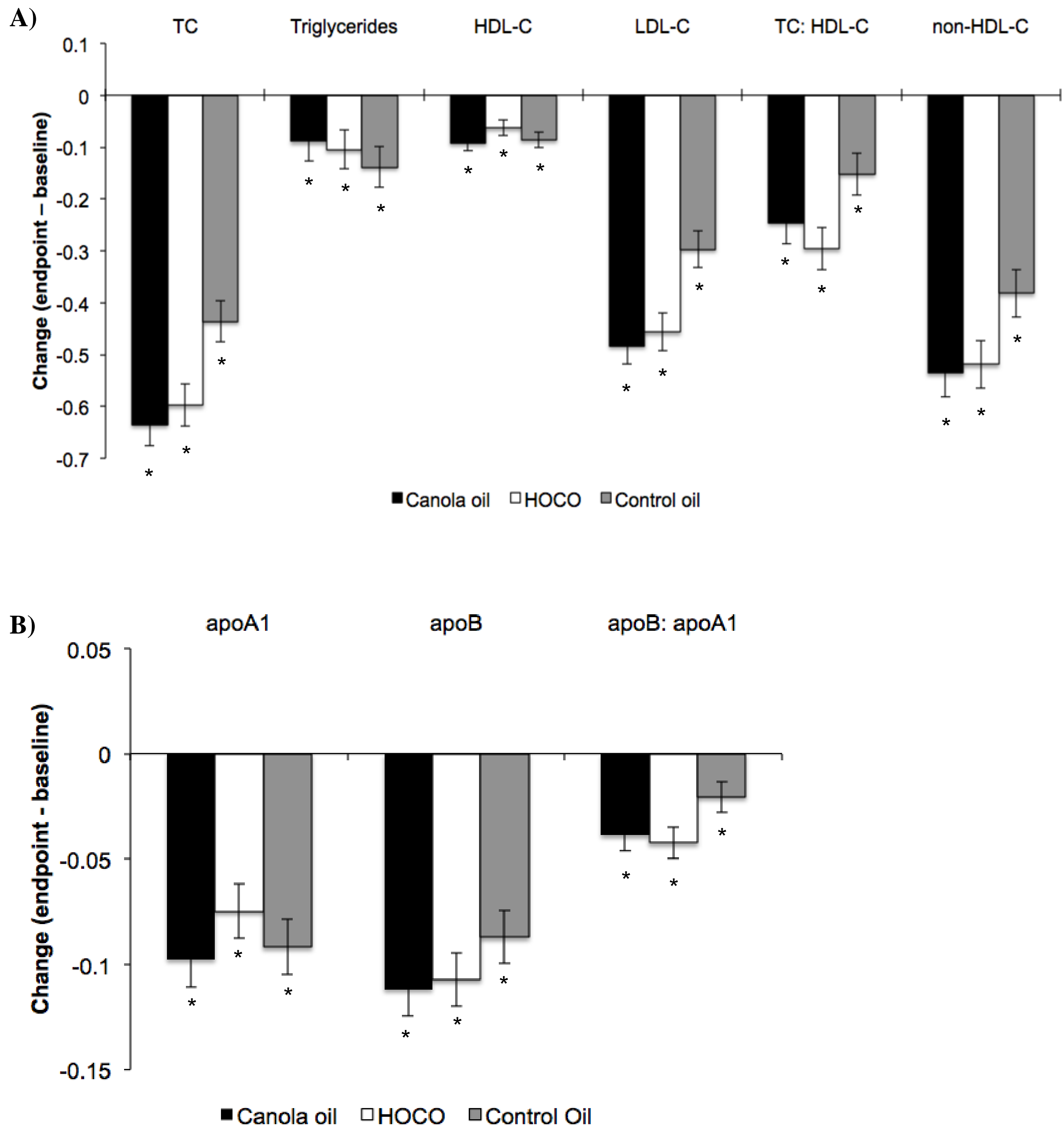


Figure 3-2. Absolute change from baseline in A) lipids and lipoproteins (mmol/L), and B) apolipoproteins (g/L) following the canola oil, HOCO, and control oil diets (n=119). The bars represent least squares means and the error bars represent standard errors of the means. * indicates significant change from 0, $P \leq 0.05$.

Abbreviations: apo A1, apolipoprotein A1; apo B, apolipoprotein B; HDL-C, high-density lipoprotein-cholesterol; high-oleic acid canola oil, HOCO; LDL-C, low-density lipoprotein-cholesterol; TC, total cholesterol.

Discussion

COMIT II is the first double blind, randomized, controlled feeding, crossover study to compare the effects of both conventional and high-oleic acid canola oil diets against a control diet with a fatty acid composition consistent with Western intakes on lipids, lipoproteins, and apolipoproteins in participants with MetS risk factors. The principal finding is that 42 days of canola oil and HOCO consumption similarly lowered endpoint total cholesterol, LDL-C, apo B, and non-HDL-C, and to a greater magnitude than the Western diet control. Further, the total cholesterol: HDL-C and apo B: apo A1 ratios were reduced after HOCO versus control. These data indicate that HOCO, with increased MUFA at the expense of decreased PUFA, elicited beneficial effects on atherogenic lipids and lipoproteins comparable to canola oil and consistent with CVD risk reduction.

High-oleic oils are being increasingly incorporated into the food supply to replace PHVO high in TFA (23, 49), although the health effects of widespread consumption of high-oleic oils remain unclear. Recently, Raatz et al. modeled the impact of replacing conventional soybean oil and canola oil with their high-oleic counterparts (modeling scenarios of 10%, 25%, or 50% replacement) on fatty acid intakes among US adults using 2007-2014 NHANES data (53). Changes in fatty acid intakes for the total population and across age- and sex-specific groups showed increases in total MUFA and reductions in total SFA and PUFA across all replacement levels; importantly, the essential fatty acids linoleic acid and α-linolenic acid were significantly reduced. Numerous age and sex subgroups were at risk of not meeting the Dietary Reference Intake Adequate Intake for linoleic and α-linolenic acid at the 25% and/or 50% replacement scenarios. This modeling analysis presents an unanticipated potential consequence of high-oleic

oil consumption. Investigation into the clinical cardiovascular impact of HOCO is necessary to identify any unfavorable effects of this novel oil on cardiovascular biomarkers.

Only two clinical trials to date have assessed the effects of HOCO on lipid and lipoprotein endpoints (67, 68), the primary biomarker targets for atherosclerotic CVD risk reduction (102). A recent study from our group, COMIT I, assessed the effects of controlled feeding of five dietary oils that varied in USFA compositions, among those including canola oil and HOCO, on lipids and lipoproteins in individuals at risk for or with MetS (n=130) (68). Endpoint values following 28 days of canola oil and HOCO were 4.81 ± 0.14 and 4.77 ± 0.14 mmol/L for total cholesterol, and 2.91 ± 0.08 and 2.86 ± 0.08 mmol/L for LDL-C, respectively. Herein, we report lower total cholesterol and LDL-C endpoint values after 42 days of canola oil and HOCO, consistent with sustained feeding resulting in greater lipid lowering effects. Analogous to the current report, the two COMIT I canola diets did not differ in endpoint values for any parameters. Gillingham et al. was the first to compare the effects of a high-oleic acid rapeseed oil diet compared to a Western control diet on lipids and lipoproteins (67). Following 28 days of controlled feeding in hypercholesterolemic participants (n=36), endpoint total cholesterol, LDL-C, and non-HDL-C were lower after the high-oleic phase (5.27 ± 0.14, 3.10 ± 0.12, and 3.94 ± 0.14 mmol/L) versus the Western diet control phase (5.65 ± 0.16, 3.53 ± 0.14, 4.28 ± 0.17 mmol/L). These findings are consistent with the COMIT II study results, with higher endpoint values likely due to the different populations studied. While these trials provide important insights into HOCO effects on CVD biomarkers compared to canola oil (68) and a Western diet (67), COMIT II is the first study to simultaneously examine conventional canola oil, HOCO, and a Western diet.

The COMIT II study participants were required to have at least two MetS risk factors at the screening visit, one of which being elevated waist circumference, rather than the syndrome definition of three factors (10). These inclusion criteria were selected to increase the generalizability of our findings to a sample that is highly representative of the North American population. An analysis of 2003-2012 NHANES data reported MetS prevalence among adults as 33%, with higher rates among women and Hispanics (11); the COMIT II sample had slightly higher rates at baseline (38%), likely due to the predominance of women (63%). MetS prevalence increases markedly with age [approximately 18% among 18-39 years and 50% among 60+ years in the US (11)], underscoring the relevance of this syndrome as the proportion of the older population rapidly grows. These rates are concerning since MetS is associated with a 5-fold greater risk of incident diabetes (103) and a 2-fold greater risk of incident CVD events and mortality (7). Thus, selecting participants with MetS biomarkers is appropriate for lifestyle intervention trials that aim to identify dietary strategies for chronic disease prevention and risk reduction among North Americans, with important implications for dietary counseling and nutrition policy recommendations. Previous investigations of the effects of canola oil on lipid and lipoprotein parameters in individuals at risk for or with MetS have been limited to three trials, including COMIT I, all of which reported lipid-lowering benefits of canola oil (68-70).

Although there is a substantial evidence base to support cardioprotective benefits of canola oil, very few trials have directly compared a canola oil-based diet to a control diet with the fatty acid composition of the average, contemporary Western diet (22). This prevents the determination of how diets enriched in canola oil fare as a replacement for a diet with the fat profile typical of Western intakes, as well as confirmation of the absence of adverse lipid effects. According to the latest NHANES food consumption data (2015-2016), the average intake of

SFA, MUFA, and PUFA among US adults is 12%, 12%, and 8% of total energy, respectively (26), percentages that the COMIT II control diet aimed to emulate (i.e., 12% SFA, 11% MUFA, and 10% PUFA). The SFA content of the control oil was roughly 2-fold that of the canola oil and HOCO diets, and the MUFA content was appreciably lower than the two canola diets, although still aligned with average intakes. Ghee, coconut oil, and flaxseed oil had to be included in the control blend to generate the targeted fatty acid profile, which was based on an exhaustive evaluation of oil combinations during the COMIT II study design. These are arguably non-traditional oils, and future research would benefit from including lipid sources more characteristic of a truly representative Western diet (i.e., butter, corn oil, animal fats).

COMIT II had numerous strengths, including a tightly regulated, controlled feeding, double-blind, multi-center, randomized, crossover design with a large sample size and inclusion of a commonly consumed oil. The crossover design allowed subjects to act as their own controls during each treatment period, while the controlled feeding aspect reduced confounding variables characteristic of free-living designs. Moreover, blood was sampled on two consecutive days at the baseline and two consecutive days at the endpoint of each experimental period, allowing for calculation of mean values and possible attenuation of intra-individual variability of lipid parameters. Further, collection of treatment-specific blood samples on days 1 and 2 ensured attainment of precise baseline values (data not shown), in contrast to assumed return to initial baseline value post-washout, for inclusion as a covariate in the primary analysis. A limitation of COMIT II is small reductions in body weight (<1kg) were observed across all treatments; however, it is not uncommon to see some degree of weight loss in controlled feeding trials, and is likely attributable to shifts from the habitual diet to a generally healthier controlled feeding eating pattern (i.e., lower sodium, lower SFA, higher fiber, among others). This may explain the

substantial differences in all measured outcomes compared to baseline, particularly in the control condition. Importantly, the magnitude of weight reduction did not differ across treatments and, thus, it is unlikely that the weight loss meaningfully mediated the lipid and lipoprotein treatment response. Further, the lack of weight gain suggests dietary compliance, with the absence of the continual consumption of calorically dense foods outside of the foods provided.

In summary, canola oil- and HOCO-based diets similarly improved the lipid and lipoprotein profile compared to a control diet with a fatty acid composition characteristic of Western intakes in individuals with at least two MetS symptoms. Incorporating high-oleic acid and/or conventional canola oils into the diet by replacing dietary sources higher in SFA is an effective strategy to improve lipid and lipoprotein parameters and, thus, reduce atherosclerotic CVD risk.

Chapter 4: Effects of canola oil diets compared to a diet with a Western fatty acid profile on expression of lipid- and lipoprotein-related genes in human PBMC

Abstract

Background: Circulating apo B-containing lipoproteins are the traditional clinical lipid targets to reduce atherosclerotic CVD risk. We have demonstrated that diets containing canola oil or HOCO improve circulating lipids, lipoproteins, and apolipoproteins compared to a control diet with a fatty acid profile characteristic of Western intakes. However, the biological mechanisms underlying these diet-induced clinical responses are unknown.

Objective: The purpose of this study was to investigate the cardiovascular health promoting mechanisms of canola oils using a PCR-based approach to assess expression of targeted lipid- and lipoprotein-related genes. We hypothesized that canola oils would cause differential expression in genes regulating lipid and lipoprotein metabolism compared to a control oil with a Western diet fatty acid profile, and that these shifts would correspond with the lipid and lipoprotein responses.

Design: In a double blind, randomized, three period crossover, multi-site, controlled feeding trial, participants with an increased waist circumference plus at least one additional criterion for MetS consumed three isocaloric, weight maintenance diets for 6 weeks separated by ≥ 4-week washouts: 1) a canola oil diet (17.5% of energy from MUFA, 9.2% PUFA, 6.6% SFA), 2) a HOCO diet (19.1% MUFA, 7.0% PUFA, 6.4% SFA), and 3) a control oil diet (10.5% MUFA, 10.0% PUFA, 12.3% SFA). Blood was collected at the end of each diet period in PAXgene Blood RNA tubes from a subset of participants (n=42). RNA was extracted from PBMC and expression of 17 gene targets that regulate lipid and lipoprotein metabolism was assessed using

real time-PCR analysis. Transcript levels were normalized for the internal control gene, β-actin, and expressed relative to the control oil.

Results: Canola oil and HOCO decreased expression of the ATP-binding cassette transporters A1 (canola: -16%, *P*=0.0006; HOCO: -11%, *P*=0.005) and G1 (canola: -9%, *P*=0.003; HOCO: -15%, *P*=0.003) in PBMC relative to control, with a trend toward an increase in lipoprotein lipase expression (*P*=0.09). No significant treatment effects on expression of the remaining genes were observed. The expression of ABCA1 and ABCG1 was not correlated with endpoint circulating lipids, lipoproteins, or apolipoproteins.

Conclusions: Canola oil diets altered expression of gene species in human PBMC compared to a diet with a fatty acid profile reflecting Western intakes. These data suggest coordinated fatty acid regulation of select genes implicated in cholesterol and phospholipid homeostasis. Future research is needed to determine the precise mechanisms by which canola-oil based diets lower atherogenic cholesterol.

Introduction

Circulating apo B-containing lipoproteins, including LDL-C and non-HDL-C are the primary targets to reduce atherosclerotic CVD risk, as recommended by the NLA patient-centered management of dyslipidemia (19, 60). The European Atherosclerosis Society concluded stated that LDL-C is unequivocally causative of atherosclerotic CVD (61), and meta-analyses of statin regimens demonstrate that every 1 mmol/L (38.67 mg/dL) reduction in LDL-C reduces the risk of major vascular events by 22% (RR: 0.78, 95% CI: 0.76-0.80), with further reductions yielding greater benefit (62). Thus, interventions to reduce LDL-C level are imperative for reduction of CVD risk.

There is substantial evidence to support that canola oil improves atherogenic lipids and lipoproteins, particularly when replacing sources of SFA (22). We have demonstrated that diets containing canola oil or HOCO as the principle source of dietary fat (i.e., 60 g per 3000 kcal) reduce the apo B-containing particles LDL-C and non-HDL-C, as well as apo B, compared to a contemporary Western diet comparatively higher in SFA and lower in USFA (*see Chapter 3*). The biological mechanisms by which canola oil-based diets lowered LDL-C and other apo B-containing particles, however, are not well defined.

Dietary fatty acid intake directly modulates the plasma fatty acid profile (74, 104). These circulating fatty acids are of physiological significance and can serve as signaling molecules that regulate expression of a variety of genes, including those implicated in lipid and lipoprotein metabolic pathways (71). PUFA are well-characterized regulators of lipid- and lipoprotein-related gene expression and function through numerous mechanisms, including ligand activation of the transcription factors PPAR to stimulate transcription of fatty acid catabolism genes, and inhibition of the processing of SREBP1 to reduce transcription of fatty acid and cholesterol

synthesis genes (71). MUFA also mediate gene expression, particularly through activation of PPAR to promote the utilization of fatty acids as an energy source, whereas SFA activate toll-like receptor 4 to promote expression of inflammatory genes (71). Thus, it is plausible that that the effects of dietary fatty acid interventions on cardiovascular health are mediated, at least in part, by regulation of gene expression.

The objective of this study is to assess the expression of target genes related to lipid and lipoprotein metabolism in PBMC using PCR analysis to investigate a potential mechanism underlying the lipid and lipoprotein response to a canola oil diet, a HOCO diet, and a diet with a Western fatty acid composition. We hypothesize the two canola oil diets will cause differential expression in genes regulating lipid and lipoprotein metabolism compared to the Western diet, and that the expression response will correspond with the lipid/lipoprotein response described in *Chapter 3*.

