The Effect of Consuming Canola and Flax Oils in Modulation of Vascular Function and Biomarkers of Cardiovascular Disease Risks

by

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A Thesis Submitted to the Faculty of Graduate Studies of The University of Manitoba

in partial fulfillment of the requirements of the degree of DOCTOR OF PHILOSOPHY

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Abstract

It is well established that replacing dietary saturated fatty acids with unsaturated fatty acids reduces cardiovascular disease (CVD) risk. Although epidemiological and clinical evidence indicate health benefits of consuming various fatty acid classes including n-9, n-6, and short- and longer-chain n-3 fatty acids, current dietary recommendations fall short of providing the optimal amounts of these fatty acids in daily diets. In addition, significant knowledge gaps remain in our understanding of the effects of, and mechanisms underpinning the action of, the various fatty acid classes on risk factors for CVD. The objective of this research was to contribute to the evaluation of health benefits of using different dietary oils, and determine how these benefits may play a role in improving public health and decreasing CVD risk. Additionally, this research examined effects of diet-gene interactions, endogenous fatty acid ethanolamides (FAEs) on body fat mass distribution as well as changes in the composition of gut microbiota following consumption of dietary oil treatments. The Canola Oil Multicenter Intervention Trial (COMIT) was conducted as a 5-phase randomized, controlled, double-blind, cross-over clinical trial. Each 4-wk treatment period was separated by 4-wk washout intervals. A total of 130 volunteers with abdominal obesity consumed each of 5 identical weight-maintaining, fixed-composition diets with one of the following treatment oils (60 g/3000 kcal) in the form of beverages: 1) conventional canola oil (Canola; n-9 rich), 2) high–oleic acid canola oil with docosahexaenoic acid (CanolaDHA; n-9 and n-3 rich), 3) a blend of corn and safflower oil (25:75) (CornSaff; n-6 rich), 4) a blend of flax and safflower oils (60:40) (FlaxSaff; n-6 and short-chain n-3 rich), and 5) high–oleic acid canola oil (CanolaOleic; highest in n-9). At endpoints, plasma fatty acid levels reflected the differences in fatty acid composition of five dietary treatments. All diets lowered
total cholesterol (TC) compared with baseline. TC was lowest after the FlaxSaff phase and highest after CanolaDHA. The CanolaDHA treatment improved HDL-C, triglycerides, and blood pressure thereby reducing Framingham risk scores compared with other oils varying in unsaturated fatty acid composition. Homozygotes minor allele carriers of rs174583 (TT) on FADS2 gene showed lower (P<0.01) plasma EPA and DPA levels across all diets, but no differences were observed in DHA concentrations after the CanolaDHA feeding. In addition, plasma FAE levels were positively associated with plasma fatty acid profiles. Minor allele A carriers of rs324420 of FAAH gene showed a higher (P<0.05) plasma FAE levels compared with major allele C carriers across all diets, and showed higher (P=0.0002) docosahexaenylethanolamide levels in response to the CanolaDHA diet. Impacts of consuming 60 g of five dietary oil treatments on gut microbiota composition were relatively minor at the phylum level and mainly at the genus level, while BMI contributed to a significant shift at the phylum level. In conclusion, consumption of a novel DHA-enriched canola oil improved blood lipid profile and largely reduced CVD risk. Diet-gene interactions might help identify sub-populations who appear to benefit from increased consumption of DHA and oleic acid. The metabolic and physiological responses to dietary fatty acids may be influenced via circulating FAEs, while the altered microbiota profile by shifts in MUFA and/or PUFA may be associated with specific physiological effect. Personalized diets varying in unsaturated fatty acids composition based on specific lifestyles, environmental factors, psychosocial factors, and genetic make-ups will become the future “healthy eating” recommendations to prevent CVD risk.
Acknowledgements

First, I would like to thank my supervisor, Dr. Peter J.H. Jones, for providing me with guidance, support, encouragement and opportunities throughout my doctoral research at the University of Manitoba’s Richardson Centre for Functional Foods ad Nutraceuticals. Peter, it has been quite an adventure and sincerely I appreciate it, thank you. I would like to thank my committee members Dr. Peter Eck for his passionate attitude and positive direction for research, as well as Dr. Grant Hatch for the mentorship and inspirational conversions. I would send a special thank to Dr. Ehsan Khafipour for the continuous support and valuable guidance in the analysis of gut microbiota.

I would also like to acknowledge Dr. Vanu Ramprasath and Dr. Vijitha Senanayake for their amusing assistance and training throughout the clinical project. I would send many thanks to Khatima Khalloufi and Haifeng Yang for the technical assistance; Hanja, Danielle and Julia for the clinical assistance. I would also like to thank the staff and my friends at Human Nutritional Sciences, and the Richardson Centre, including Louise Grapentine, Yanan Wang, Caitlin McFadyen, Dylan MacKay, Mohammad Abdullah, Ale Serrano, Sandra Castillo, Chenxi Cai, Resa Wan and many others.

Moreover, this could not have been possible without my parents Jianyuan and Zhengle. You have been the best parents I can ever imagine. Whenever I need you, you are always there for me. Most importantly, I will never forget my beloved soul mate, my wife, Xiaobei. Thank you for your companionship for the past 8 years, from Louisiana bayou to South China bay, and to snowy Winterpeg.
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<td>AA</td>
<td>Arachidonic acid</td>
</tr>
<tr>
<td>ACAT</td>
<td>Acyl-CoA cholesterol acyltransferase</td>
</tr>
<tr>
<td>ACE</td>
<td>Abundance based coverage estimation</td>
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<tr>
<td>AEA</td>
<td>Anandamide (arachidonoyl ethanolamide)</td>
</tr>
<tr>
<td>AHA</td>
<td>American Heart Association</td>
</tr>
<tr>
<td>ALA</td>
<td>Alpha-linolenic acid</td>
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<tr>
<td>ALEA</td>
<td>Alpha-linolenoyl ethanolamide</td>
</tr>
<tr>
<td>ALKP</td>
<td>Alkaline phosphatase</td>
</tr>
<tr>
<td>Apo</td>
<td>Apolipoprotein</td>
</tr>
<tr>
<td>BED</td>
<td>Binge eating disorder</td>
</tr>
<tr>
<td>BMI</td>
<td>Body mass index</td>
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<tr>
<td>Canola</td>
<td>Conventional canola oil</td>
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<tr>
<td>CanolaDHA</td>
<td>High-oleic acid canola oil with docosahexaenoic acid</td>
</tr>
<tr>
<td>CanolaOleic</td>
<td>High-oleic acid canola oil</td>
</tr>
<tr>
<td>CB</td>
<td>Cannabinoid receptor</td>
</tr>
<tr>
<td>CDC</td>
<td>Centers for Disease Control</td>
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<tr>
<td>CHD</td>
<td>Coronary heart disease</td>
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<tr>
<td>COMIT</td>
<td>Canola oil multicenter intervention trial</td>
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<tr>
<td>CornSaff</td>
<td>A blend of corn and safflower oils (25:75)</td>
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<tr>
<td>CVD</td>
<td>Cardiovascular disease</td>
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<td>D5D</td>
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D6D          Delta-6-desaturase
DBP          Diastolic blood pressure
DEXA         Dual energy x-ray absorptiometry
DHA          Docosahexaenoic acid
DHEA         Docosahexaenoyl ethanolamide
DPA          Docosapentaenoic acid
EDTA         Ethylenediaminetetraacetic acid
ELOVL        Elongation of very-long-chain fatty acid enzyme
EPA          Eicosapentaenoic acid
FAAH         Fatty acid amide hydrolase
FAE          Fatty acid ethanolamide
FADS         Fatty acid desaturase
FAME         Fatty acid methyl ester
FAO          Food and Agriculture Organization
FlaxSaff     A blend of flax and safflower oils (60:40)
FMD          Flow mediated dilatation
FRS          Framingham risk score
FSR          Fractional synthesis rate
GAPDH        Glyceraldehyde 3-phosphate dehydrogenase
GC-FID       Gas chromatography equipped with flame ionization detection
GC-IRMS      Gas chromatography with combustion isotope-ratio mass spectrometry
GWAS         Genome-wide association studies
HDL-C        High-density lipoprotein cholesterol
PLS-DA  Partial least square discriminant analysis
PPAR-α  Peroxisome proliferator-activated receptor alpha
PSU    Pennsylvania State University
PUFA    Polyunsaturated fatty acid
TC      Total cholesterol
TC/EA-IRMS  High temperature conversion elemental analyzer with combustion isotope-ratio mass spectrometry
TFA    Trans fatty acid
TG      Triglycerides
RCFFN  Richardson Centre for Functional Foods and Nutraceuticals
RFMC   Risk Factor Modification Centre
RHI    Reactive hyperemia index
SBP    Systolic blood pressure
SFA    Saturated fatty acid
SGOT   Serum glutamic oxaloacetic transaminase
SGPT   Serum glutamate pyruvate transaminase
SMOW   Standard Mean Ocean Water
SNP    Single nucleotide polymorphism
SREBP-1 Sterol response element-binding protein 1
UPLC-MS/MS Ultra-performance liquid chromatography tandem Quattro micro API mass spectrometry
VA     Virodhamine
VIP    Variable influence on projection
Chapter I