Methods

Participants

The criteria for inclusion in the COMIT II study were previously described in *Chapter 3*. In brief, the COMIT II participants included adults with an increased waist circumference (men ≥94 cm, women ≥80 cm) plus at least one additional MetS criteria: elevated fasting blood glucose (≥5.6 mmol/L), triglycerides (≥1.7 mmol/L), systolic blood pressure (≥130 mmHg), diastolic blood pressure (≥85 mmHg); and/or decreased HDL-C (men <1 mmol/L, women <1.3 mmol/L).

The genetic sub-study of COMIT II received funding to analyze 50 participants and was originally to be conducted only on participants at the INAF site; however, the protocol was modified to also include subjects from PSU in order to reach the target sample size. All individuals provided written consent to participate in the genetic component of the study during the informed content process prior to study enrollment.

Study design and interventions

The study design details of COMIT II were previously described in *Chapter 3*. Briefly, COMIT II was a double blind, randomized, controlled, 3 period crossover, controlled feeding clinical trial conducted at four centers in North America. Participants consumed isocaloric, weight maintenance diets of fixed macronutrient composition (matched for percentage of energy from carbohydrate, protein, and total fat) and a smoothie containing a treatment oil for 6 weeks, separated by ≥ 4-week washout periods. Treatment oils included conventional canola oil, HOCO, or control oil (blend of ghee, safflower oil, coconut oil, and flaxseed oil with the fatty acid composition of a Western-type diet). The fatty acid compositions (% of total energy) of three diets were as follows: canola oil [17.5% MUFA (15.6% oleic acid), 9.2% PUFA (2.1% α-linolenic acid, 6.4% linoleic), 6.6% SFA], HOCO [19.1% MUFA (17.9% oleic acid), 7.0% PUFA (0.8% α-linolenic acid, 5.6% linoleic), 6.4% SFA), or control oil [10.5% MUFA (5.9% oleic acid), 10.0% PUFA (1.7% α-linolenic acid, 7.3% linoleic), 12.3% SFA].

Sample collection and analyses

Blood was collected at the endpoint of each diet period from a subset of the COMIT II participants into a PAXgene Blood RNA Tube (PreAnalytiX GmbH; Hombrechtikon, Switzerland). The PAXgene Blood RNA Tube with its vacuum is designed to collect precisely 2.5 mL of blood, with the stop of blood flow once the required volume is obtained (105). Following blood collection, the tube was gently inverted 8-10 times and incubated upright at room temperature for a minimum of 2 hours, transferred to -20°C for a minimum of 24 hours for freezing, then stored at -80°C. Samples were shipped overnight from PSU and INAF to the University at Buffalo on dry ice for analysis.

PBMC RNA was purified from the whole blood stabilized in PAXgene Blood RNA Tubes. Intracellular RNA is highly unstable *in vitro*; post-phlebotomy, RNA rapidly degrades as a result of cell death or enzymatic degradation, and certain species of RNA increase through gene induction (105). PAXgene Blood RNA Tubes are prefilled with a RNA stabilization reagent (2.76 mL or additive per ml of blood) to inhibit these processes and ensure stability of the intracellular RNA profile (minimum of 50 months at -80°C), preventing the under or overestimation of relative gene transcript *in vivo*. Thus, use of PAXgene Blood RNA Tubes provides an accurate snapshot of the intracellular RNA at the time point of blood collection. Intracellular total RNA was purified from stabilized whole blood using the PAXgene Blood RNA Kit (PreAnalytiX GmbH; Switzerland). To ensure high-quality RNA yields for reliable analysis, RNA purity was analyzed by UV spectroscopy using a nanodrop spectrophotometer (A_{260}/A_{230}; pure RNA ratio ~2) and integrity was analyzed by agarose gel electrophoresis (visualization of 18S/28S ribosomal RNA bands; ratio <2:1 indicates degradation). mRNA was converted to cDNA using an iScript advanced cDNA synthesis kit (Bio-rad; USA).

Twenty gene targets that regulate blood lipid and lipoprotein metabolism were selected (US National Library of Medicine, National Center for Biotechnology Information, Gene; www.ncbi.nlm.nih.gov/gene) and assessed in the cohort. Details of each gene are described in **Table 4-1**, including the common abbreviation, full name, and description of the function of the encoded protein specific to lipid/lipoprotein metabolism. The relative transcript levels of the target gene mRNA were determined by real-time PCR analysis. The housekeeping genes were β-actin, glyceraldehyde 3-phosphate dehydrogenase, and large ribosomal protein.

The specific methods to analyze the circulating lipid, lipoprotein, and apolipoprotein levels were described in *Chapter 3*. Values were determined from fasting blood draws collected on two consecutive days at the end of each diet period (i.e., day 41 and 42).

Table 4-1. Target genes related to lipid and lipoprotein metabolism assessed by PCR in the COMIT II subgroup. [1]

Abbreviation	Full name	Description of encoded protein as it relates to lipid/lipoprotein metabolism
ABCA1	ATP-binding cassette subfamily A member 1	Membrane-associated transporter; functions as a cholesterol efflux pump for cellular lipid removal; important to cholesterol/phospholipid homeostasis
ABCG1	ATP-binding cassette subfamily G member1	Involved in macrophage cholesterol and phospholipid transport and regulates cellular lipid homeostasis in other cell types
ABCG8	ATP-binding cassette subfamily G member 8	Facilitates transport of sterols back into the intestinal lumen for reduced absorption and elimination in feces; promotes sterol incorporation into bile for release into the intestine; expression biased to liver, duodenum, small intestine, and gallbladder
CETP	cholesteryl ester transfer protein	Plasma protein that catalyzes the transfer of cholesteryl ester and triglyceride between HDL and lower density lipoproteins, specifically movement of cholesteryl ester from HDL to VLDL/LDL and of triglyceride from VLDL to HDL; expression biased to spleen and fat
CYP7A1	cytochrome P450 family 7 subfamily A member 1	Endoplasmic reticulum membrane protein that catalyzes the conversion of cholesterol to bile acids for cholesterol catabolism in the liver; rate-limiting enzyme of bile acid synthesis and cholesterol removal from the body; expression restricted to liver
DGAT1	diacylglycerol O-acyltransferase 1	Transmembrane protein that catalyzes the conversion of diacylglycerol and fatty acyl CoA to triacylglycerol for the final step in triglyceride synthesis; highly expressed in the small intestine and duodenum
HMG-CoAR	3-hydroxy-3-methglutaryl-CoA reductase	Rate-limiting enzyme for endogenous cholesterol synthesis; negative feedback regulation by sterols and metabolites from products of the HMG-CoA reaction; target of statin action
LCAT	lecithin-cholesterol	Extracellular enzyme that esterifies free cholesterol; required for cholesteryl ester

	acyltransferase	transport via CETP; expression biased to liver
LDLR	low-density lipoprotein receptor	Cell surface protein involved in receptor-mediated endocytosis; binds apolipoprotein B 100 on LDL
LIPC	lipase C, hepatic type	Triglyceride lipase expressed in the liver that hydrolyzes triglyceride and also acts as a ligand for receptor-mediated lipoprotein uptake
LIPE	lipase E, hormone-sensitive type	Hydrolyzes stored triglyceride to free fatty acids in adipose tissue
LIPG	lipase G, endothelial type	Phospholipase that may be related to lipoprotein metabolism and vascular biology
LPL	lipoprotein lipase	Triglyceride hydrolase and ligand for receptor-mediated lipoprotein uptake; biased expression in fat and heart; provides free fatty acids for adipose and cardiac tissue
MTTP	microsomal triglyceride transfer protein	Protein that plays a central role in assembly of lipoproteins; expressed biased to the small intestine, duodenum, and liver
PCSK9	proprotein convertase subtilisin/kexin type 9	Constitutively secreted as a zymogen into the extracellular matrix and trans-Golgi network for autocatalytic processing in the endoplasmic reticulum; plays a role in cholesterol and fatty acid metabolism through escorting LDL receptors for lysosomal degradation; inhibition of this protein reduces circulating cholesterol; expressed in the liver, intestine, and lung
PPARGC1A	peroxisome proliferator-activated receptor γ, coactivator 1 α	Transcriptional coactivator that interacts with PPARγ; allows PPARγ to modulate transcription of genes involved in blood pressure, cholesterol homeostasis, and obesity
PLTP	phospholipid transfer protein	Lipid transfer protein in human plasma; mediates transfer of phospholipids from triglyceride-rich lipoproteins to HDL; regulates the size of HDL particles and may be involved in cholesterol metabolism
SREBF1	sterol regulatory element	Nuclear transcription factor that, in the presence of low cellular sterols, binds to and

	binding transcription factor 1	activates the sterol regulatory element-1, a motif in the promoter of genes involved in sterol biosynthesis and uptake (i.e., LDLR gene); encoded protein is synthesized as a precursor that once cleaved translocates to the nucleus to activate transcription (cleavage inhibited by sterols); regulates cholesterol homeostasis and fatty acid metabolism
SREBF2	sterol regulatory element binding transcription factor 2	Transcription factor that controls cholesterol homeostasis by regulating transcription of sterol-regulated genes; binds the sterol regulatory element-2
VLDLR	very low-density lipoprotein receptor	Surface protein involved in receptor-mediated endocytosis; plays a role in metabolism of VLDL triglyceride

[1] Summary of gene function adapted from the gene repository, available in the US National Library of Medicine, National Center for Biotechnology Information (www.ncbi.nlm.nih.gov/gene).

Abbreviations: HDL, high-density lipoprotein; LDL, low-density lipoprotein; VLDL, very low-density lipoprotein.

Statistical methods

The Livak method, or $2^{-\Delta\Delta CT}$ method, was used to analyze the treatment effect on the real-time PCR data (106). This approach determines the relative changes in gene expression of a treatment group compared to a reference group. The PCR instrument provides threshold cycle values (C_T) that indicate the cycle number at which the fluorescence levels of the amplified gene target reached an arbitrary threshold. The numerical C_T value is inversely related to the amount of amplicon (i.e. greater PCR signal or higher gene transcript, lower C_T value). Gene expression values for all treatments were first normalized to the internal control β-actin (ΔC_T), then to the Western diet control oil ($\Delta\Delta C_T$). The latter value was calculated by subtracting the control group from the canola and HOCO for each gene. $\Delta\Delta C_T$ was converted to $2^{-\Delta\Delta C}{}_T$, to express the fold change in gene expression relative to control and eliminate the inverse relationship for appropriate interpretation. All gene expression outcomes were log transformed (i.e., $\log_2{}^{-\Delta\Delta C}{}_T$) prior to assessment of treatment effects to ensure normally distributed data.

Statistical analyses were performed using SAS 9.4 (SAS Institute Inc., Cary, NC, US). The primary outcome was the effect of treatment on $\log_2{}^{-\Delta\Delta C}{}_T$ values of relative expression. This was assessed for each gene using a repeated measures mixed model procedure, with subject, treatment sequence, and study center as random effects, and visit as the repeated effect. The main effect of treatment oil (canola oil, HOCO, control oil) and sex, as well as their interaction was assessed; sex and the sex-by-treatment interaction were not significant for any genes. Because PAXgene Blood RNA tubes were not collected at baseline, baseline value of the dependent variable could not be used as a covariate. Tukey-Kramer adjusted *P*-values were used for multiple pairwise comparisons between treatments, only when there was a significant effect of

treatment. Normality of the residuals from the final models was assessed. The distribution of the residuals was skewed for the LIPE gene; one outlier was removed to meet the normality assumption. Data are presented as mean fold changes ($2^{-\Delta\Delta CT}$) ± standard error of the mean from the control, and $P \leq 0.05$ was considered significant. Because of the exploratory nature of this analysis, no adjustments were made for testing of multiple genes.

Pearson correlation coefficients between endpoint gene expression (ΔC_T) and endpoint plasma lipids, lipoproteins, and apolipoproteins (average day 41 and 42) were investigated. Gene expression values normalized to the internal control (ΔC_T) were utilized, rather than relative expression ($\log_2 {}^{-\Delta\Delta C_T}$), because the relative expression of each gene following the control diet had a value of 1 ± 0. The univariate procedure was used to assess the normality of the distribution for all variables. Five genes did not meet the normality assumption and were transformed as indicated in the correlation table prior to analyses. The lipid, lipoprotein, and apolipoprotein parameters of interest were those in which we saw a significant effect of diet in *Chapter 3* (i.e., LDL-C, total cholesterol, non-HDL-C, and apo B). No adjustments were made for multiple comparisons due to the exploratory nature of the study.

Results

Baseline characteristics

The flow of participants through COMIT II and inclusion for the gene expression analyses is shown in **Figure 4-1**. Eight subjects were removed from the final analyses due to insufficient and/or low quality RNA, incomplete sample sets, and a weight change of >5% during any diet period. **Table 4-2** presents the baseline characteristics of the participants

included in the genetic analysis. The cardiovascular risk profile of the subgroup was overall similar to the full sample, with the exception of a higher percentage of males (*see Chapter 3, Table 3-3*). The subgroup was 50% male, middle-aged, and obese, with an increased waist circumference. The remaining MetS criteria and cardiovascular biomarkers were, on average, within healthy ranges.

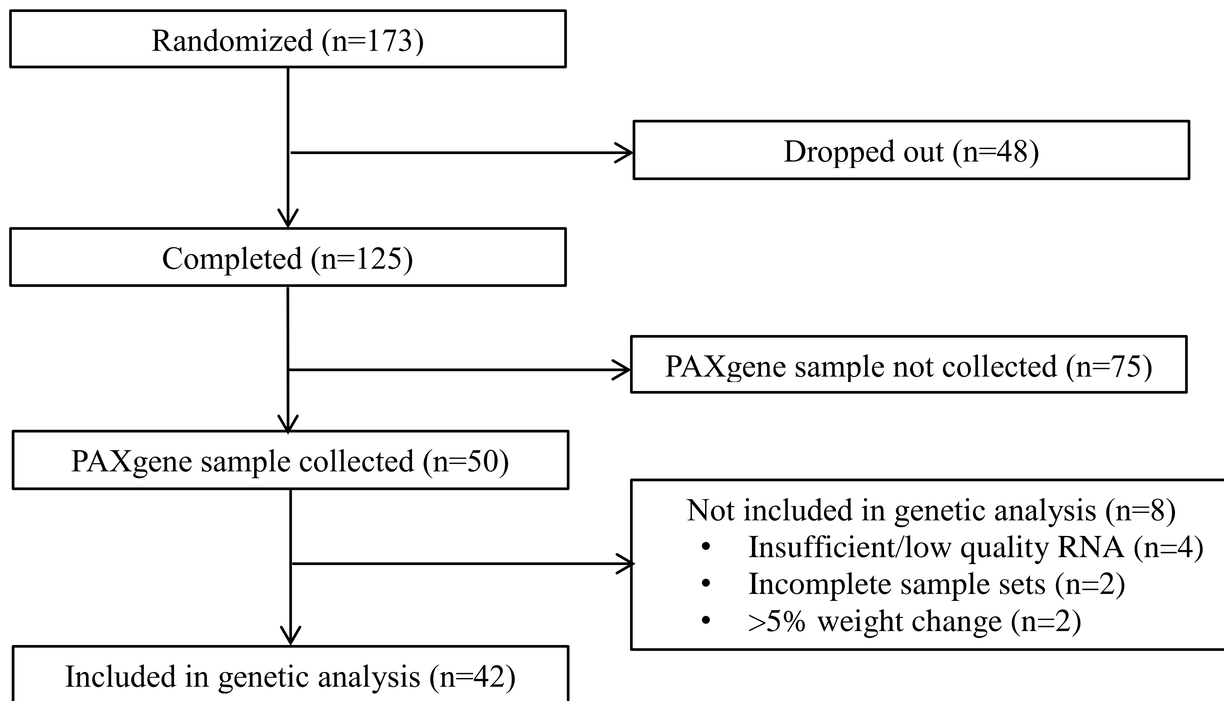


Figure 4-1. Flow diagram of the COMIT II participants for inclusion in the gene expression analyses (n=42).

Table 4-2. Baseline characteristics of the COMIT II participants in the subgroup for genetic analyses (n=42). [1]

Variable	n=42
Sex (female)	21, 50%
Anthropometric measures	
Age (years)	43.33 ± 13.98 (22.00 – 65.00)
Weight (kg)	89.12 ± 19.60 (60.40 – 146.35)
BMI (kg/m^2)	30.31 ± 5.22 (22.84 – 42.11)
MetS criteria	
Waist circumference (cm)	101.79 ± 11.45 (80.40 – 131.65)
Female	99.48 ± 11.71 (80.40 –121.65)
Male	104.10 ± 10.98 (93.85 –131.65)
Triglycerides (mmol/L)	1.43 ± 0.68 (0.33 – 3.67)
HDL-C (mmol/L)	1.39 ± 0.36 (0.74 – 2.18)
Female	1.51 ± 0.34 (0.96 – 2.18)
Male	1.26 ± 0.34 (0.74 – 1.97)
Glucose (mmol/L)	5.16 ± 0.36 (4.43 – 6.27)
Blood pressure (mmHg)	
Systolic	113.67 ± 11.64 (87.67 – 141.67)
Diastolic	73.48 ± 10.97 (53.67 – 96.00)
Additional cardiovascular risk biomarkers	
Total cholesterol (mmol/L)	5.06 ± 0.86 (3.44 – 6.71)
LDL-C (mmol/L)	3.02 ± 0.66 (2.01 – 4.28)

[1] Data collected on days 1 and 2 of diet period 1. Categorical variables are expressed as frequency, percent and continuous variables expressed as mean ± standard deviation (minimum – maximum).
Abbreviations: BMI, body mass index; HDL-C, high-density lipoprotein-cholesterol; LDL-C, low-density lipoprotein-cholesterol; MetS, metabolic syndrome.