Overall introduction

1.1 Background

Metabolic syndrome (MetS) represents a cluster of factors that increase the risk of developing cardiovascular disease, type 2 diabetes mellitus, and all cause mortality\(^{(1)}\). Although numerous definitions of MetS exist, the most commonly used criteria include elevated waist circumference, high triglycerides, reduced HDL-C levels, increased blood pressure, and impaired glucose tolerance as underlying core components. The prevalence of MetS in the U.S. has increased from 2003 to 2012 but ranges from 18.3\% to more than 50\%, depending on the composition of population (sex, race/ethnicity, and age groups) being studied\(^{(2)}\). Overall, it is estimated that about 35\% of all adults and 50\% of individuals aged over 60 years old possess MetS in the U.S. while a significant higher prevalence was observed in women compared with men (35.6\% vs. 30.3\%). Given the fact that there is one most obvious link between dietary intake and risk of developing MetS, eating healthy or keeping the balance of energy intake has become the most effective environmental solution that helps the prevention of MetS within general population.

Dietary fat, because it is a richer source of calories compared to two other macronutrients carbohydrate and protein, is often perceived to be responsible for weight gain and obesity. Current dietary strategies suggest that low fat diets aid in weight control and substitution of dietary fat with carbohydrate or protein improve the metabolic profile. However, fats serve as one of the essential energy sources and contribute both structural and metabolic functions of
whole body metabolism. Only if energy intake exceeds energy expenditure over a prolonged period, will obesity develop and increase the risks of developing MetS. Thus, a prudent diet has to include the fat component with a healthy fatty acid composition, but will limit total fat to 30% or less of total energy. Therefore, the more important question focuses on the healthy fatty acid composition, or the type of fatty acids in the diet, which could be an important tool in managing body composition and/or weight, and prevention of MetS.

It is well established that consumption of saturated (SFA) and trans fat (TFA) should be minimized in Western diet (3; 4), because these types of fats have been linked to increased risk of cardiovascular disease (CVD). Debate is ongoing regarding what other fats should replace SFA and TFA in an effort to prevent risk of developing MetS and related CVD events. However, current dietary recommendations fall short of providing specific guidelines for optimal dietary amounts of monounsaturated (MUFA) and polyunsaturated fatty acids (PUFA), including n-9, n-6, short- and long-chain n-3 fatty acids (5).

During the past few decades, MUFA has become of special interest in regulating CVD risk factors and body weight, since researchers started comparing Mediterranean diets with Western diets. As the primary MUFA in human diet, oleic acid (OA) has been associated with several beneficial effects including decreasing LDL-C levels, increasing HDL-C levels, and possibly improving body composition. Kien et al. (6) recently showed that replacing dietary palmitic acid with OA resulted in significant reduction in blood LDL-C levels and increase in whole-body fat oxidation in human subjects. It is believed that OA is the preferred substrate for acyl-CoA cholesterol acyltransferase (ACAT) in the liver where it stimulates cholesterol ester formation and increases expression of LDL receptor in the liver, resulting in lower LDL-C levels in the
circulation\(^{(7)}\). Meanwhile, evidence shows that MUFA diets appear to induce greater diet-induced thermogenesis, energy expenditure, fat oxidation and weight loss compared to SFA diets\(^{(8)}\). One possible explanation is that a derivative of OA named oleylethanolamide (OEA), an endogenous chemical signaling lipid, can modulate PPAR-\(\alpha\) activity to stimulate lipolysis\(^{(9)}\), which leads to food intake reduction\(^{(10)}\) and shifts in body composition\(^{(11)}\). The recent trial on hypercholesterolemic participants demonstrated that dietary OA may modulate regional and total fat mass via OEA through lipid-signaling mechanisms\(^{(12)}\).

On the other hand, dietary guidance for PUFA intake has also progressed significantly in the past decade. Recommendations have been made both based on a large numbers of studies from epidemiologic and controlled clinical studies, indicating a range of beneficial physiological effects of PUFA\(^{(13)}\). Two series of PUFA, n-6 and n-3 fatty acids, exist with both being essential nutrients in human diets. Foods rich in n-6 PUFA are predominant in Western diets over the past 100 years\(^{(14)}\). The most common n-6 PUFA is linoleic acid (LA) which is needed for many physiological functions of whole body metabolism and well known for its reduction on major risk factors for CVD, including reduced LDL-C levels, improved insulin sensitivity, and lower blood pressure profiles. Thus, replacing SFA with n-6 PUFA has long been recommended to prevent CVD. However, because n-6 fatty acids are commonly abundant in Western diets, arguments exist surrounding the adverse effects of excessive consumption of n-6 PUFA in terms of their increased pro-inflammatory and immunoactive properties\(^{(15)}\). In addition, LA can be converted endogenously to arachidonic acid (AA) and further to a variety of pro-inflammatory eicosanoids which are considered harmful for heart health\(^{(16)}\). Therefore, the current recommendation for n-6 PUFA intake is above 5 \%, but not to exceed 10 \% of total energy\(^{(17)}\).
n-3 fatty acids are an important component of human physiology. In the late 1970s, the first epidemiologic studies began accumulating evidence on the potential functions of n-3 fatty acids in the prevention of CVD\(^{(18; 19)}\). The benefits of n-3 fatty acid consumption on CVD have long been a central theme in nutrition research. Studies illustrated that the high intakes of long-chain n-3 fatty acids can effectively reduce multiple CVD risk factors. The most common n-3 fatty acids for the prevention of CVD include one plant derived alpha-linolenic acid (ALA), and marine derived eicosapentaenoic acid (EPA), and docosahexaenoic acid (DHA), all of which have demonstrated an effective impact on the modulation of multiple cardiovascular risk factors in epidemiological\(^{(20)}\), animal models\(^{(21)}\), and human clinical investigations\(^{(22)}\). Overall, abundant evidence has shown that the associations between dietary ALA consumption and the prevention of heart disease are positive. The consumption of ALA improves cardiovascular health by modulating circulating lipid concentrations, altering membrane structure and function by enhancing the total n-3 fatty acid content of cell membrane phospholipids, and reducing inflammatory reactions by blocking the formation of AA-derived eicosanoids\(^{(23)}\). However, since dietary EPA and DHA also show great efforts on CVD prevention, the lack of understanding is in which specific fatty acids EPA, DHA or their precursor, ALA plays the key role in lowering lipid levels and reducing anti-inflammatory effects in CVD.

Therefore, an increasing volume of literature has been directed towards defining the optimal ratio between n-3 and n-6 fatty acids in order to achieve nutrient adequacy and prevention of CVD events. Novel modified vegetable oils are designed and manufactured to contain the balanced proportions of SFA, MUFA and n-6/n-3 PUFA; they provide opportunities for consumers to prevent risk of MetS or CVD events. The knowledge gaps are how to evaluate the efficacy of
those vegetable oils with various combinations of n-9, n-6, and n-3 fatty acids on common risk factors in human clinical trials.

In this study, five oils with different n-9: n-6: n-3 ratios were selected as the intervention. The treatment oils were conventional canola oil, which is famous for its richness of OA and ALA; high oleic canola oil, which has additional OA contents compared with conventional canola oil; DHA-enriched high oleic canola oil, which blends additional long-chain n-3, DHA, in high oleic canola oil; a blend of flax oil with safflower oil, which contains an n-9: n-6: n-3 ratio of 1: 1: 1; and a blend of corn oil with safflower oil, which represents a typical n-6 rich oil in North America. The three canola oil-based treatments were defined as high MUFA treatments, while two safflower oil-based treatments were made as high PUFA oils. Because the study was specifically designed to compare the unsaturated fatty acids in different formats, all treatment oils were made low in SFA according the common dietary recommendations.

1.2 Rationale

The consequence of total fat consumption on circulating lipids and the incidence of CVD has long been a central theme in nutrition research. Consumption of MUFA and PUFA favorably modulates circulating lipids and arterial health, but there is confusion surrounding the specific health benefits of the common dietary vegetable oils with varying fatty acid profiles. Therefore, examining the independent health benefits of these novel modified vegetable oils is paramount to strengthening our understanding of the role of MUFA and PUFA consumption in human health and disease prevention. This research investigated the health benefits of ALA from consumption of diets rich in canola oil, novel MUFA and DHA enriched canola oils, and flax oil compared with a control diet representative of North American diets rich in n-6 fatty acid. Treatment oils
were examined for their potential influence on plasma fatty acid response, sera lipid parameters, lipoprotein characterization, and blood pressures profiles. Furthermore, in an effort to elucidate the genetic factors that promote ALA conversion to EPA/DHA and strengthen the role of ALA in cardiovascular health, this research examined common genetic variants in the *FADS* and *ELOVL* gene with ALA conversion to EPA/DHA and n-3 fatty acid composition of serum in response to consumption of the treatment oils. Also, this trial provided a platform to investigate the corresponding changes in fatty acid derivatives, fatty acid ethanolamides (FAEs), which are associated with lipid signaling, fat oxidation and appetite control following a series of n-3 fatty acid dietary interventions in humans *in vivo*. In addition, the study provided an opportunity to examine gut microorganisms and metabolic mechanisms underpinning the health benefits after the treatment oil consumption.