Endpoint-to-endpoint gene expression

The relative endpoint values for 17 lipid- and lipoprotein-related genes following the canola oil and HOCO diets are presented in **Table 4-3**. All values are expressed as the mean fold change relative to control, or $2^{-\Delta\Delta C_T}$, and the *P*-values were from assessment of the treatment

effects on the $\log_2$ transformed variables. The expression of the ABCG8, MTTP, and PPARGC1A genes were not assessed due to limited observations. ABCA1 and ABCG1 gene expression was lower after canola oil (*P*=0.0004) and HOCO (*P*=0.0007) relative to the control oil. There was a trend toward a treatment effect on the LPL gene (*P*=0.09). No effects on the remaining genes were observed. The effects of the three oils of gene expression of the ATP-binding cassette transporters ABCA1 and ABCG1 are presented in **Figure 4-2**. ABCA1 gene expression was lower after canola vs. control (*P*=0.0006), as well as HOCO vs. control (*P*=0.005), with no difference between canola oils (*P*=0.79). In addition, ABCG1 gene expression was lower after canola vs. control (*P*=0.003) and HOCO vs. control (*P*=0.003), with no difference between canola oils (*P*=0.99).

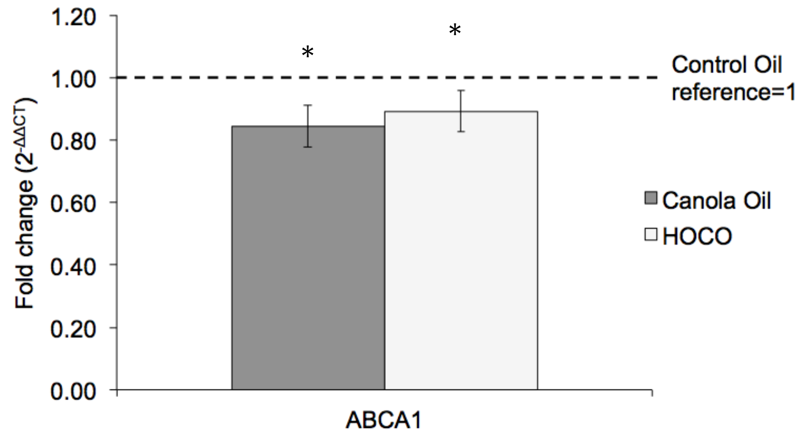
Table 4-3. Relative expression of genes related to lipid and lipoprotein metabolism in PBMC following canola and high-oleic acid canola oils (n=42). [1]

Gene	Canola Oil	HOCO	*P*-value for treatment [2]
ABCA1	0.84 ± 0.07	0.89 ± 0.07	0.0004
ABCG1	0.91 ± 0.07	0.85 ± 0.04	0.0007
CETP [3]	1.31 ± 0.13	1.48 ± 0.27	0.78
CYP7A1	1.55 ± 0.32	1.43 ± 0.19	0.83
DGAT1	1.12 ± 0.10	1.10 ± 0.10	0.97
HMG-CoAR	1.05 ± 0.06	1.10 ± 0.07	0.68
LCAT	1.11 ± 0.05	1.07 ± 0.05	0.30
LDLR	1.20 ± 0.12	1.18 ± 0.11	0.86
LIPC	1.23 ± 0.17	1.44 ± 0.33	0.82
LIPE [4]	1.21 ± 0.14	1.17 ± 0.09	0.84
LIPG	1.23 ± 0.22	1.84 ± 0.45	0.26
LPL	1.14 ± 0.17	1.36 ± 0.23	0.09
PCSK9	1.54 ± 0.29	1.96 ± 0.52	0.34
PLTP	1.56 ± 0.33	1.52 ± 0.28	0.99
SREBF1	1.36 ± 0.21	1.51 ± 0.34	0.35
SREBF2	1.11 ± 0.07	1.20 ± 0.08	0.20
VLDLR	1.07 ± 0.13	1.25 ± 0.26	0.32

[1] Data are expressed as the mean fold change relative to control ($2^{-\Delta\Delta CT}$) ± standard error of the mean. The value for all genes after the control diet is 1 ± 0.
[2] *P*-value for the main effect of treatment. The treatment effect on $\log_2{}^{-\Delta\Delta C_T}$ values of relative expression was assessed for each gene using a repeated measures mixed model, with subject, treatment sequence, and study center as random effects, and visit as the repeated effect. The main effects of treatment oil and sex, as well as their interaction, were assessed. Final models included only treatment. Sex and sex-by-treatment were not significant for any dependent variables. Normality of the residuals from the final models was assessed. $P \leq 0.05$ was considered significant and $P \leq 0.10$ was considered a trend.
[3] n=41 for HOCO (125 observations).
[4] One observation skewed the distribution of the residuals and was removed to meet the normality assumption (125 observations).

Abbreviations: HOCO, high-oleic acid canola oil. All gene abbreviations listed in Table 4-1.

A)



B)

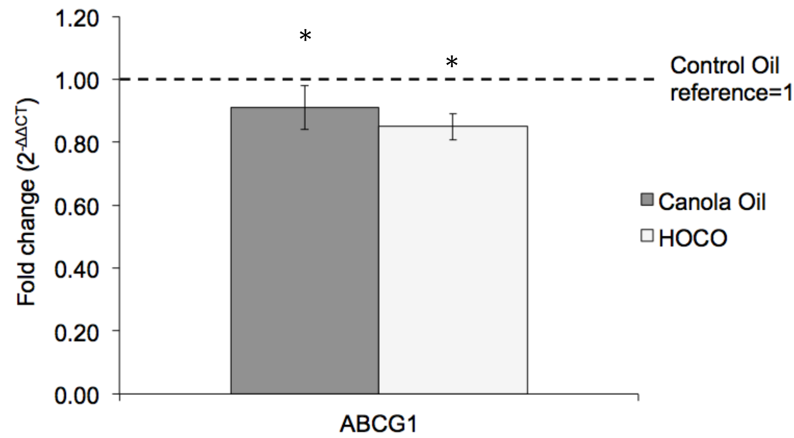


Figure 4-2. Canola and high-oleic acid canola oils reduced endpoint **A)** ABCA1 and **B)** ABCG1 gene expression compared to the Western diet control oil. Bars represent the mean fold change relative to control ($2^{-\Delta\Delta C_T}$) and error bars are the standard error of the mean. Control values for the genes are 1 ± 0. The treatment effect on $\log_2{}^{-\Delta\Delta C_T}$ values of relative expression was assessed for the two genes using a mixed model, with subject, treatment sequence, and study center as random effects, and visit as the repeated effect. The final models included treatment as the main effect. Tukey-Kramer adjusted *P*-values were used for multiple pairwise comparisons between treatments. * indicates statistically significant difference between canola or HOCO and control, $P \leq 0.05$.

Abbreviations: ABCA1, ATP-binding cassette transporter A1; ABCG1, ATP-binding cassette transporter G1; HOCO, high-oleic acid canola oil.

Correlations between lipids/lipoproteins and gene expression

Pearson correlation coefficients between the endpoint gene expression and endpoint plasma lipids, lipoproteins, and apolipoproteins stratified by diet are presented in **Table 4-4**. Only those lipid/lipoprotein parameters in which there was a significant treatment effect in *Chapter 3* are presented in the table. Following the HOCO diet, LDL-C (r=0.32, *P*=0.04) and non-HDL-C (r=0.33, *P*=0.03) were positively associated with SREBF2; LDL-C, total cholesterol, and non-HDL-C were negatively correlated with LIPE (r=-0.31 to -0.40, P= 0.01 to 0.049). Following the canola oil diet, all lipids/lipoproteins were positively correlated with LCAT (r=0.35 to 0.46, P= 0.002 to 0.02); non-HDL-C (r=-0.34, P=0.03) and apo B (r=-0.32 P=0.04) were negatively correlated with LIPE. Following the control diet, total cholesterol was positively associated with HMG-CoAR (r=0.33, *P*=0.03) and SREBF2 (r=0.35, *P*=0.03), and all lipids/lipoproteins were positively correlated with LCAT (r=0.37 to 0.48, P= 0.001 to 0.01). Because of the significant treatment effect on ABCA1 and G1 described above, we assessed correlations between these genes and HDL-C and apo A1 (data not shown). There was a trend toward an association with HDL-C and ABCG1 (r=0.26, *P*=0.10) after the control diet, with no associations in the canola or HOCO diets.

Table 4-4. Pearson correlation coefficients between PBMC gene expression and blood lipids, lipoproteins, and apolipoproteins, stratified by the three test diets (n=42). [1]

Gene	Diet	LDL-C	Total cholesterol	Non-HDL-C	apo B
ABCA1	HOCO	0.08	0.08	0.01	0.01
	Canola	0.00	-0.01	-0.09	-0.08
	Control	0.02	0.03	-0.07	-0.09
ABCG1	HOCO	0.21	0.18	0.10	0.07
	Canola	0.10	0.09	-0.01	0.00
	Control	0.27	0.27	0.17	0.10
CETP [2]	HOCO	-0.10	0.02	-0.04	-0.05
	Canola	-0.03	0.01	-0.08	-0.11
	Control	-0.03	-0.05	-0.13	-0.15
CYP7A1	HOCO	0.03	0.07	0.12	0.06
	Canola	-0.14	-0.01	0.01	-0.04
	Control	-0.09	-0.08	-0.03	-0.05
HMG-CoAR	HOCO	0.03	0.14	0.08	0.05
	Canola	0.19	0.25	0.20	0.14
	Control	0.22	0.33**	0.17	0.13
PCSK9	HOCO	-0.04	-0.01	0.03	-0.06
	Canola	-0.17	-0.18	-0.04	-0.11
	Control	-0.02	0.01	0.06	0.02
PLTP	HOCO	-0.15	-0.17	-0.10	-0.12
	Canola	-0.06	-0.14	-0.11	-0.08
	Control	0.02	-0.11	0.10	0.14
SREBF2	HOCO	0.32**	0.28	0.33**	0.29
	Canola	0.13	0.15	0.23	0.16
	Control	0.27	0.35**	0.29	0.20
VLDLR	HOCO	-0.05	-0.05	-0.02	-0.07
	Canola	-0.24	-0.22	-0.12	-0.16
	Control	0.05	-0.02	0.08	0.05
DGAT1	HOCO	0.06	0.00	-0.01	-0.05
	Canola	0.05	0.03	0.03	0.00
	Control	0.06	0.18	0.00	-0.10
LIPC	HOCO	0.05	0.13	0.10	0.04
	Canola	-0.05	-0.01	-0.03	-0.08
	Control	0.01	0.10	0.06	0.00
LIPG	HOCO	0.07	0.02	0.14	0.08
	Canola	-0.15	-0.06	-0.01	-0.04
	Control	0.04	0.05	0.15	0.10
LPL	HOCO	-0.09	0.02	0.02	-0.04
	Canola	-0.18	-0.10	-0.05	-0.10
	Control	-0.02	0.01	0.05	0.02
LCAT [3]	HOCO	0.24	0.16	0.14	0.11
	Canola	0.38**	0.46**	0.40**	0.35**

	Control	0.42**	0.48**	0.39**	0.37**
LDLR [3]	HOCO	-0.15	-0.15	-0.10	-0.13
	Canola	-0.09	-0.12	-0.08	0.00
	Control	-0.23	-0.22	-0.15	-0.14
LIPE [3]	HOCO	-0.32**	-0.40**	-0.31**	-0.26*
	Canola	-0.26	-0.29	-0.34**	-0.32**
	Control	-0.18	-0.18	-0.12	-0.09
SREBF1 [4]	HOCO	-0.01	0.00	-0.13	-0.10
	Canola	-0.19	-0.17	-0.19	-0.18
	Control	-0.08	-0.18	-0.09	-0.03

[1] Correlations between endpoint gene expression normalized to the internal control (ΔC_T) and endpoint plasma lipids, lipoproteins, and apolipoproteins (average day 41 and 42). The latter parameters were selected based on those with a significant treatment effect in *Chapter 3*.
[2] n=41 for all correlations on the HOCO diet.
[3] Square root transformation.
[4] Log transformation.
** indicates significance, $P \leq 0.05$

Discussion

This study investigated the underlying mechanisms through which canola oil may function to improve circulating cholesterol levels and promote CV health. A PCR-based approach was utilized to determine the relative gene expression of target genes related to lipid and lipoprotein metabolism in PBMC from a subset of participants in the COMIT II study, a randomized, controlled, double blind, three period crossover, controlled feeing clinical trial. The principle finding is that canola oil- and HOCO-based diets down-regulate expression of ABCA1 and ABCG1 genes compared to a control diet with a Western diet fatty acid composition. These genes did not significantly correlate with any of the lipid, lipoprotein, or apolipoprotein endpoints. These data suggest coordinated fatty acid regulation of select gene species implicated in cholesterol and phospholipid homeostasis in human PBMC.

ABCA1 is an integral membrane lipid transporter that belongs to the ATP-binding cassette transporter superfamily and is a crucial player in modulation of plasma HDL-C levels, HDL biogenesis, and cellular cholesterol homeostasis (107). ABCA1 mediates the translocation of phospholipids and free cholesterol from the inner to outer leaflet of the plasma membrane, binding and unfolding of lipid-free extracellular apo A1 at the cell surface, insertion of apo A1 into the lipid bilayer for lipidation, and release of nascent HDL (107, 108). Mutations in the ABCA1 gene are implicated in Tangier disease, a rare autosomal recessive disorder with a clinical phenotype of severely decreased or absent circulating HDL-C and accumulation of cholesterol in various tissues, including tonsils and spleen (109). ABCG1 is also a member of the ATP-binding cassette transporter family and contributes to efflux of cholesterol to mature HDL subclasses (i.e., HDL2 and HDL3) that make up the bulk of plasma HDL, but does not interact with lipid-poor apo A1 (110).

Transcription of the ABCA1 and ABCG1 genes is regulated by LXR, an oxysterol-activated nuclear transcription factor that forms an obligate heterodimer with RXR and binds to direct repeat 4 elements within the promoter region for targeted gene induction (111-113). Treatment of human THP-1 macrophages with oxidized cholesterol derivatives (i.e., oxysterols) or retinoic acid increased ABCA1 mRNA (7- and 8-fold, respectively), with a potent additive effect when combined (37-fold) (111). Similarly, treatment of human THP-1 macrophages with oxysterols (112) and of mouse macrophages with an LXR-specific activator (110) led to marked increases in ABCG1 mRNA. ABCA1 and ABCG1 are expressed in a wide variety of cell types, but highly expressed in macrophages (114).

USFA suppress ABCA1 and ABCG1 gene expression in *vitro*, with PUFA demonstrating stronger inhibitory effects than MUFA (113, 115). Following of incubation of HEPG2

hepatocytes or RAW264.7 macrophages with oxysterols and various fatty acids, EPA and oleic acid, but not palmitic acid, suppressed the oxysterol up-regulation of ABCA1 mRNA levels (115). EPA and linoleic acid incubation also reduced endogenous ABCG1 mRNA levels in RAW264 cells, while oleic acid numerically lowered mRNA, though not significantly (113). Linoleic acid (-80%), arachidonic acid (-75%), EPA (-70%), and oleic acid (-40%), suppressed the ABCA1 promoter activity, with a modest suppressive effect by palmitic acid (-10%) and an increase by stearic acid (+10%) (115). Similarly, EPA (-70%), linoleic acid (-62%), arachidonic acid (-57%), and oleic acid (-57%) suppressed ABCG1 promoter activity, with no effect of palmitic acid and an increase from stearic acid (113). Further, decreased ABCA1 and ABCG1 protein abundance was observed in macrophages incubated with n-3 PUFA, n-6 PUFA, and oleic acid (113, 115). The proposed mechanism by which USFA interfere with LXR/RXR binding to the promoter and suppress gene expression is through interaction with co-regulators (113, 115). Although fatty acids can induce LXR gene transcription indirectly through PPAR activation, Uehara et al. did not find an effect of USFA on LXR or RXR transcription (113, 115). Together, these data demonstrate that USFA downregulate ABCA1 and ABCG1 expression, perhaps as a function of degree of hydrocarbon chain saturation, with corresponding shifts in protein level.