### 1.3 Objectives

Taken together, the study contains five major objectives:

1. Examine the post-treatment plasma fatty acid profiles in response to the consumption of canola oil, DHA enriched canola oil, high oleic acid canola oil, a blend of flax oil with safflower oil, and a blend of corn oil with safflower oil.

2. Test the post-treatment lipid profile, lipoprotein, and blood pressures in response to the consumption of canola oil, DHA enriched canola oil, high oleic acid canola oil, a blend of flax oil with safflower oil, and a blend of corn oil with safflower oil.

3. Investigate the association between genetic variants in the *FADS1 & FADS2* and *ELOVL2 & ELOVL5* gene clusters with ALA conversion to EPA/DHA and n-3 fatty acid composition in human plasma in response to consumption of canola oil, DHA enriched canola oil, high
oleic acid canola oil, a blend of flax oil with safflower oil, and a blend of corn oil with safflower oil.

4. Investigate the impacts of consumption of canola oil, DHA enriched canola oil, high oleic acid canola oil, a blend of flax oil with safflower oil, and a blend of corn oil with safflower oil on circulating FAE levels and their associations with body composition, energy expenditure and anti-inflammatory properties.

5. Determine the effects of consumption of canola oil, DHA-enriched canola oil, high oleic acid canola oil, a blend of flax oil with safflower oil, and a blend of corn oil with safflower oil on gastrointestinal microbiota profiles and diversity.
1.4 References


Bridge to chapter II

The following chapter comprises a literature review which provides a broad overview of the current knowledge of associations between dietary fatty acids and cardiovascular disease risk. In particular, the review is focused on health benefits of replacing saturated fats with unsaturated fats and the argument on the optimal amounts of different fat types in a healthy diet. In addition, this chapter also reviewed the current findings on topics including effects of diet-gene interactions, endogenous fatty acid ethanolamides, and changes in gut microbiota composition in response to dietary intakes. This chapter is an introduction to the background of the Canola Oil Multicenter Intervention Trial.
Chapter II

Literature review

2.1 Dietary fatty acids and cardiovascular disease

Cardiovascular disease (CVD) is a class of disorders of the heart and blood vessels. The most common type of CVD is coronary heart disease (CHD) which includes stable angina, unstable angina, myocardial infarction, also known as heart attack, and sudden coronary death. Other types of CVD include cerebrovascular disease i.e. stroke, hypertensive heart disease, peripheral artery disease, rheumatic heart disease, atherosclerosis, atrial fibrillation, and venous thrombus.

Heart disease, stroke and other types of CVD are the leading causes of death for both men and women in the world, and more people die from CVD than from any other causes every year \(^{(1)}\). The WHO data shows an estimated 17.5 million people died from CVD in 2012, representing 31% of all global deaths. Over 80% of CVD deaths take place in low- and middle-income countries. In addition, it has been estimated that by 2030 more than 23 million people will die annually from CVD. Data from American Heart Association (AHA) showed that in 2011 the age-standardized death rate from all CVD was 229.6 per 100 thousand people, down 11.5% compared to the rate of 259.4 per 100 thousand in 2007. However, CHD, stroke and other CVD events combined were still the leading causes of death. CHD remained the first killer of CVD events, accounting for 47.7% of total CVD deaths, followed by stroke (16.4%) and heart failure (7.4%). CVD is also major cause of illness and disability and are estimated to cost over $300 billion annually in the U.S. in health care expenditures and lost productivity. And by 2030, these
total medical costs of CVD could increase to over $900 billion per year \(^{(2)}\). In Canada, the latest data from Statistics Canada in 2008 showed that 29% of all causes of deaths were attributed to CVD \(^{(3)}\). Among all CVD caused deaths, 54% were due to CHD, followed by heart failure (23%) and stroke (20%).

The causes of CVD events are usually the presence of a combination of risk factors, including age, gender, family history, smoking, alcohol consumption, physical inactivity, unhealthy diet, and other raised blood biomarkers. Some of these risk factors such as age, gender, and family history are unmodifiable, while many others are behavioral risk factors and considered to be improved by lifestyle change.

One of the most important behavioral risk factors of CVD is the unhealthy diet which is directly linked to the cause of many chronic diseases. Thus, improvements in diet may favorably lower the risk of CVD events. Intakes of different types of nutrients can largely influence the burden of chronic disease, particularly CVD and energy intake should be in balance with energy expenditure. The three primary macronutrients carbohydrate, protein and fat are all consumed in large quantities and provide with the bulk of energy in daily diets. Ultimately, because dietary fats are richer energy sources for the body compared to other macronutrients and can be stored for energy in excess of what the body requires it is essential to both structural and metabolic functions of whole body metabolism. However, excessive intake of fat is often perceived to be responsible for obesity and higher risk of CVD. It is suggested that a healthy diet has to contain fat which should not exceed 30% of total energy intake to avoid unhealthy weight gain or related chronic diseases like CVD. Therefore, the key question is how to determine the dietary fatty acid
composition, including saturated fats (SFA), trans fat (TFA) and unsaturated fats, in a healthy diet in order to reduce the risk of CVD.

It has been well established that consumption of SFA and TFA should be minimized in Western diets. The Food and Agriculture Organization (FAO) advises that SFA and TFA are risk factors for CVD, and the risk of developing CVD can be lowered by reducing SFA to less than 10% of total energy intake, and TFA to less than 1%, and replacing both with unsaturated fats \(^{(4)}\). There is no argument that TFA is not part of a healthy diet, but the effect of SFA consumption on CVD events has become relatively controversial over time. A number of recent systematic reviews stated that no associations between consumption of SFA and risk of CVD and all-cause mortality were observed in observational studies and randomized controlled trials \(^{(5; 6; 7)}\). Interestingly, despite their results, these reviews also clearly stated that replacing SFA with unsaturated fats can significantly reduce serum cholesterol levels. Given the fact that a high serum cholesterol level is a risk factor for CVD, the AHA in 2006 suggested that the intake of SFA should be limited to <7% of total energy, and TFA to <1% of total energy, and these fat contents should be replaced by unsaturated fats \(^{(8)}\). The most recent guideline kept this suggestion and recommended that a healthy diet should replace SFA with monounsaturated fats (MUFA) and polyunsaturated fats (PUFA), as high MUFA and/or PUFA diet appeared to be more metabolically beneficial than a high SFA diet \(^{(9)}\). However, these dietary recommendations fell short of providing specific guidelines for the optimal dietary amounts of MUFA and PUFA, including n-9, n-6, short- and long-chain n-3 fatty acids \(^{(10)}\).
2.1.1 Monounsaturated fatty acids

MUFA is classified as a group of fatty acids containing only one double bond in the carbon chains with all the remaining carbons single-bonded. The common MUFA includes palmitoleic acid (16:1n-7), cis-vaccenic acid (18:1n-7), and oleic acid (OA, 18:1n-9). OA is the predominant MUFA in the diet, representing up to 92% of dietary MUFA \(^{(11)}\). In 1999, the AHA suggested that MUFA intake should make up as much as 15% of total energy content when the total fat content of the diet is at 30% \(^{(11)}\). In 2007, the American and Canadian Dietetic Association provided a recommendation of dietary MUFA intake to be less than 25% of total energy intake \(^{(12)}\).

Although the total fat intake in Western diets is similar to that of Mediterranean diets, the greatest difference between these two types of diets are the composition of fat types, especially the amount of MUFA content. In the U.S., MUFA intake is 13% to 14% of total energy while SFA intake is in excess at 11-12% of energy \(^{(11)}\). The typical Mediterranean diet is characterized by its high MUFA content, ranging from 16 to 29% of energy with olive oil being the principal source of fat, and SFA comprising less than 8% of energy \(^{(11)}\). Epidemiological data showed an inverse relationship in CVD risk between people consuming a Mediterranean diet and those consuming a Western diet \(^{(13)}\). In fact, the prevalence of metabolic syndrome in the U.S. is three times higher than its prevalence in Mediterranean countries \(^{(14)}\). Therefore, over the last decade, the concept of high OA modified dietary oils with improved stability has been introduced into the commercial production of oil industry. While olive oil is the most commonly consumed MUFA rich oil, MUFA rich oils in North America, including conventional canola oil and canola oil with even higher OA content, have been widely marketed.
Dietary MUFA provide beneficial effects in prevention of CVD risk. Numerous controlled clinical studies have shown MUFA consumption favorably affects a number of risk factors for CVD events, including serum lipid profile, blood pressure, weight control, insulin sensitivity, and glycemic index.