Consistent with the in *vitro* findings, we reported down-regulation of ABCA1 and ABCG1 in human PBMC following the canola diets compared to the Western diet control. This fatty acid regulation of gene expression is likely attributed to the higher MUFA (canola: 18% of total energy, HOCO: 19%, control: 11%) rather than PUFA content (canola: 9%, HOCO: 7%, control: 12%), given the marginal difference in the latter. The functional significance of this attenuated gene expression is unknown, as we did not measure cholesterol efflux. Cholesterol efflux to apo A1 stimulated by oxysterols was entirely inhibited in RAW264.7 macrophages

incubated with oleic acid or EPA, with no effect of palmitic acid (115), whereas ABCA1-mediated efflux was decreased, yet still apparent, in J774 and THP-1 macrophages incubated with oleic acid (116). However, cholesterol efflux assays performed in clinical trials of dietary interventions high in USFA, including vegetable oils (117, 118) and nuts (119, 120), report increased cholesterol efflux. In a previous study from our group, COMIT 1, participants at risk for MetS (n=101) were fed five oils (60 g/day per 3000 kcal) high in USFA and low in SFA (<7%) for 4 weeks in a randomized, crossover, controlled feeding trial matched for macronutrients (118). Ex *vivo* cholesterol efflux capacity was assessed at the trial enrollment and after each of the five diets using THP-1 macrophages incubated with participant whole sera; cholesterol efflux increased from baseline following all USFA oils (canola oil: +39%, HOCO: +34%), with no differences between diets. Because whole serum was utilized, the reported increase is an assessment of global efflux of free cholesterol to numerous extracellular acceptors (i.e., lipid poor apo A1, mature HDL particles, and/or apo B-containing lipoproteins) and, thus, mediated by ABCA1, ABCG1, scavenger receptor class B type 1 (SR-B1), and/or passive diffusion. Although ABCA1 and ABCG1 gene expression was not assessed in COMIT I, extrapolation of our findings of decreased ABCA1 and G1 expression suggest complementary pathways contributed to the elevated cholesterol efflux following the five USFA oils, particularly considering SR-B1 mRNA is not influenced by fatty acids in *vitro* (115). In a post-prandial study, Berryman et al. found a 3.3% increase in whole serum ex *vivo* cholesterol efflux from baseline 12-hours after walnut consumption in adults with moderate hypercholesterolemia (n=15) (120). However, cells cultured with apo B-depleted serum (i.e., HDL serum) did not change efflux capacity, indicating walnuts modulated global, but not HDL-specific, efflux. Helal et al. fed 12 weeks of extra-virgin olive oil (25 ml/day) in health adults (n=26) and found human

monocyte derived macrophages from the participants had increased ABCA1 and ABCG1 mRNA and protein level, with decreased SR-B1 (117). Cellular-based assays revealed this increase was driven by specific polyphenols in the extra-virgin olive oil, supported by evidence that polyphenols upregulate LXR expression in THP-1 macrophages and human monocyte-derived macrophages (121). Together, these data suggest that efflux capacity following USFA interventions may be maintained or improved through complementary mechanisms, and confounding nutrients with genetic regulation capabilities must be considered.

Although the fold changes in ABCA1 and ABCG1 following HOCO and canola oil were statistically significant, the gene expression suppression may not be biologically relevant, particularly considering diet-induced changes in gene expression are smaller than the effects of pharmacological agents or the pathophysiologic effects of disease (122). There was no diet effect on endpoint HDL-C, as described in *Chapter 3*, and ABCA1 and ABCG1 relative gene expression were not significantly correlated with any lipid, lipoprotein, or apolipoprotein parameters after the diets. There was a trend toward a treatment effect on the LPL gene (P=0.09), with higher expression following canola diets compared with control. This finding is consistent with the mechanism by which USFA suppress ABCA1 gene expression. LPL is a secreted lipolytic enzyme that is highly expressed in macrophages and functions to hydrolyze triglyceride to release free fatty acids (123). LPL levels are inversely correlated with ABCA1 gene expression; silencing of LPL induced ABCA1 gene expression, as well as increased ABCA-1 mediated cholesterol efflux, in THP-1 macrophages (123). The addition of LPL replenishes the fatty acid products, which consequently can inhibit ABCA1 gene expression (123). Thus, our findings are consistent with regulation of this lipase and sterol transporters in *vitro*.

The correlation analyses were performed to identify relationships between lipids/lipoprotein levels and gene expression after the three test diets. Total cholesterol was positively associated with both HMG-CoAR (r=0.33, *P*=0.03) and SREBF2 (r=0.35, *P*=0.03) after the control diet only. These findings are biologically consistent, given HMG-CoAR is the rate-limiting enzyme in cholesterol biosynthesis and its expression is regulated by SREBP2. However, we did not see a significant treatment effect between the three diets on endpoint HMG-CoAR expression and, thus, cannot conclude that the higher atherogenic cholesterol after the control diet is due to increased expression of genes involved in cholesterol biosynthesis. Vallim et al. reviewed the evidence from animal models and hypothesized that dietary SFA increases circulating cholesterol through modulation of hepatic uptake, rather than biosynthesis, and regulates SREBP2 to decrease LDLR expression (124). In contrast, our findings indicate no effect of the diets on LDLR expression and no association between LDLR gene expression and cholesterol outcomes in any diet. Future work is necessary to determine which aspect of cholesterol homeostasis is modulated in humans following dietary fatty acid manipulations.

The purpose of this study was to explore the potential mechanism of action of fatty acids causing lowering of LDL-C and other apo B-containing particles. Although the COMIT II study was designed to differ only in fatty acid composition, with constant macronutrient compositions across diets, a limitation of COMIT II is that we cannot exclude an effect of other unintended nutrient differences between diets. It is plausible that the phytosterol composition differed between the two-canola diets and the Western-type control diet, although this was not directly measured in the COMIT II interventions. According to the USDA nutrient database, canola oil is a source of the plant sterols and stanols stigmasterol, (3 mg/100 g), campesterol (241 mg/100 g), β-sitosterol (413 mg/100 g), β-sitostanol (0.925 mg/100 g), campestanol (0.811 mg/100 g) and

Δ-5 avenasterol (11.721 mg/100 g) (36). Phytosterols have a dose response LDL-C-lowering effect (81) and exert their effects through multiple mechanisms (19, 82). Previous studies designed to assess the effects of replacement of SFA with vegetable-based sources of USFA on lipid and lipoprotein parameters have been confounded by differences in these plant-derived compounds (82). We utilized the Katan Calculator to predict differences in blood lipids and lipoproteins following replacement of the COMIT II control diet with the canola oil and HOCO diets, and compared these values to the observed differences between mean endpoints in *Chapter 3* (**Table 4-5**). The near equivalent values between predicted and observed support that the fatty acids, rather than phytosterol content, were the primary nutritional component differing between the diets underlying the lipid and lipoprotein response, as this predictive equation was populated with only the COMIT II fatty acid compositions. Moreover, an ancillary study of COMIT II assessed sterol and stanol markers of cholesterol absorption (i.e., cholestanol/cholesterol, campesterol/total cholesterol, and sitosterol/cholesterol) and synthesis (i.e., lathosterol/cholesterol, desmosterol/cholesterol) and did not demonstrate a consistent pattern of plant sterol regulation that might explain the lipid-lowering results (*data not published, MS thesis of COMIT II student Xiang Chen*).

Table 4-5. Predicted versus observed differences in lipid and lipoprotein parameters after replacement of the control diet with the canola oil or HOCO diet.

Outcome	Predicted changes following replacement of Control Oil [1]		Actual differences between Canola Oil or HOCO and Control Oil [2]	
	Canola Oil	HOCO	Canola Oil	HOCO
Total cholesterol (mmol/L)	-0.23	-0.20	-0.26	-0.19
LDL-C (mmol/L)	-0.23	-0.21	-0.25	-0.22
TC: HDL-C (ratio)	-0.17	-0.15	-0.17	-0.20
apo B (g/L)	-0.04	-0.03	-0.04	-0.04
HDL-C (mmol/L)	-0.01	-0.01	-0.01	0.02
Triglycerides (mmol/L)	0.01	0.04	0.02	0.03
apo A1 (g/L)	0.00	0.01	-0.01	0.02

[1] Determined using the Katan calculator (125).
[2] Values calculated as: (endpoint mean of canola or HOCO) – (endpoint mean of control)

Abbreviations: apo, apolipoprotein; HDL-C, high-density lipoprotein-cholesterol; HOCO, high-oleic acid canola oil; LDL-C, low-density lipoprotein-cholesterol; TC, total cholesterol.

A strength of this study is the use of PAXgene Blood RNA Tubes that contain a RNA stabilization reagent that provides an accurate snapshot of the intracellular RNA at the time point of blood collection. We isolated RNA from PBMC and assessed gene expression to assess the effects of canola oil, HOCO, and the Western diet control oil from a mechanistic perspective. Ideally, adipose, muscle, or liver biopsies from study participants are the best measure of tissue-specific metabolic shifts in response to diet, though these methodologies are invasive, elevate participant burden, and, in the case of liver, are arguably impossible. PBMC are a systemically circulating class of cells that interact with peripheral tissues and are easily and repeatedly accessible in human subjects; thus, it has been proposed that these cells may be a good surrogate marker of the metabolic profile of adipose, endothelium, and hepatic tissue (80). PBMC are currently used as a tool in dietary intervention studies with nutrigenomic research questions (80),

as well as in gene expression pattern profiling of various physiological states such as chronic metabolic disease (126) and fasting (122). PBMC and whole blood transcript levels of various lipid metabolism genes have been shown to correlate with WAT and liver expression levels in rat and mouse models (127, 128). Furthermore, comparison of transcript levels in mononuclear leukocytes and liver biopsy samples from humans (n=13) who underwent a cholecystectomy for gallstones showed strong correlations between LDLR (r=0.89) and HMG-CoAR (r=0.83) mRNA levels in these two compartments (129). However, because PBMC are a mixture of white blood cells, primarily lymphocytes and also monocytes, gene expression response may vary according to proportion of cell type within the total PBMC sample.

Determination of gene expression profiles does not provide insight into protein level or functional significance, as described above. Future research would benefit by investigating the COMIT II serum efflux capacity, including whole and apo B-depleted serum, in global and ABCA1-specific assays. This would address if there are clinically significant implications of the reported ABCA1 and ABCG1 mRNA reductions following canola oils, as well as identify the proportion of efflux subscribed to various cholesterol acceptors. Future research should assess associations between gene expression and circulating plasma fatty acids to provide insight into which specific fatty acid species may be influencing gene expression.

In summary, 6 weeks of canola oil- and HOCO-based diets reduced expression of LXR-regulated ABCA1 and ABCG1 genes compared to a control diet with a Western diet fatty acid profile. The diets failed to exert statistically significant effects on the remaining lipid- and lipoprotein-related genes. The specific mechanisms by which canola and HOCO lower apo-B containing lipoproteins cannot be explained by our PBMC gene expression data and future research is needed.

Chapter 5: Effects of diets that vary in fatty acid composition on fecal short-chain fatty acid levels and their relationship with blood lipids and lipoproteins

Abstract

Background: The SCFA acetic acid, propionic acid, and butyric acid are microbial-produced metabolites that can influence host physiology through regulation of hepatic cholesterol metabolism. These biologically relevant gut metabolites may play a role in the hypocholesterolemic effects of select dietary components. We previously reported that diets containing canola oil and HOCO improved circulating lipids, lipoproteins, and apolipoproteins compared to a macronutrient-matched diet with a Western-like fatty acid profile. The biological mechanisms driving this cholesterol-lowering response are unknown.

Objective: The objective of this exploratory study was to determine the effects of three controlled feeding test diets that differ only in fatty acid composition on fecal SCFA levels, and to assess the correlations between endpoint fecal SCFA levels and endpoint circulating lipids, lipoproteins, and apolipoproteins.

Design: In a double-blind, randomized, controlled, three period crossover, controlled feeding clinical trial, participants with ≥2 MetS measures (n=20) were provided with an isocaloric, weight maintenance diet plus conventional canola oil, HOCO, or a control oil (control diet formulated to represent a Western diet fatty acid profile) for 6 weeks followed by washout periods of ≥4 weeks. Fecal samples were collected at the study enrollment and the end of each diet period. SCFA were extracted with ethyl acetate and quantified by gas chromatography-mass spectrometry. Blood was collected at the same time points for analysis of circulating lipids, lipoproteins, and apolipoproteins.

Results: After 6 weeks, a trend toward a treatment effect on endpoint fecal propionic acid was observed (*P*=0.09), with a trend toward a higher concentration following the control compared to the canola diet (*P*=0.09). Acetic acid was increased from baseline following the control diet (*P*=0.04). After the control diet only, fecal levels of propionic acid were positively correlated with blood levels of LDL-C, non-HDL-C, and apo B (r=0.52 to 0.64, *P*=0.003 to 0.02), with a trend in total cholesterol (r=0.39, *P*=0.10), and acetic acid was positively correlated with LDL-C and apo B (r=0.48 to 0.49, *P*=0.03 to 0.04), with a trend in non-HDL-C (r=0.44, *P*=0.06). No significant correlations between fecal SCFA and lipids and lipoproteins were observed after the two canola-based diets.

Conclusion: These data suggest that the adverse effects of a contemporary Western diet fatty acid profile (i.e., higher-SFA/lower-USFA) on circulating lipid and lipoprotein parameters compared to diets higher in USFA/lower in SFA may be mediated by gut-derived SCFA. Future research is warranted to confirm these exploratory findings.

Introduction

The role of the intestinal microbiota in human health and disease has garnered substantial attention as of late, particularly the relationship between the gut and pathogenesis of cardiometabolic disease (130). Resident gut microbial composition is shaped by a variety of environmental factors, including diet, that, in turn, alter the production of gut microbial metabolites and influence host physiology. SCFA are a by-product of microbial carbohydrate fermentation and are absorbed across the intestinal epithelium, travel through the hepatic portal vein to the liver, and modulate host energy metabolism (85). SCFA can regulate hepatic cholesterol metabolism to lower blood cholesterol through a myriad of pathways, including suppression of cholesterol biosynthesis (86-89) and promotion of bile acid synthesis (86, 131) and cholesterol uptake (131). Thus, these biologically relevant metabolites may play a role, at least in part, in the hypocholesterolemic effects of select dietary components.

Human studies that assess the effects of macronutrients on the gut microbiota underscore the role of carbohydrate and protein interventions (130). Although limited in number, clinical trials of dietary fat interventions provide evidence to suggest that the fatty acid profile can influence the gut microbial composition across the lifespan and in various disease states (96, 132-137). However, only three studies have assessed the effects of dietary fat interventions on the biologically relevant SCFA, none of which maintained macronutrient composition across interventions preventing determination of the effects of dietary fatty acids (95, 96, 138). SCFA concentrations can be measured in the excreted fecal sample to provide a snapshot of the processes within the lower intestine. Although there is an established relationship between SCFA and cholesterol lowering, there is a paucity of human trials that investigated the associations between fecal SCFA and circulating lipids/lipoproteins in dietary intervention studies (97, 98).

We previously reported that canola oil diets improve atherogenic lipid and lipoprotein parameters compared to a macronutrient-matched control diet with a Western-like fatty acid profile (*Chapter 3*), though the underlying mechanisms of this response are unknown. Because dietary fat can alter the gut microbial composition, which therefore modulates gut microbial metabolites, it is possible that microbial-derived SCFA may mediate the effects of dietary fatty acids on lipid parameters. Therefore, we conducted an exploratory analysis to: 1) determine the effects of the three COMIT II diets (i.e., canola oil diet, HOCO diet, and control oil diet with a Western diet fatty acid composition) on fecal SCFA levels, and 2) assess the associations between fecal SCFA levels and circulating lipid and lipoprotein CVD biomarkers. We hypothesize that dietary fat composition will differentially affect fecal SCFA levels, with significant associations between endpoint SCFA and circulating endpoint total cholesterol, LDL-C, non-HDL-C, and apoB.

Methods

Study design and interventions

COMIT II was a multi-site, double-blind, randomized, controlled, three period crossover, controlled feeding clinical trial in which participants were provided with an isocaloric, weight maintenance diet plus conventional canola oil, HOCO, or a control oil blend (control diet formulated to represent a Western diet fatty acid profile) for 6 weeks followed by washout periods. Details of the COMIT II study design and interventions can be found in *Chapter 3*.

Sample collection

Fecal samples were collected at the study enrollment and at the end of each diet period (4 total) in a subset of the participants at the PSU site (n=20). These participants were identified during the informed consent process, voluntarily elected to provide samples, and received an additional $50 in study compensation.

Participants were provided with a fecal collection kit that included: a fecal hat, two pairs of laboratory gloves, a plastic spoon, a collection tube (comparable in dimensions to a prescription medicine bottle) with a spoon attached to the lid and labeled with the participant's study details (i.e. participant number, diet period, and visit), an opaque bag, a clear bag with the participant's study details, and a large plastic disposal bag (**Figure 5-1**). A small transport cooler with ice packs was also provided. Participants were instructed to place the fecal hat on the toilet seat and provide the sample, apply gloves and transfer the sample to the collection tube using the plastic spoon, screw the cap on the collection tube and place it in the opaque bag, place the opaque bag in the clear bag, place all used collection items into the large plastic bag, and dispose. Immediately following collection, participants were instructed to store the sample in their home freezer. The samples were brought to the clinic in the cooler with frozen ice packs the following day and stored at -80°C until analysis.

Fecal Sample Collection Instructions for Participants

At baseline and at the end of each diet period you will be provided with a collection kit which will include: a fecal sample collection device (a "stool hat"), large handled spoon, two pairs of disposable gloves, a sample container, 2 Ziploc bags, a cooler and ice packs. Please place the ice packs in your freezer as soon as possible so they will be frozen when transporting your sample.

At the time of sample collection:

1. Wash your hands.

2. Put on disposable gloves.

3. Lift the toilet seat. Align the "stool hat" with the rear of the toilet rim (see Photo A). Put the seat back down. Use the toilet as you normally would, letting the sample collect in the "hat".