Evidence from randomized controlled trials has substantiated that when SFA was replaced by MUFA, favorable effects were observed on LDL-C levels as well as LDL-C: HDL-C ratio\textsuperscript{(11; 15)}. As the major MUFA in human diet, OA has been associated with several beneficial effects including decreased LDL-C, increased HDL-C, and possible improvement of body composition. Kien et al.\textsuperscript{(16)} recently showed that replacing dietary palmitic acid with OA resulted in significant reductions in blood LDL-C levels and increases in whole-body fat oxidation rates in human subjects. It is believed that OA is the preferred substrate for acyl-CoA cholesterol acyltransferase (ACAT) in the liver, and that it stimulates cholesterol ester formation and increases expression of LDL receptor in the liver, resulting in lower LDL-C levels in the circulation\textsuperscript{(17)}. Recently, data from a crossover intervention study of 16 subjects by Kien et al.\textsuperscript{(18)} showed that total cholesterol level was decreased by 13% with a significant lower LDL-C level and LDL: HDL ratio when a 3-wk high palmitic acid diet was replaced by a high OA diet. To date most meta-analyses of MUFA related intervention studies have reported that MUFA consumption has been associated with LDL-C reduction, and possibly increased HDL-C levels\textsuperscript{(19; 20; 21)}, while a number of other meta-analyses claimed no changes on LDL-C levels\textsuperscript{(22; 23)}. Although there is no clear rationale on blood lipids improvement for MUFA recommendations, it is obvious that side effects of MUFA consumption have not been reported in the literature.
The positive associations between MUFA consumption and blood pressure control have been well established according to data from numerous randomized controlled trial studies. An early meta-analysis investigating long-term effects of MUFA consumption by Shah et al. \cite{24} suggested that MUFA rich diets significantly reduced both systolic and diastolic blood pressure in humans. In the recent meta-analysis by Schwingshackl et al. \cite{15}, more promising data showed that consumption of high MUFA (>12%) diets improved blood pressure profiles compared with low MUFA (<12%) diets. Besides long-term beneficial effects, evidence shows that MUFA consumption can also significantly improve postprandial blood pressure profile. A crossover double-blind intervention trial demonstrated that high MUFA intake produced a more pronounced postprandial decrease in blood pressure in both lean and obese volunteers compared with both SFA and n-3 PUFA treatments \cite{25}. Results of the OmniHeart randomized trial suggested that a high MUFA diet significantly decreased systolic blood pressure by 1.3 mm Hg and by 2.9 mm Hg compared to baselines among those with hypertension \cite{26}. Therefore, it is well established that MUFA consumption exerts a favorable effect on blood pressure control in humans.

High MUFA diets may also be associated with regulating body weight and preventing obesity. Obesity or overweight is one major problem globally, especially in North America \cite{27}. According to the national survey data, the prevalence of adult obesity in the US is approximately 35% by the year 2010 \cite{28}. One favorable implication of Mediterranean diets is that people following such dietary pattern are less obese compared with those following Western diets, indicating the potential association between the key element, MUFA in Mediterranean diets and body weight. An early study in obese type 2 diabetes patients showed that dietary MUFA intake effectively induced the glycemic response and resulted in maintenance of body weight \cite{29}. In
another weight maintenance study, a 4-wk controlled feeding of high MUFA diet tended to favorably shift adipose tissue from android region to gynoid region compared to an ALA rich diet (30). A review by Gillingham et al. (14) providing a critical assessment of the studies surrounding efficacy of dietary MUFA for reduction of CVD risk, suggested that the metabolism and preferential oxidation of dietary MUFA can play a role in influencing body composition and decrease the risk of obesity.

Other studies have also shown that high MUFA diets improved insulin sensitivity and glycemic index (26; 31). A meta-analysis by Schwingshackl et al. (15) investigating long-term high MUFA dietary intervention trials on biomarkers of obesity and CVD risks concluded that MUFA represented a beneficial component in the design of dietary regimens for managing risk factors in the prevention and treatment of CVD. Nonetheless, further investigations are needed to determine whether high MUFA intake helps with calorie control and improve weight maintenance with regard to prevent CVD risk.

In summary, although some suggestion exists that MUFA rich diets elicit less cholesterol-lowering effects compared with PUFA rich diets, epidemiological evidence suggests that high MUFA intakes can provide a beneficial effect in the prevention of CVD. Future dietary guidance given with regard to MUFA might instruct a balanced energy intake with regular physical activity to achieve the benefits of MUFA consumption.

2.1.2 Polyunsaturated fatty acids

PUFA is classified as a group of fatty acids containing two or more double bonds in their carbon chains. Two classes of PUFA are commonly presented in the diet, including n-3 PUFA which
have a double bond at n-3 position and n-6 which have a double bond at n-6 position. PUFA constitutes an important component of all cell membranes and regulates a wide range of physiological functions. A large body of literature suggests that higher intakes of both n-6 and n-3 PUFA can reduce risk for CVD. However, because of excessive consumption of n-6 PUFA in Western countries in last few decades, some have recommended substantial reductions in n-6 PUFA intake. Therefore, in the human diet, an n-6 to n-3 ratio of 4:1 is currently recommended to be optimal to health based on comparative studies (32).