4. Unscrew the cap from the collection vial. Using the large handled sterile spoon provided, remove enough fecal sample to fill approximately 2/3 of the collection container (Photo B). You may use the small spoon attached to the end of the cap to assist in removing the sample from the sterile spoon. Do not collect any urine in the container. Screw the cap back onto the container. Make sure the cap is tightly closed. Wipe off container.

5. Dispose of used gloves, spoon and stool hat.

6. Wash hands and put on second pair of gloves.

7. Place the sample container into the black plastic Ziploc bag provided. Seal the bag and place in your **FREEZER immediately.**

8. On the day when you are returning sample, remove the black Ziploc bag from the freezer and place it into the clear plastic bag with your ID number on it. Seal the bag. Place one ice pack on the bottom of the cooler and surround the sample with the other ice packs; drop off as soon as possible.

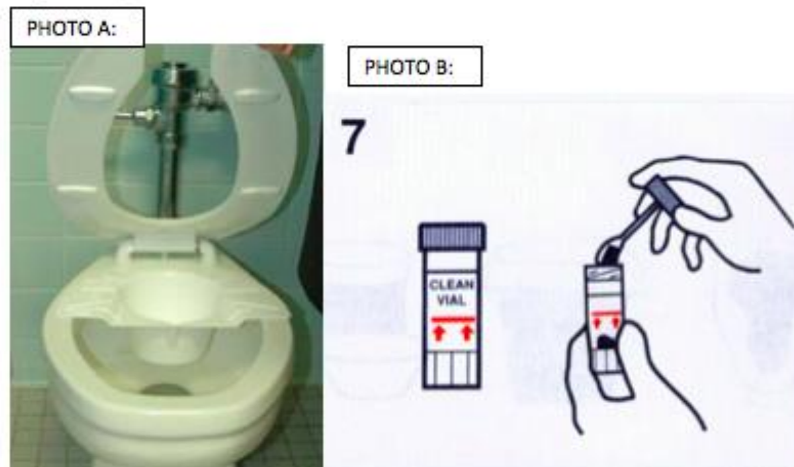


Figure 5-1. Fecal sample collection instructions provided to participants at study enrollment and the endpoint of each diet period.

Fecal sample SCFA extraction and quantification methodology

Fecal samples were processed individually to avoid cross-contamination. The collection containers were thawed on ice under the fume hood to allow for ventilation and, once thawed, were mixed with the plastic spoon attached to the container lid to homogenize the sample. Samples were weighed in 100-150 mg aliquots in sterile eppendorf tubes and stored at -80°C until analysis.

To determine the SCFA levels in the participant fecal samples, the following protocol was followed. Samples were thawed on ice and transferred to reinforced tubes. Approximately 1/3 of each tube was filled with 0.1 mm beads (Zirconia/silica beads, BioSpec). Samples were suspended with 1 mL of Millipore water with 0.5% phosphoric acid per 100 mg of sample, and homogenized using a bullet blender (Next Advance, Inc.; NY, USA) for 2 minutes at medium-high setting. The sample was vortexed for 1 minute at medium-high speed to re-homogenize the fecal suspensions and centrifuged for 10 minutes (17,000 x g; 4°C). The supernatant was collected (800 µL) and 800 µL of ethyl acetate solution was added (i.e., 1:1). The ethyl acetate solution contained the internal standard, heptanoic acid 1mM. Samples were vortexed for 2 minutes and centrifuged for 10 minutes at 17,000 x g to extract the fatty acids with ethyl acetate. A volume of 600 µL was transferred from the ethyl acetate phase into a gas chromatography (GC) vial. Samples were stored at -20°C until analysis.

To identify the concentration of each SCFA in the fecal samples, a calibration curve ranging from 3 to 30,000 µM was obtained by preparing a stock solution containing a mixture of the fatty acid standards in ethyl acetate with subsequent dilutions. The following protocol was used to generate the standards, standard mix, and serial dilutions. Each standard was added to a 15 mL falcon tube containing 10 mL of Millipore water with 0.5% phosphoric acid for a

concentration of 30 mM. The standards were added in the following volumes: acetic acid (17.16 µl), propionic acid (22.45 µl), isobutyric acid (26.51 µl), butyric acid (27.56 µl), 2- methylbutyric acid (32.73 µl), isovaleric (33.12 µl), valeric (32.95 µl), caproic (37.47 µl), and heptanoic acid (42.54 µl). Falcon tubes were vortexed for 1 minute. 800 uL was transferred into a 2 mL tube and 800 µL of ethyl acetate (with the heptanoic acid internal standard) was added. Standards were vortexed for 2 minutes and centrifuged for 10 minutes at 17,000 x g to extract the fatty acid with ethyl acetate. A volume of 600 µL was transferred from the ethyl acetate phase into a GC vial for each standard. Next, the standard mix was prepared by adding 200 uL from each SCFA GC vial into a single tube (the remaining volume in the vials was stored at -20°C until analysis). The ethyl acetate and heptanoic acid solution was added (400 uL) and serial dilutions were performed. 500 uL of the ethyl acetate and heptanoic acid solution was added to 6, 2 mL tubes and 500uL was transferred from the stock standard mix to tube 1 and vortexed; 500 uL was transferred from tube 1 to tube 2; etc. All dilutions (3, 1.5, 0.75, 0.375, 0.1875, 0.094 mM) were transferred in a volume of 600 µL to the GC vial and stored at -20°C.

Fecal SCFA levels were quantified by gas chromatography-mass spectrometry (GC-MS) analysis (Agilent 7890A GC coupled to Agilent 5975C MS). Samples, standards, and standard mix dilutions were injected in duplicate. The peak areas were normalized to the internal standard of heptanoic acid in each sample to correct for injection variability between samples and minor variability in the instrument response. The instrument output for each sample was analyzed manually using the generated standard curves from the standard mixes. Herein, we report acetic acid, butyric acid, and propionic acid, as well as the sum of the three SCFA, because they are the predominant SCFA in human feces (≥ 95%) (85).

Circulating lipid, lipoprotein, and apolipoprotein levels were assessed in blood samples. Blood was collected on two consecutive days at the study enrollment and the endpoint of each diet period following a 12 hour fast from food or drink (besides water) and 48 hour fast from alcohol. The methods to analyze the lipid, lipoprotein, and apolipoprotein levels are described in *Chapter 3*.

Statistical methods

Statistical analyses were performed using SAS 9.4 (SAS Institute Inc., Cary, NC, US). The primary analysis was endpoint-to-endpoint comparisons of SCFA (i.e., acetic acid, propionic acid, butyric acid, and the sum total of the three SCFA) between the three diets. A secondary analysis was performed to assess absolute change in SCFA from baseline within each diet. Data were analyzed per-protocol and missing data were not imputed. $P \leq 0.05$ was considered significant and $P \leq 0.10$ was considered a trend.

The normality of each dependent variable was assessed using the univariate procedure. The effects of the diets on SCFA were investigated using a repeated measures mixed model, with subject and treatment sequence as random effects and visit as the repeated effect. The model included the main effect of diet (canola oil diet, HOCO diet, control oil diet) with the study baseline value of the dependent variable as a covariate. Tukey-Kramer adjusted *P*-values were used for multiple pairwise comparisons between treatments. Normality of the residuals from the final models was confirmed. Change from baseline within a treatment was assessed by the least squares means *P*-values from the mixed model output.

Pearson correlation coefficients were used to for associations between endpoint SCFA

and endpoint lipids, lipoproteins, and apolipoproteins, stratified by diet. Total cholesterol, LDL-C, non-HDL-C, and apo B were the endpoints of interest because of the significant treatment effect on these endpoints in *Chapter 3*. The normality of each variable was confirmed using the univariate procedure. A general linear model procedure was used to test whether the slopes of the regression lines were equal across diets. There was a significant interaction between SCFA and diet for all dependent lipid, lipoprotein, and apolipoprotein variables; thus, the null of hypothesis of equal slopes was rejected and pooled correlations could not be utilized. The correlation coefficients are presented by diet. Due to the exploratory nature of this study, we did not adjust for multiple comparisons.

Results

Baseline characteristics

The baseline characteristics of the participants included in the fecal sample analyses (n=20) are present in **Table 5-1**. Overall, the sample was similar to the full COMIT II sample (*see Chapter 3, Table 3-3*) with a few exceptions; this subgroup had a higher percentage of males (55%), was on average younger, and 25% of the subgroup met the criteria for MetS (i.e., ≥ 3 MetS measures) in contrast to approximately 40% of the full sample.

Table 5-1. Baseline characteristics of the COMIT II participants included in the fecal sample analyses (n=20). [1]

Variable	Value [2]
Sex (female)	9, 45%
Anthropometric measures	
Age (years)	34.05 ± 9.63 (24.00 – 57.00)
Weight (kg) [3]	94.61 ± 18.77 (66.35 – 146.35)
BMI (kg/m^2)	30.57 ± 4.52 (24.34 – 42.11)
MetS criteria	
Waist circumference (cm)	103.36 ± 8.83 (87.25 – 125.00)
Female	101.08 ± 7.73 (87.25 – 114.00)
Male	105.23 ± 9.59 (94.30 – 125.00)
Triglycerides (mmol/L)	1.55 ± 0.82 (0.49 – 3.67)
HDL-C (mmol/L)	1.26 ± 0.33 (0.74 – 2.00)
Female	1.42 ± 0.29 (1.17 – 2.00)
Male	1.12 ± 0.31 (0.74 – 1.83)
Glucose (mmol/L)	5.15 ± 0.43 (4.43 – 6.37)
Blood pressure (mmHg)	
Systolic blood pressure	118.93 ± 13.39 (98.67 – 141.33)
Diastolic blood pressure	81.57 ± 9.25 (68.00 – 96.00)
Number of MetS Criteria [4]	
0	0, 0%
1	3, 15%
2	12, 60%
3	3, 15%
4	2, 10%
5	0, 0%
Additional cardiovascular risk biomarkers	
Total cholesterol (mmol/L) [4]	5.13 ± 1.04 (3.60 – 7.36)
LDL-C (mmol/L) [4]	3.17 ± 0.78 (1.88 – 4.65)

[1] Data collected on days 1 and 2 of diet period 1.
[2] Categorical variables are expressed as frequency, percent and continuous variables expressed as mean ± standard deviation (minimum – maximum).
[3] Weight was measured using a scale at each participating center (i.e., not DXA weight).
[4] Enrolled participants met the requirements of an increased waist circumference plus one additional factor at the screening visit; values present here are from the baseline visits of diet period 1.

Abbreviations: BMI, body mass index; HDL-C, high-density lipoprotein-cholesterol; LDL-C, low-density lipoprotein-cholesterol; metabolic syndrome, MetS

Treatment effects on fecal SCFA and correlation analyses

Fecal SCFA compositions per 100 mg of fecal sample were measured at baseline and endpoint (**Table 5-2**) and adjusted to the internal standard of heptatonic acid. There was a trend toward a diet effect on endpoint propionic acid (*P*=0.09), and Tukey's pairwise comparisons indicated a trend toward a higher concentration following control compared to canola (*P*=0.09; control vs. HOCO *P*=0.22). There was also a trend toward a treatment effect on endpoint butyric acid (*P*=0.10). Change score analyses indicate acetic acid was increased from baseline after the control diet (*P*=0.04), whereas butyric acid (P=0.89), propionic acid (P=0.16) and total SCFA (P=0.12) were not significantly changed from baseline following control.

Table 5-2. SCFA composition of fecal samples collected at study baseline and endpoint of each diet period (n=20). [1]

SCFA	Baseline	Endpoint			*P*-value	
		HOCO	Canola	Control	Diet	Baseline SCFA
Acetic acid	2099 ± 189	2256 ± 221	2236 ± 172	2647 ± 244 ¶	0.23	0.0007
Butyric acid	1543 ± 145	1702 ± 204	1307 ± 126	1656 ± 158	0.10*	0.009
Propionic acid	1148 ± 117	1126 ± 105	1078 ± 101	1362 ± 134	0.09*	0.001
Total SCFA [2]	4790 ± 394	5358 ± 426	4622 ± 337	5665 ± 495	0.17	0.006

[1] Values presented as mean ± SEM in µg/g of fecal sample. A mixed model was used to assess the main effect of diet on endpoint SCFA levels, using the baseline value for each respective SCFA as a covariate.
[2] Sum of acetic acid, butyric acid, and propionic acid.
* indicates a trend toward significant main effect of diet, *P*≤0.10.
¶ indicates significant change from baseline, *P*≤0.05.

Abbreviations: HOCO, high-oleic acid canola oil; SCFA, short-chain fatty acid.

Correlations coefficients between endpoint circulating lipids and lipoproteins and endpoint fecal SCFA are presented in **Table 5-3** and stratified by the three diets. LDL-C, total cholesterol, non-HDL-C, and apo B were the outcomes with a significant treatment effect in *Chapter 3* and, thus, were selected for correlation analyses. There were no significant correlations between lipids/lipoproteins and SCFA for the HOCO and canola oil diets. After the control oil diet, LDL-C and apo B were positively correlated with acetic acid, propionic acid, and total SCFA ($P \leq 0.05$ for all). In addition, non-HDL-C was correlated with propionic acid ($P=0.01$), with a trend toward a correlation with acetic acid ($P=0.06$) and total SCFA ($P=0.07$). There was a trend toward a correlation between total cholesterol and propionic acid ($P=0.10$) after the control diet.

Table 5-3. Pearson correlation coefficients between endpoint circulating lipids and lipoproteins with endpoint fecal SCFA levels, stratified by each diet.

	HOCO		Canola		Control	
	r_P	*P*-value	r_P	*P*-value	r_P	*P*-value
LDL-C						
Acetic acid	-0.33	0.16	0.04	0.86	0.48**	0.04
Butyric acid	-0.01	0.97	-0.07	0.76	0.27	0.27
Propionic acid	-0.20	0.38	0.30	0.20	0.52**	0.02
Total SCFA	-0.16	0.54	0.08	0.72	0.46**	0.05
Total cholesterol						
Acetic acid	-0.36	0.13	0.02	0.92	0.33	0.16
Butyric acid	-0.09	0.72	-0.20	0.40	0.14	0.57
Propionic acid	-0.22	0.34	0.19	0.43	0.39*	0.10
Total SCFA	-0.20	0.42	-0.01	0.98	0.31	0.12
Non-HDL-C						
Acetic acid	-0.30	0.20	-0.02	0.92	0.44*	0.06
Butyric acid	-0.04	0.88	-0.18	0.46	0.18	0.46
Propionic acid	-0.06	0.81	0.32	0.17	0.56**	0.01
Total SCFA	-0.17	0.50	0.02	0.94	0.42*	0.07
apo B						
Acetic acid	-0.28	0.25	-0.05	0.83	0.49**	0.03
Butyric acid	-0.05	0.84	-0.16	0.49	0.22	0.36
Propionic acid	-0.07	0.77	0.27	0.25	0.64**	0.003
Total SCFA	-0.17	0.51	-0.01	0.98	0.48**	0.04

* indicates a trend towards significance, $P \leq 0.10$.
** indicates significance, $P \leq 0.05$.

Abbreviations: apo, apolipoprotein; HDL-C, high-density lipoprotein-cholesterol; HOCO, high-oleic acid canola oil; LDL-C, low-density lipoprotein-cholesterol; SCFA, short-chain fatty acid.

Discussion

In this exploratory analysis, we investigated the effects of 6 weeks of a canola oil diet, a HOCO diet, and a control diet with a Western-like fatty acid profile on fecal SCFA, and assessed the associations between these fecal SCFA and blood lipids and lipoproteins. The fecal and blood specimens were collected in a subset of participants (n=20) from the COMIT II study, a randomized, controlled, three period crossover, double blind, controlled feeding clinical trial. There was a trend toward a treatment effect on endpoint fecal propionic acid (P=0.09), and acetic acid was increased from baseline after the control diet (P=0.04). Correlations between endpoint circulating lipids/lipoproteins and endpoint fecal SCFA were observed following the control diet; propionic acid was positively correlated with LDL-C, non-HDL-C, and apo B, with a trend in total cholesterol. Moreover, LDL-C and apo B were positively correlated with acetic acid and total SCFA, with a trend in non-HDL-C. These data suggest the possibility that the effects of a Western diet fatty acid profile (i.e., higher-SFA/lower-USFA) on circulating lipid and lipoprotein parameters may involve modulation of the gut environment.

To our knowledge, this is the first clinical trial to assess the effects of diets that have a constant macronutrient profile and differ only in fatty acid composition on levels of SCFA in excreted fecal samples. Our finding of a trend toward higher endpoint propionic acid in the higher-SFA control diet compared to the lower-SFA canola oil diet matched for other macronutrients suggests independent effects of fat quality on the gut environment. Previous trials with dietary fat manipulation report null effects on endpoint fecal SCFA between a high-SFA diet, high-MUFA/low-glycemic diet, high-MUFA/high-glycemic, high-carb/low-glycemic diet, or high-carb/high-glycemic diet after 24 weeks (96), as well as no differences between an energy-restricted high-fat/low-carb diet (58% and 5% of total energy) and an energy-restricted

low-fat/high-carb (28% and 46% of total energy) after 8 weeks (95). In contrast, Duncan et al. reported lower endpoint butyrate after a higher-fat/lower-carb diet (125 g/ 24 g) compared a lower-fat/higher-carb (74 g/ 164 g) diet, and concluded the results were a function of carbohydrate intake (138). We cannot determine the independent effects of dietary fat on SCFA from these trials because the simultaneous shifts in various nutrients confound the result interpretation.

We also report increased acetic acid from baseline following the COMIT II higher-SFA control diet. Similarly, Fava et al. reported increased fecal acetic acid from baseline following 24 weeks of a high-SFA diet (SFA 17.5% of total energy; food exchange model) in adults aged 30-65 years with at least two MetS measures (n=11) (96). The result of elevated fecal SCFA content following higher-SFA diets may be due to increased microbial production of SCFA or decreased intestinal absorption, with evidence to support the latter. A SCFA rectal infusion study in humans reported that fecal acetate content was inversely related to percent of absorption in the rectum and distal colon, suggesting that fecal acetate content may reflect absorption (139). In contrast, Brinkworth et al. reported reductions from baseline in fecal SCFA after 8 weeks of an energy-restricted very high-fat/low-carb diet, including acetate and butyrate, but not propionate (95). Notably, the reductions in fecal SCFA were attributed to the substantially reduced dietary substrate available for microbial colonic fermentation on the high-fat/low-carb diet (i.e., fermentable carbohydrate). Thus, the interpretation of the SCFA content within human fecal samples may be dependent on the known modulatory effects of the diet interventions on microbes.

Interestingly, we found that both fecal acetic and propionic acids were highly, positively correlated with circulating atherogenic lipids and lipoproteins only after the control oil diet. We

were not surprised to see no significant relationship between lipids/lipoproteins and butyric acid because the majority of this SCFA is consumed by colonocytes for energy, rather than absorbed into the hepatic portal vein for regulation of metabolism (140). Our findings are directionally opposite of associations reported in probiotic and fiber interventional trials. Lee et al. investigated the effects of *Bifidobacterium animals* subsp. Lactis BB12 on circulating lipids/lipoproteins and fecal SCFA in healthy adults (n=30) with a decreased a transit time between bowel movements (98). In a randomized, crossover design, participants consumed a yogurt smoothie with no BB-12, a yogurt smoothie with BB-12 added pre-fermentation, a yogurt smoothie with BB-12 added post-fermentation, or BB-12 in a capsule for 4 weeks with 2 week washouts. Associations between lipid parameters and fecal SCFA showed significant negative correlations between total cholesterol, LDL-C, and non-HDL-C with total SCFA, acetate, propionate, and butyrate. Consistent with these findings, Fechner et al. compared a higher fiber diet with citrus fiber, a high-fiber diet with lupin fiber, and a low-fiber diet for 4 weeks in a crossover design and reported a negative correlation in change in circulating LDL-C and change in fecal propionate (r_s=-0.45, n=156, $p<0.001$) and acetate (r_s=-0.44, n=156, $p<0.001$) (97). However, as described above, it is difficult to compare our findings to these studies because of the major intervention differences. Fecal excretion rates of SCFA are higher following high- versus low-fiber diets (141). Fibers and probiotics may increase fecal SCFA content as a result of elevated substrate and/or fermenter within the gut environment increasing the total SCFA pool, leading to both increased absorption for beneficial cholesterol effects and increased loss given the greater pool. Future research is warranted to determine intervention-specific influences on fecal SCFA levels.

Nonetheless, significant correlations between SCFA and lipids are intriguing, as numerous lines of evidence indicate that SCFA regulate hepatic cholesterol metabolism to lower plasma cholesterol levels (85). In rodent models, intake of SCFA lowers circulating cholesterol and the hepatic cholesterol pool, with arguably more evidence of a cholesterol-lowering effect following acetic and propionic acids (86, 87, 90, 91, 131). Evidence from human studies indicates a beneficial lipid-modifying effect of SCFA supplementation, including total cholesterol lowering from baseline after 12 weeks of various acetic acid dosages in vinegar (92), as well as total cholesterol and LDL-C lowering from baseline after 24 weeks of propionate ester (94). The mechanisms underlying this response are multifactorial and include regulation of hepatic cholesterol biosynthesis through decreased cholesterol synthesis rates (87, 88), down-regulation of genes within the cholesterol biosynthesis pathway such as HMG-CoA reductase and synthase (89), reduced concentrations of biosynthetic intermediates (86), decreased enzyme activity (86), as well as regulation of cholesterol removal through promotion of conversion to bile acids (86). Recent insights into the plasma-lowering response suggest increased conversion of cholesterol to bile acids to eliminate excess cholesterol and increase cholesterol uptake from circulation, rather than modulation of cholesterol biosynthesis (131). After 6 weeks of feeding in hypercholesterolemic Golden hamsters, a basal high-cholesterol diet plus acetate, propionate, or butyrate (dose mimicking 10% energy in humans) lowered plasma total cholesterol and non-HDL-C and increased fecal bile acid excretion compared to the basal high-cholesterol diet. Further, hepatic gene expression indicated upregulation of genes involved in hepatic cholesterol uptake (i.e., SREBP2, LDLR) and bioconversion of cholesterol to bile acids (i.e., CYP7A1), with verification of increased protein levels. No effect of the SCFA of HMG-CoA protein or intestinal

cholesterol absorption was observed (131). Although discrepancies remain in the precise mechanisms, the data conclusively indicate that SCFA lower circulating cholesterol.

Because our diets differ only in fatty acid composition, we hypothesize that the increased SFA content of the control diet (or decreased USFA content) alters the gut environment such that less microbial-produced SCFA are absorbed and, thus, more are lost in the feces. This decreased absorption results in less SCFA, particularly acetate and propionate, available for regulation of hepatic cholesterol metabolism, including involvement in suppression of biosynthesis and/or promotion of uptake and excretion. This hypothesis might explain the strong positive correlations between fecal SCFA and lipid/lipoprotein parameters following the higher-SFA diet only. Although intriguing, we underscore that this is speculative and future work is necessary to corroborate our findings and elucidate the details of this proposed pathway.

Limitations of this study include a lack of information on SCFA flux, including the interaction between diet-induced changes in microbial production, colonocyte absorption, and fecal excretion. Assessment of solely fecal SCFA levels provides an incomplete assessment of the gut environment; approximately 95% of all SCFA produced by microbes within the large intestine are absorbed by colonocytes, leaving only 5% in the feces (85). However, this is an overarching drawback of the human fecal microbial metabolite field and not unique to this study. While we were able to easily access fecal samples to measure excreted SCFA levels, future human studies should also consider concomitant use of rectal dialysis bags to measure intestinal absorption. Rahat-Rozenbloom et al. utilized this technique to determine if the increased fecal SCFA concentration in obese versus lean adults was due to differences in intestinal absorption, and was able to conclude no differences in absorption, with higher fecal SCFA due to increased production from the presence of more efficient microbial fermenters in the obese phenotype

(142). Further, gut transit time can also influence SCFA composition and assessment of fecal consistency is a reasonable, low-burden measure (142). In addition, measurement of fecal bile acids is of interest to validate findings in animal models to elucidate the proposed cholesterol-lowering pathway in humans (131). Future studies designed to assess the effects of dietary fatty acids on fecal SCFA and their relationship to lipid parameters should incorporate the aforementioned measures into the study protocol.

Limitations in fecal processing and participant collection are as follows. The distribution of SCFA throughout a fecal sample is highly variable, with a greater concentration on the inside compared to the perimeter, likely due to increased exposure of the periphery to the intestinal epithelial cells for absorption and/or greater evaporation of the volatile fatty acids (143). Thus, to reduce the error of variability by sampling region, we homogenized the fecal sample before weighing for analyses. Although antibiotics can modulate the type and composition of fecal SCFA (144), we did not remove participants who took antibiotics during any time point in the study because of the small sample size. All samples once returned to the Clinical Research Center were immediately frozen at -80°C. While participants were provided with detailed instructions for sample collection, participant non-compliance with an extended period from defecation to freezing of the sample in the at-home freezer cannot be ruled out. Because extended duration at room temperature can alter the fecal SCFA composition, future studies should require the participants to immediately treat the samples with 70% ethanol for SCFA stability (145).

In summary, in this exploratory study, we investigated the effects of a canola oil diet, a HOCO diet, and a control diet with a Western-like fatty acid profile on endpoint fecal SCFA, and assessed the relationship between these microbial metabolites and blood lipids and lipoproteins.

We report a trend toward a treatment effect on endpoint fecal propionic acid following the control diet compared to the canola oil diet, and an increase in acetic acid from baseline after the control diet. Correlation analyses showed positive correlations between fecal SCFA and blood lipids and lipoproteins following the control diet only, particularly propionic acid and acetic acid with LDL-C and apo B. These data indicate a relationship between microbial-derived SCFA and circulating lipids and lipoproteins following a higher-SFA/lower-USFA diet, although we cannot conclude causation from our data. Future work is necessary to confirm our findings in a larger cohort and should manipulate only dietary fatty acids to identify their unique effects.

Chapter 6: Summary, limitations, and future directions

6.1. Summary of results

This dissertation adds novel data to the existing dietary oil and cardiometabolic disease prevention literature by employing a scholarly approach to simultaneously investigate diet-induced outcomes and explore mechanistic underpinnings in human participants. The results are from COMIT II, a multi-site, randomized, double blind, controlled, 3 period crossover, controlled feeding clinical trial that was designed to evaluate the effects of canola and high oleic canola oil on cardiometabolic risk factors, including lipids, lipoproteins and apolipoproteins which were the focus of my dissertation. Participants were fed an isocaloric, macronutrient matched (approximately 15% protein, 50% carbohydrate, 35% fat), weight maintenance diet for 6 weeks containing conventional canola oil, oleic acid-enriched canola oil, or an oil blend (49% butter oil/ghee, 29% safflower oil, 14% flaxseed oil, 8% coconut oil). The oils provided 18% of total energy in the diet (60 g per 3000 kcal) and the fatty acid profiles of the diets were as follows: canola oil diet 17% MUFA, 9% PUFA, 7% SFA; HOCO diet 19% MUFA, 7% PUFA, 6% SFA; control diet 11% MUFA, 10% PUFA, 12% SFA. The control diet was designed to emulate the fatty acid profile of contemporary Western intakes. The participants had an increased waist circumference with ≥ 1 additional MetS criteria.

In Objective 1, we assessed the effects of three test diets on circulating lipid, lipoprotein, and apolipoprotein CVD biomarkers. Although it is well established that conventional canola oil confers benefit on the atherogenic lipid and lipoprotein profile, evidence of the cardiovascular impacts of oleic acid-enriched canola oil are sparse. Because HOCO has displaced PHVO, intakes of this oil and other high-MUFA oils are widespread; therefore, it was necessary to determine non-inferiority of HOCO to canola oil and superiority of HOCO to a higher SFA,

lower USFA diet. The primary findings were 6 weeks of canola oil- and HOCO-containing diets similarly lowered endpoint total cholesterol, LDL-C, non-HDL-C, and apo B compared to the control diet. These data indicate that HOCO, with increased MUFA at the expense of decreased PUFA, elicits beneficial effects on the CVD lipid profile equivalent to canola oil and greater than a higher SFA, lower USFA Western-like diet.

In Objectives 2 and 3, we aimed to elucidate the biological mechanisms in humans that underlie the atherogenic lipid- and lipoprotein-lowering response when canola oils as sources of USFA replace sources of SFA in the diet. In Objective 2, we assessed the expression of 17 genes that regulate lipid and lipoprotein metabolism in circulating PBMC collected at the end of each test diet. These genes are involved in a myriad of pathways including cholesterol and phospholipid efflux, lipid core transfer between lipoproteins, cholesterol catabolism and bile acid synthesis, lipid biosynthesis, lipoprotein uptake, phospholipid and triglyceride lipases, and sterol-regulated transcription factors. After 6 weeks, the canola oil- and HOCO-based diets down-regulated expression of the ABCA1 and ABCG1 genes compared to the Western diet control, with no treatment effects on the remaining genes. These data provide evidence of coordinated shifts in cholesterol and phospholipid homeostasis in PBMC in response to diet, particularly regulation of genes implicated in sterol efflux. However, these data do not support dietary fatty acid regulation of expression of genes in PBMC involved in the atherogenic lipid-lowering response.

In Objective 3, we assessed the effects of the three diets on microbial-derived SCFA levels in excreted fecal samples. Because diet, including fat, can alter the intestinal microbial composition, we sought to explore if our diets modulated metabolites that influence host metabolism. A trend toward a treatment effect on fecal propionic acid was observed, and acetic

acid increased from baseline after the control diet. Propionic acid and acetic acid were positively associated with LDL-C and apo B in the control diet only. These data suggest that the gut environment may mediate the negative effects of a higher-SFA, lower-USFA diet on lipid and lipoprotein outcomes. Together, these three studies provide information to add to the existing knowledge of the role of dietary fatty acids in modulating circulating cholesterol levels and in the delicate balance of cholesterol absorption, biosynthesis, uptake, and excretion in humans.

6.2. Limitations and future directions

Limitations specific to each objective were described in detail in their respective chapters and will be summarized herein, with an emphasis on overall COMIT II limitations. A limitation of the COMIT II experimental design was that the oil blend in the control diet contained oils non-traditional to the Western diet. The aim of the investigators was to design a control diet that emulated the total percentage of calories from SFA, MUFA, and PUFA in the Western diet, and the oils used were required to achieve such targets. Some of the individual fatty acids in the control diet were not directly congruent with Western intake. However, approximately 50% of the control oil blend was butter-based (i.e., butter oil/ghee), a major source of animal fat in the Western diet, and only 8% was from coconut oil. Future research should incorporate fats and oils more representative of Western sources (i.e., corn oil, animal fats) when designing a control arm with a Western diet fatty acid profile.

Although HOCO and canola oil have unique fatty acid profiles when analyzed as independent oils, the COMIT II study design diluted assessment of the proportional fatty acid difference. The intervention oils provided approximately 50% of the total daily fat; thus, the

remaining 50% was provided by other fat sources (i.e., mayonnaise, salad dressing, dairy fat) in equivalent amounts across diets, resulting in very modest fatty acid differences between the canola oil diet and HOCO diet. The conclusion of a lipid and lipoprotein benefit of HOCO similar to canola oil and relative to control is in the context of 6 weeks of intake when incorporated as approximately 18% of total energy (60 g per 3,000 kcal). Treatment effects on lipids and lipoproteins following higher intakes of HOCO and canola oil (i.e., >18% of total energy) are unknown. A higher oil dosage is not within the DGA recommendations in a healthy US-style eating pattern (i.e., 2000 kcal, 27 g oil; 3000 kcal, 44 g oil) (18) and modeling exercises suggest risk of essential fatty acid deficiency following elevated intake of high-oleic acids oils (53). Thus, we cannot conclude the longer-term implications of high-oleic oil consumption or the effects of higher dosages, and future research should consider the potential adverse effects of overconsumption for pertinent dietary recommendations.

We reported modest reductions in body weight from baseline, with no differences in the magnitude of weight loss between the diets. Although designed as a weight-maintenance trial, mild weight loss is common in controlled feeding trials if participants have an unhealthy habitual diet prior to study enrollment. To thwart the effects of weight loss, participants with a weight change of >5% during any diet period were excluded from analyses. The average <1 kg loss over 6 weeks may have contributed to the reduction from baseline in lipid parameters in the control arm in Objective 1. Alternatively, the PUFA content of the control diet was similar to that of the canola diet because the diets were designed based on the primary outcome with a MUFA-driven hypothesis. Given the established beneficial effects of n-6 PUFA on lipids and lipoproteins, this may have also played a role in the beneficial change from baseline following the control diet. Nonetheless, it should be underscored that the lipid/lipoprotein benefit was greatest following the

two canola-oil based diets. Although we report no differences between the canola oil diets in endpoint total cholesterol, LDL-C, apo B, or non-HDL-C in Objective 1, we were not powered to detect an effect between these two diets. The difference in endpoint LDL-C between the canola diets was approximately 1% (LSM difference 0.03 mmol/L, SD 0.44); the actual differences across lipid and lipoprotein endpoints were equivalent to those calculated in fatty acid-based predictive equations. Post-hoc power calculations indicate a sample size of 1,690 is necessary to detect a 1% difference in mean endpoint LDL-C, with 80% power and alpha=0.05.

In our assessment of gene expression in Objective 2, we are only able to draw conclusions regarding PBMC gene expression. Although there are numerous lines of evidence to support that PBMC serve as a surrogate for assessment of hepatic gene expression, we cannot state unequivocally that expression shifts do not occur in the liver following diets with varied fatty acid compositions. Further, states of fasting can substantially alter the gene expression environment (122) and all blood was drawn following a 12-hour minimum fast. We also cannot infer from our mRNA data information on translation to protein, post-translational modifications, or functional significance, and regulation may not be at the transcriptional level. Future studies should assess cholesterol efflux capacity in the COMIT II serum to determine if there are clinical implications of attenuated ABCA1 and ABCG1 expression after canola oil-based diets. Our mRNA analyses only included endpoint data and future studies should also assess expression at enrollment in order to co-vary for the baseline value in statistical models.

A limitation of Objective 3 is assessment of fecal SCFA levels provides an incomplete assessment of the gut environment, without information on microbial production or colonocyte absorption of SCFA. Future studies should include concomitant measures to determine overall SCFA flux in response to diet. Our study was the first to assess the effects of dietary fatty acid

quality on fecal SCFA levels while maintaining macronutrient composition; confirmatory studies are necessary. Because this study was hypothesis generating in nature, we did not adjust for multiple assessments in our correlation analyses and significant correlations in some instances may have been by chance. In addition, SCFA measurement was a post-hoc analysis and fecal samples were not collected with this in mind. Future studies should limit variability in participant fecal sample collection and storage.

Although beyond the scope of this dissertation, there remains a dearth of research on the effects of canola oil on cardiovascular events (i.e., non-fatal myocardial infarction or stroke, cardiovascular mortality). Given the beneficial effects of extra-virgin olive oil on primary prevention in the PREDIMED trial (58), future cardiovascular incidence trials should assess incorporation of canola oil into a Mediterranean-style dietary pattern for prolonged periods. Demonstrable benefit of canola oil would likely increase North American acceptability of the dietary pattern because of the oil's neutral flavor profile. Future ad libitum study designs should be utilized to determine whether oleic acid-enriched diets regulate appetite and the role of these oils in weight reduction in overweight participants.

In conclusion, this dissertation research incorporates numerous scientific domains, including assessment of intermediate clinical biomarkers, intracellular gene expression, and fecal microbial metabolites, to comprehensively address pertinent nutritional science research questions with implications for intake recommendations and CVD prevention. We are indisputably limited in our ability to assess biological mechanisms in response to dietary interventions in humans, but our findings described herein provide data to begin to uncover the profound effects of dietary fat quality on human physiology. Research is warranted to further delineate the role of dietary fatty acids in modulating cholesterol homeostasis in humans.

Appendix

A. Informed consent form

CONSENT FOR RESEARCH
The Pennsylvania State University

Title of Project: Canola Oil Multi-center Intervention Trial II (COMIT II): Effects of Oleic Acid Enriched and Regular Canola Oil on Body Composition and Lipid Metabolism in Participants with Metabolic Syndrome Risk Factors

Principal Investigator: Penny Kris-Etherton, PhD
Address: 319 Chandlee Lab
Telephone Number: 814-863-8056

Subject's Printed Name: ____________________________

We are asking you to be in a research study. This form gives you information about the research.

Whether or not you take part is up to you. You can choose not to take part. You can agree to take part and later change your mind. Your decision will not be held against you.

Please ask questions about anything that is unclear to you and take your time to make your choice.

1. Why is this research study being done?

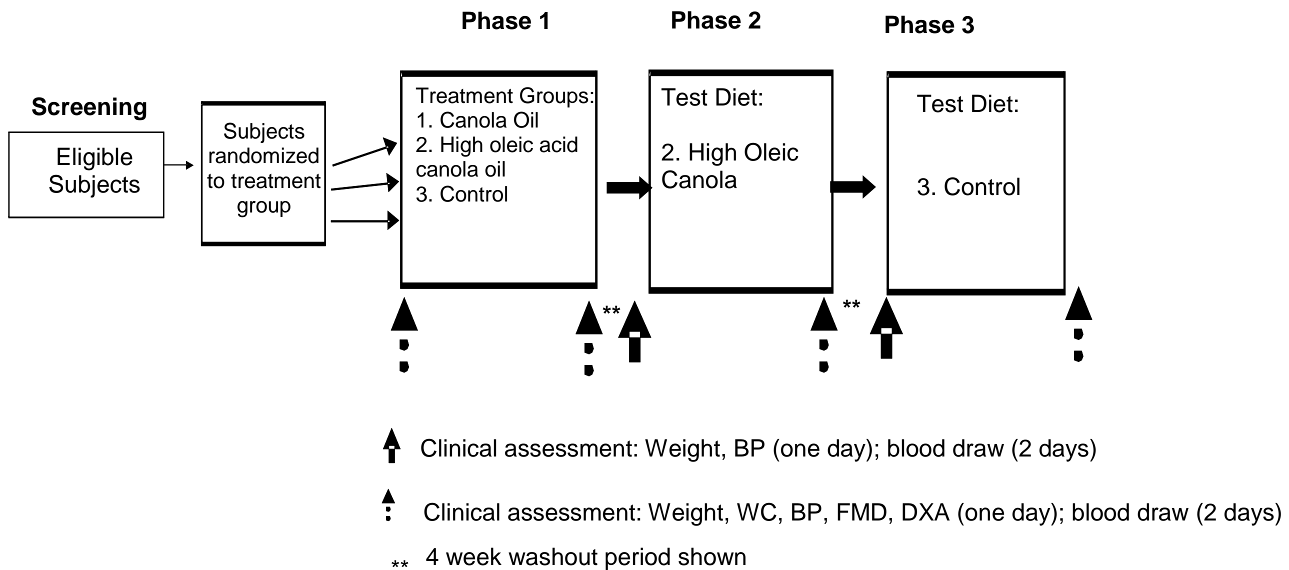
We are asking you to participate in this research study to examine how the consumption of different dietary oil varieties affects a broad range of metabolic responses that are important in the development of cardiovascular diseases. Specifically, this study will examine the relationship between dietary oil consumption and body composition, vascular function, and blood markers of cardiovascular disease risk. Additionally, we will examine the efficiency of your body in converting fat from dietary oils into other specific fat compounds with known health benefits.

2. What will happen in this research study?

<u>General Overview of the Study</u>
Diet Design
If you agree to participate in this study, your participation will last 26 weeks total, consisting of 3 diet treatment phases each lasting 6 weeks and separated by an approximate 4 week break. During each treatment phase, you will be provided with a balanced, precisely controlled heart

healthy diet (35% energy from fat, 50% carbohydrate, and 15% protein). Calorie levels will be estimated for weight maintenance, this is not a weight loss study therefore calories will be adjusted as needed to ensure that you do not lose or gain weight over the course of the study. All diet phases contain foods that are commonly found at a grocery store and differ only in the dietary oils provided as follows:

1) Control phase: Dietary fat consumed will provide 35% of total energy and will be comprised of up to 60% canola oil.
2) High oleic acid canola oil phase: Dietary fat consumed will provide 35% of total energy and will be comprised of up to 60% high oleic acid canola oil. Oleic acid is a specific dietary fatty acid with health benefits.
3) High oleic acid canola + DHA phase: Dietary fat consumed will provide 35% of total energy and will be comprised of up to 60% DHA enriched canola-oil. DHA is a specific dietary long-chain fatty acid with health benefits.



<u>Procedures to be Followed</u>

Screening Tests

If you decide to participate in the study and are considered eligible after the telephone screening, you will be further screened during a visit to the Clinical Research Center (CRC) at Penn State to determine eligibility to participate. This visit will consist of filling out standard forms (informed consent, medical history, personal information); questionnaires (i.e. attitudes toward food and eating); measuring height and weight so your body mass index (BMI) can be calculated; and measuring blood pressure (BP). If, after these measurements, it is determined you are still eligible to continue in the research, a blood sample will be taken from a forearm or hand vein and a complete blood count, including liver and kidney function and a blood fat panel will be performed (approximately 15 ml of blood or 1 tablespoon will be taken). You will feel a small pinch or

discomfort when the needle is inserted. If the initial blood draw is unsuccessful it may need to be repeated, with your permission. If you take thyroid medicine, and do not have a current (within 6 months) lab test, we will draw 3.5 ml (0.2 Tbsp) more blood to conduct a thyroid test. If you are female, you will be given a urine pregnancy test. You will be contacted within 3-5 days with the results of the screening blood sample. A clinician at the CRC will review all of the screening data and if you are still eligible for the study, you will be contacted to schedule your start date and baseline data collection appointments. There will be no charge for the screening blood work or measurements and you will get these results. If you agree to participate in this study, you will agree to check with the study staff before participating in any other research studies; the study coordinator will let you know if it is alright to participate.

Feeding Study

If you agree to participate in the study you will agree to eat only those foods (3 meals and a snack every day) and beverages provided to you (some non-caloric beverages are allowed for free choice) during the feeding periods of the study. You will come to one of the Diet Centers on campus Monday through Friday for breakfast, lunch, or dinner (you choose which fits your schedule better), where meals will be prepared and provided for you. Your other two meals and a snack will be packed for you to take and eat at a place of convenience. On Fridays, you will be given a cooler that contains your remaining Friday meals and Saturday and Sunday meals and snacks. You will be required to appropriately refrigerate and store all foods provided to you for take-out.

You will be weighed regularly at your mealtime and you will provide the study staff with information about any non-study foods you may have eaten, any study foods not eaten and caffeine [limited to five (8oz), caffeine-containing beverages/day] and alcohol consumption (limited to 2 drinks/week). You are supposed to eat only the foods given to you and nothing else. You must eat all of the food given to you. If for some reason you fail to do this, it is important that you tell the study staff that you did not follow protocol so they can make a note of it in your records. The information you provide to the study coordinators will be collected on two separate forms; one to be completed daily and one to be completed weekly. It should only take about 5 minutes to complete these forms each day. Your calorie intake may be adjusted over the course of the study in order to maintain your screening body weight. You understand that this is **not a weight-loss study**. The diets are designed to meet your calorie needs and keep your body weight constant. Calorie intake will be adjusted up or down as necessary to maintain your weight. Also, you understand that you must keep your exercise level constant throughout the whole study.

Baseline and Endpoint Testing

Blood sampling:

You cannot consume any food or drinks except water for 12 hours, and cannot drink alcohol during the 48 hours prior to having your blood taken. You also cannot engage in vigorous physical activity 12 hours prior to having your blood taken.

In addition to the blood taken at screening, blood samples also will be taken on two consecutive days at the start and end of each treatment phase (i.e. days 1, 2 and 41, 42) for a total of 12 times. After a twelve hour fast (consumption of no food or drinks except water), a blood sample will be taken from your arm. If the initial blood draw is unsuccessful it may need to be repeated, with your

permission. Your weight and blood pressure also will be recorded. Approximately 65 ml (about 4 tablespoons) of blood will be collected at the start of each treatment period over two days (31 ml, or 2 Tbsp., on days 1,2), and approximately 75 ml of blood will be collected at the end of each treatment period over two days (41 ml on day 41 and 34 mls on day 42). An additional 10 ml of blood will be collected at the start of the study (day 1 of treatment 1). Therefore, over the 26-week study, blood will be taken 12 times with 430 ml of total blood taken. A typical American Red Cross blood donation is 1 pint (500 mls.) Blood samples will be frozen and analyzed at the end of the study (when all subjects have completed the study). The results of the study will only be available at the end of the entire study (which may take up to 3 years). Your blood may be tested for the following: blood fats (total cholesterol, LDL-cholesterol, HDL-cholesterol, triglycerides, LDL particle size and apolipoproteins), blood sugar (glucose, insulin), inflammatory markers and possibly how the blood fat is being metabolized (e.g. lipid hydroperoxides, fatty acid oxidation), protein expression from DNA and RNA extraction. NO personal information will be kept with any sample – only ID# will be assigned and only the Primary Investigator and the Study Coordinator will have access to the ID# assignments with the study files. If you are female, you will be asked to provide a urine sample at each blood draw. If you become pregnant during this time, you will be asked to leave the study.

DXA Scans and Waist Circumference

At the beginning and end of each diet period, body composition will be determined by dual energy x-ray absorptometry (DEXA/DXA). DXA is the state-of-the-art method for measurement of body composition. This procedure requires you to remove all jewelry and change into shorts and a t-shirt (provided to you at the research facility) before lying flat on your back on a padded table while an x-ray scanner moves across your body. The scan takes approximately 20 minutes and requires you to be completely still for the duration of the scan. This procedure will be conducted in the CRC and will be administered by a qualified operator. If you are a female of childbearing age you will be required to undergo a urine pregnancy test, the result of which must be negative before having the DXA scan. Your waist circumference measurements will be taken at this time to track how much fat is lost from the abdominal area of the body.

Vascular Ultrasound Test by Flow-mediated dilation (FMD):

In addition to collecting blood samples, you may be asked to undergo a test that determines the health of your arteries and their reactivity to mild stressors, which are described below. This test will be performed using an ultrasound machine. Ultrasound is often used to see images of babies in the womb. We will use ultrasound to measure the diameter of an artery in your upper arm, before and after the inflation of a blood pressure cuff on the forearm. In most people, this procedure produces dilation (opening up) of the artery. The purpose of this test is to assess the effects of different dietary oils on blood vessel function. Due to the influence of menstrual cycle on this measure of vascular health only individuals who are male or females who are postmenopausal or have had a hysterectomy (with ovaries removed) will be asked to participate in this measurement.

Ultrasound will be used to collect images of the brachial artery in your right arm. This procedure is known as Flow Mediated Dilation (FMD). The FMD test takes about 30 minutes and is performed as follows:

1. In a private room, you may be asked to remove your shirt and put on a hospital gown. (You will not be asked to remove any clothing below the waist). You will lie quietly on a bed in a quiet, darkened room.
2. Your right arm will be extended at a 45-degree angle from your shoulder and rest on some foam cushion supports. A blood pressure cuff will be placed on your forearm.
3. A research assistant will place 3 EKG electrodes (stickers) on your upper chest and stomach.
4. You will be asked to rest for 10 minutes.
5. A technician, trained in medical ultrasound, will sit at the head of the bed. The technician will apply ultrasound gel on your arm and will place an ultrasound probe (which looks like a microphone) on that arm.
6. An image of the blood vessels in your arm will be viewed on the ultrasound equipment next to the bed. The technician may need to move the probe over a small area of your upper arm to obtain the clearest image. The image of the artery will be videotaped for 5 minutes.
7. Next, the blood pressure cuff on your lower arm will be tightly inflated (to a pressure of 250 mmHg) and it will remain inflated for 5 minutes.
8. While the cuff remains inflated, the technician will continue to record the image of the artery in your upper arm. At the end of 5 minutes, the cuff will be deflated and images will be captured and recorded for an additional 2.5 minutes. It is very important throughout the recording that you rest quietly and keep your arm as still as possible.

Stable isotope analysis
On the 41st day of each diet phase, a fasting blood draw will be taken. You will not have eaten or drank anything, except water, for the previous 12 hours. Afterwards you will be asked to consume ~ three tablespoons of tagged water (known as deuterium). The movement of these tagged materials will allow us to assess the quantity of long chain fatty acids (EPA and DHA) that your body is producing in response to your diet. All of the above tagged materials are non-radioactive, non-toxic, and do not pose any health risk to you. Another fasting blood sample will be obtained when you return the next morning.

<u>Optional Study Components</u>:
Activity Monitoring
Your physical activity will be monitored using Actigraph GT3X+ activity monitors from days 28-35 of each phase. The activity monitor is worn on your waist and will monitor your activity and sleep using a multi-axial accelerometer and an ambient light sensor. The data from the device will be downloaded on day 35 of each period and saved under your study ID# and period, then the data on the device will be deleted. Data will then be processed using Actilife 6 software. This software will provide outcome measures of 24 hour physical activity including steps taken, raw acceleration, activity counts, energy expenditures, physical activity intensity, body position, and sleep/wake measurements.

Stool Sample Collection:
At baseline (start of treatment phase 1 only) and at the end of each time point you will be asked to collect a stool sample (10-20g) in the week prior to attending your clinic visit. You will be provided with a stool sample kit and detailed instructions for collection of a clean sample. You will be asked to freeze the sample immediately and keep them frozen until your scheduled endpoint visit.

Compliance with Study Protocol
******Please note: Successful completion of this study depends on the total cooperation of the participants. If during the study, you cannot eat the food provided or comply with other study procedures (such as attending clinic visits), you will be asked to leave the study. Every effort will be made to give you a chance to comply with the study requirements, but if you do not follow the above study protocol you may be dropped from the study.***
In addition, please advise us of any medical events (such as illness, injury, surgery etc) that arise during the course of the study. Depending on the event, we may require you to obtain a medical clearance before continuing with the study. Some medications may also interfere with our study outcomes so please inform us of any medication changes. ***

3. What are the risks and possible discomforts from being in this research study?

<u>Discomforts and Risks</u>

Feeding Study

The diets used in this study are nutritionally adequate, whole-food diets. Foods will be prepared according to accepted standards of sanitation and provisions are made to ensure the safety of foods provided for off-site consumption. However, it is possible that incorrect food handling during shipping, storage or preparation, if not detected, could result in food-borne illness. Every effort will be made to safeguard against this possibility. Feeding studies that require on-site eating of meals and strict adherence to the diets provided may interfere with social activities centered around eating such as dining in restaurants. While the menus will provide some variety in the diets, the number of food items will be more limited than that available in an average grocery store. The limited variety may become repetitious over the course of the study. In addition, you may experience a GI (stomach) upset from the change of diet, due to the increased/altered fat content. Symptoms also may include diarrhea or nausea but this will likely subside once you become accustomed to the new diet.

Blood Sampling

Blood draws often cause mild pain, swelling or bleeding. There may be some bruising (blood under the surface of the skin), which will be minimized by pressing on the site after the needle is removed. There is also a slight chance of infection, dizziness or fainting. These risks will be minimized and most likely eliminated by having trained medical staff draw the blood in a clinical setting using sterile supplies. If dizziness or fainting occurs, the symptoms will be alleviated by having you lie flat with your feet raised. The medical staff will ask that you remain at the clinic until your blood pressure has been checked and they are sure that you are OK.

DXA Scans

The DXA bone density procedure exposes you to a small amount of radiation where the x-ray beam crosses your body. This radiation dose is not necessary for your medical care and is for research purposes only. The total radiation dose for the 6 DXA scans planned over the course of a year is equivalent to a whole-body radiation dose of approximately 9 mrem (millirem). A mrem is a unit of whole-body radiation dose. For comparison purposes, 300 mrem is about what the average person in the U.S. receives from natural background sources such as the sun, outer space and materials found naturally in the air and soil. 9 mrem is less than you would receive from ~2

weeks worth of natural background radiation in central Pennsylvania. For the test, you will be asked to remove all clothing (except underpants) and wear shorts and a t-shirt during this procedure. A room will be available for you to change and efforts will be made to make sure you are comfortable with this arrangement.

Vascular Ultrasound Test (FMD)
There are no known risks associated with ultrasound. However, because the blood pressure cuff on your right forearm is inflated tightly, it is likely that your hand and arm below the blood pressure cuff will experience "pins and needles" (tingling and pricking sensations) while the cuff is inflated and for a few minutes after it is released. This feeling is similar to what you feel when your hand or foot "fall asleep." During the 5 minutes that the blood pressure cuff is inflated on your forearm, your arm could become numb and we will ask you not to move it. This might be moderately painful. However, any discomfort or numbness should go away within minutes of cuff deflation and there are no known long-term risks associated with this test. There is a possibility for red blotching or mild bruising (petechiae) to appear on the skin above and below the location of the blood pressure cuff. Studies indicate that petechiae are rare (occurring in less than ½ of 1% of patients) and it is typically not uncomfortable and it does not require treatment. There are no risks associated with measurement of blood pressure, heart rate, or EKG as long as the participant is not allergic to adhesive tape. Temporary redness at the site of the electrode placement is possible.

All video tapes from the ultrasound will have no personal identifying information associated with them and will be stored in a locked closet indefinitely since there is no indication on the tape of who the subject is.

Activity Monitoring
If you choose to participate in this optional component of the study you will be instrumented with an Actigraph GT3X+ activity monitor to be worn for 1 week at the end of each treatment period. The risks involved with wearing this device are minimal and may include interference with daily tasks (such as sleeping and bathing).

Stool collection
If you elect to participate in this optional component you may experience some level of embarrassment or discomfort from being asked to collect stool samples. However, you will be provided with detailed instructions on how to collect the samples within the comfort of your own home, and at your convenience, to help reduce any concerns you may have.

Loss of Confidentiality
Your participation in this research is confidential. However, there is always a potential for loss of confidentiality despite our best efforts. To prevent this from occurring all records are coded with a unique ID number and no names are used. Records containing names or other identifying information are kept under lock at the PI's research office. All records associated with your participation in the study will be subject to the usual confidentiality standards applicable to medical records. In the event of publication of this research, no personal identifying information will be disclosed.

4. **What are the possible benefits from being in this research study?**

4a. What are the possible benefits to you?
You will have a chance to learn the principles of good nutrition practices. You also will receive the results of your screening blood work and information about how your biomarkers for cardiovascular disease risk changed in response to the dietary oils. At the end of the study it will be explained how various fatty acids have beneficial effects on your vascular function, lipid levels and body composition. The final results of the study will not be available until all of the analysis is completed. This may take up to three years. However, no benefit from participation in this study is guaranteed.

4b. What are the possible benefits to others?
It is hoped that the information gained from this study will increase our understanding of the effects of various fatty acids on vascular function and biomarkers for cardiovascular disease risk. In addition it may help to explain why individuals respond differently to a certain types of dietary oils.

5. **What other options are available instead of being in this research study?**

Your participation in this study is voluntary. You may withdraw from this study at any time by notifying the investigators or other study personnel.

6. **How long will you take part in this research study?**

<u>Time Commitment for the Study</u>
Total time for study, after initial screening, is approximately 3980 minutes or 66.5 hours. Times may vary and females will require an additional 5 minutes for a urine pregnancy test at screening and the start and end of each treatment period. The following is an estimate of the amount of time you will spend in study activities:

- Screening: 60 min [forms, BP, weight, blood draw]

- Baseline: (Start of Phase 1) Blood draw, weight, BP, FMD - 30-45 min*
  Blood draw, WC, DXA scan – 20-30 min

- End of Phase 1: Blood draw, weight, BP, FMD – 30 – 45 min*
  Blood draw, WC, DXA scan – 20-30 min

For Phases 2-3:

- Days 1 & 2: Blood draw, weight & BP– 30 min*
  Blood draw, WC, DXA scan – 20-30 min

- Days 41 & 42: Blood draw, weight, BP, FMD – 30 min*
  Blood draw, WC, DXA scan – 20-30 min

Eating at the clinic/filling out forms/picking up food – 30-45 min / 5 days per week for a total of 20 weeks: total of 3000 min or about 50hrs

* Urine pregnancy testing is performed on women of child bearing potential only. Premenopausal women and women who have had a hysterectomy but still have their ovaries will not perform the FMD

Note: Fecal collection is optional. We estimate the time to collect, package and mail the samples at each time point to be 1.5 hours (for the entire study duration).

7. How will your privacy and confidentiality be protected if you decide to take part in this research study?

Your participation in this research is confidential. Efforts will be made to limit the use and sharing of your personal research information to people who have a need to review this information. All records are coded with a unique ID number and no names are used. Records containing names or other identifying information are kept under lock at the PI's research office. All records associated with your participation in the study will be subject to the usual confidentiality standards applicable to medical records. In the event of publication of this research, no personal identifying information will be disclosed. Your blood specimens will be coded with your unique ID number and will be maintained until three years after the date from when the study is published, and then destroyed unless (see end of document) you give permission for use to keep your blood samples for future use. At the end of the study (after all subjects have completed the study), you will be given your laboratory results without cost, and informed of the study results.

As a multi-center trial some of your blood may be analyzed by investigators in Canada, however no Canadian agency or other entity will have access to any confidential data or personal information collected during this study.

We will do our best to keep your participation in this research study confidential to the extent permitted by law. However, it is possible that other people may find out about your participation in this research study. For example, the following may review records related to this research:
- The Office for Human Research Protections in the U. S. Department of Health and Human Services
- The Penn State Institutional Review Board (a committee that reviews and approves research studies)
- The Penn State Office for Research Protections.

Some of these records could contain information that personally identifies you. Reasonable efforts will be made to keep the personal information in your research record private. However, absolute confidentiality cannot be guaranteed.

8. What are the costs of taking part in this research study?
8a. What will you have to pay for if you take part in this research study?

You will not bear any costs as a result of your participation in this study.

8b. What happens if you are injured as a result of taking part in this research study?
In the unlikely event you become injured as a result of your participation in this study, medical care is available. It is the policy of this institution to provide neither financial compensation nor free medical treatment for research-related injury. By signing this document, you are not waiving any rights that you have against The Pennsylvania State University for injury resulting from negligence of the University or its investigators.

9. Will you be paid to take part in this research study?
For your time and participation in the study you will receive monetary compensation of $300.00, prorated as follows and paid at the completion of your participation in the study. If you drop out of the study for any reason before its completion the following compensation will be provided upon retrieval of any study equipment:
$150 for completion of period 1
$150 for completion of period 2
$150 for completion of period 3
Total =$450 to be given at the completion of the participant's involvement in the study.

Those subjects who choose to participate in the fecal sample collection will receive an additional $50 or $10 per collection (baseline, end of treatments 1-3). The total compensation will be $500 to be paid at the completion of the participant's involvement.

If you are not a Penn State employee you will asked to provide your SSN for tax reporting purposes. Total payments within one calendar year that exceed $600 will require the University to annually report these payments to the IRS annually. This may require you to claim the compensation that you receive for participation in this study as taxable income.

10. Who is paying for this research study?
The funding for this study is provided by the Canola Council of Canada and Ag-Canada. However the funding source will not be involved in data analysis. They will have the right to review all publications before submission however there are no contractual agreements that allow them to have influence on, or restrict, the publication of results. Dr. West, who is an investigator on this study, has a business relationship with one of the sponsors, The Canola Council of Canada.

11. What are your rights if you take part in this research study?

Taking part in this research study is voluntary.

- You do not have to be in this research.
- If you choose to be in this research, you have the right to stop at any time.
- If you decide not to be in this research or if you decide to stop at a later date, there will be no penalty or loss of benefits to which you are entitled.

You may be asked to leave the study at any time if you do not comply with the study protocol. During the course of the research you will be provided with any new information that may affect your health, welfare or your decision to continue participating in this research.

12. If you have questions or concerns about this research study, whom should you call?
Please contact Dr. Kris-Etherton at (863-2923 or 863-8056) with any questions, complaints or concerns about the research. You can also call this number if you feel this study has harmed you. You may also contact the Office for Research Protections at (814) 865-1775, ORProtections@psu.edu if you:

- Have questions regarding your rights as a person in a research study.
- Have concerns or general questions about the research.
- You may also call this number if you cannot reach the research team or wish to talk to someone else about any concerns related to the research

INFORMED CONSENT AND AUTHORIZATION TO TAKE PART IN RESEARCH

Signature of Person Obtaining Informed Consent

Your signature below means that you have explained the research to the subject or subject representative and have answered any questions he/she has about the research.

______________________________ _________ ______ __________
Signature of person who explained this research Date Time Printed Name
(Only approved investigators for this research may explain the research and obtain informed consent.)

Signature of Person Giving Informed Consent and Authorization
Before making the decision about being in this research you should have:

- Discussed this research study with an investigator,
- Read the information in this form, and
- Had the opportunity to ask any questions you may have.

Your signature below means that you have received this information, have asked the questions you currently have about the research and those questions have been answered. You will receive a copy of the signed and dated form to keep for future reference.

Signature of Subject

By signing this consent form, you indicate that you voluntarily choose to be in this research and agree to allow your information to be used and shared as described above.

______________________________	___________	_______	__________________
Signature of Subject	Date	Time	Printed Name

Optional part(s) of the study
In addition to the main part of the research study, there is another part of the research. You can be in the main part of the research without agreeing to be in this optional part.

<u>Optional Storage of Blood Samples for Future Research</u>
As part of this study, we are obtaining blood from you. If you agree, the research team would like to store leftover samples of your blood that is collected so that your blood can be studied in the future after this study is over. These future studies may provide additional information that will be helpful in understanding cardiovascular disease, but it is unlikely that these studies will have a direct benefit to you. Neither your doctor nor you will receive results of these future research tests, nor will the results be put in your health record. If you have any questions, you should contact Dr. Kris-Etherton at 814-863-2923.

Your leftover samples will be labeled with a code number and stored in Dr. Kris-Etherton's locked laboratory. If you consent to the storage of leftover samples of your blood for future research, the period for the use of the samples is unknown. If you agree to allow your blood to be kept for future research, you will be free to change your mind at any time. You should contact Dr. Kris-Etherton at 814-863-2923 and let her know you wish to withdraw your permission for your blood to be used for future research. Should you choose not to allow for future testing of your blood it will be destroyed 3 years after publication of study results.

You should initial below to indicate your preferences regarding the optional storage of your leftover blood for future research studies.

a. Your samples may be stored and used for future research studies to learn about, prevent, treat or cure cardiovascular disease and obesity and other health problems.
   ______ Yes _____ No

b. Your samples may be shared with other investigator/groups without any identifying information.
   ______ Yes _____ No

Signature of Person Obtaining Informed Consent

Your signature below means that you have explained the optional part(s) to the research to the subject or subject representative and have answered any questions he/she has about the research.

______________________________ __________ _______ __________

Signature of person who explained this research Date Time Printed Name

Signature of Subject

By signing below, you indicate that you have read the information written above and have indicated your choices for the optional part(s) of the research study.

___________________________ __________ ______ ________________

Signature of Subject Date Time Printed Name

Do we have permission to keep your personal information and contact you about your interest in participating in future studies for Dr. Kris-Etherton and her collaborators?

______ Yes _____ No ______ Initials

<u>Person Explaining the Research</u>: Your signature below means that you have explained the optional part of the research to the participant/participant representative and have answered any questions he/she has about the research.

_____________________________ ________ _____ __________________

Signature of person who explained this optional research Date Time Printed Name

CONSENT FOR RESEARCH IN OPTIONAL STUDY COMPONENTS

The optional components of the research study (activity monitoring and fecal sample collection) have been explained to you. You have had a chance to ask questions to help you understand what will happen. You Do Not have to be in the optional part(s) of the research study. If you agree to participate and later change your mind, you can tell the researchers, and the optional part(s) of the research will be stopped.

You have decided: **(Initial one)**

Activity monitoring:

_____To take part in the activity monitoring part of the research.

_____NOT to take part in the activity monitoring of the research.

Fecal sample collection:

_____To take part in the fecal collection part of the research.

_____NOT to take part in the fecal collection part of the research.

______________________________ __________ __________________________
Signature of subject Date Printed Name

B. 7-day cycle menus for COMIT II

Day 1	Day 2	Day 3	Day 4
BREAKFAST	**BREAKFAST**	**BREAKFAST**	**BREAKFAST**
Shake	Banana	English muffin	Skim milk
Oatmeal	Mini bagel	Egg Beaters	Cracklin' Oat Bran
	Cream cheese	American cheese	Shake
	Shake	Canadian bacon	
		Shake	
LUNCH	**LUNCH**	**LUNCH**	**LUNCH**
Tuna salad	Turkey sandwich	Chicken salad	Ham & Swiss sandwich
Hamburger bun	Hummus	Cherry tomatoes	Carrots
Apple	Broccoli	Fruit blend	Celery
Baby carrots	Carrots	Crackers	Fruit blend
	Yogurt		Chips
	Orange		
DINNER	**DINNER**	**DINNER**	**DINNER**
Spaghetti	Turkey taco salad	Jambalaya	Beef tips & noodles
Roll	Corn	Salad	Veggies
Margarine		Roll	Roll
Green beans		Applesauce	Margarine
SNACK	**SNACK**	**SNACK**	**SNACK**
Shake	Banana muffin	Banana muffin	Mini cinnamon bagel
	Shake	Shake	Cream cheese
			Shake

Day 5	Day 6	Day 7
BREAKFAST	**BREAKFAST**	**BREAKFAST**
Pancakes & syrup Shake	English muffin Egg Beaters Canadian Bacon American Cheese Orange Shake	Mini Bagel Cream Cheese Banana Shake
LUNCH	**LUNCH**	**LUNCH**
Chicken BBQ sandwich Carrots Fruit blend Chips	Chicken salad Grapes Yogurt Pretzels	Egg salad sandwich Chips Fruit blend Pretzels
DINNER	**DINNER**	**DINNER**
Pork loin Mac & Cheese Broccoli Roll Applesauce Margarine	Vegetarian chili Salad Roll	Thai noodles Chicken Veggies
SNACK	**SNACK**	**SNACK**
Yogurt Shake	Banana muffin Shake	Shake

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EDUCATION

The Pennsylvania State University, Ph.D., Nutritional Sciences 2018
Bucknell University, B.S., Biology 2012

PUBLICATIONS

Bowen KJ, Kris-Etherton PM, West SG, Fleming JA, Connelly PW, Lamarche B, Couture P, Jenkins DJ, Taylor C, Zahradka P, Hammad SS, Sihag J, Chen S, Guay V, Maltais-Giguère J, Perera D, Wilson A, Castillo S, Jones PJH. Diets enriched with conventional or high-oleic acid canola oils lower atherogenic lipids and lipoproteins compared to a diet with a Western fatty acid profile in adults with central adiposity. *Accepted, The Journal of Nutrition.*

Bowen KJ, Richter CK, Skulas-Ray AC, Mozaffarian D, Kris-Etherton PM. Projected long chain n-3 fatty acid intake post-replacement of vegetables oils with stearidonic acid-modified varieties: results from a National Health and Nutrition Examination Survey 2003-2008 analysis. *In press, Lipids.*

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Bowen KJ, Kris-Etherton PM, Shearer GC, West SG, Reddivari L, Jones PJH. Oleic acid-derived oleoylethanolamide: a nutritional science perspective. Prog Lipid Res. 2017;67:1-15.

Bowen KJ, Harris WS, Kris-Etherton PM. Omega-3 fatty acids and cardiovascular disease: are there benefits? Curr Treat Options Cardiovasc Med. 2016;18(11): 69.

AWARDS

Ruth L. Pike Nutritional Sciences Graduate Fellowship Award, Penn State, 2018-2019
Pre-Doctoral Fellowship, USDA Agriculture and Food Research Initiative, 2018-2019
2nd place (out of 50) poster competition, STEM Exchange, PepsiCo and the NY Academy of Sciences, 2018
Department of Nutritional Sciences Travel Grant, Penn State, 2018
Contributions to Science Award, Penn State, 2018
Lifestyle & Cardiometabolic Health Early Investigator Travel Award, American Heart Association, 2018
John A. Milner Endowment Award, Penn State, 2017-2018
Travel Grant, Global Organization for EPA and DHA Omega-3s, 2017
Graduate Fellowship, NASA Pennsylvania Space Grant Consortium, 2016-2017