2.1.2.1 n-3 polyunsaturated fatty acids

n-3 PUFA represents an important component of human physiology. In the late 1970s, the first epidemiologic studies began accumulating evidence on the potential functions of n-3 fatty acids in the prevention of CVD (33; 34). The benefits of n-3 fatty acid consumption on CVD have long been a central theme in nutrition research, indicating that high intakes of long-chain n-3 fatty acids can effectively lower the risk of CVD events. The three most common n-3 fatty acids are alpha-linolenic acid (ALA, 18:3n-3), eicosapentaenoic acid (EPA, 20:5n-3), and docosahexaenoic acid (DHA, 22:6n-3). Mounting data from epidemiological studies, animal studies, and human clinical trials continue to demonstrate that the cardioprotective activity of the all the n-3 PUFA contents (35; 36; 37).

ALA is commonly found in vegetable oils such as canola and flaxseed oils. For instance, canola is a good source of ALA with a content of approximately 11%, and ALA content in flaxseed oil is about 50% (38). As an essential fatty acid, ALA cannot be synthesized within the human body and must be acquired through diets. ALA is also known as the metabolic precursor of EPA and DHA through n-3 fatty acid metabolism. Theoretically, both EPA and DHA can be either
directly absorbed from daily food intake or synthesized endogenously from ALA. However, most studies suggest that the conversion rates of ALA to EPA and DHA are less than 5-10% and 0.5-5%, respectively, and vary throughout different regions, population, ages, and genders (39; 40). Therefore, all three major n-3 fatty acids ALA, EPA, and DHA are recommended to be consumed within daily diets. For instance, in North America, the recommended daily intake of dietary ALA from vegetable oils for women and men aged 19 to 70 years are 1.1 and 1.6 g/d, respectively. Moreover, the recommended daily intake of EPA and DHA from fatty fish, algae or fish-oil supplements is 500 mg/d (12). However, considerable evidence from clinical and epidemiological studies suggests that increased consumption of n-3 fatty acids EPA and DHA from fish or fish-oil supplements but not ALA is beneficial in cardioprotection, particularly in CVD outcomes including all-cause mortality, cardiac and sudden death (41; 42).

It is very well recognized that n-3 PUFA consumption reduces circulating triglycerides (TG) levels, but fails to impact on cholesterol levels (43). Harris concluded in an early review of clinical studies that consumption of 4g/d of n-3 fatty acid from fish oil can significantly reduce serum TG levels by approximately 25 to 30% (44). The TG-lowering effect is positively dose-dependent across n-3 PUFA consumption with little variation in individual responses (42). A placebo-controlled, randomized, crossover trial investigating the dose-response effects of n-3 PUFA on plasma TG showed that the high dose of 3.4g/d of EPA+DHA significantly decreased TG levels over 8 weeks in healthy adults with moderate hypertriglyceridemia (45). Therefore, the AHA recommends 1g of EPA+DHA daily for cardioprotection and 2 to 4 g/d for patients needing TG lowering under a physician’s care (46).
In addition, randomized controlled intervention studies clearly demonstrated that n-3 PUFA from fish or fish oils can significantly lower blood pressure \(^{(42)}\). In a meta-analysis on 32 placebo controlled trials by Morris et al. \(^{(47)}\), a significant reduction of -3.0/-1.5 mmHg with an average dose of 4.8g/d was shown in the overall population, while a greater reduction of -3.4/-2.0 mmHg was observed in untreated hypersensitive subjects. A population-based observational study investigating 4,680 men and women aged 40-59 years showed an inverse association between blood pressure and n-3 PUFA consumption \(^{(48)}\). Likewise, a cross-sectional analysis by Virtanen et al. \(^{(49)}\) reported 396 men and 372 women in Finland, aged 53-73 years, who mainly consumed fish or fish oil intakes, had a lower systolic blood pressure if serum n-3 PUFA concentrations were higher in average. In addition, a large body of evidence also demonstrated that there was an inverse association between the incidence of heart failure or atrial fibrillation (higher blood pressure burden) and higher circulating DHA levels but not higher EPA levels \(^{(50; 51)}\). Overall, while the effects of n-3 PUFA consumption on blood pressure-lowering have been well recognized, further confirmation by interventional studies with appropriate numbers of subjects will better explain the dose-response effect and specific benefits of DHA consumption on preventing risk of CVD \(^{(52)}\).

It is acknowledged that EPA and DHA play direct or indirect roles in preventing the development of inflammatory diseases \(^{(53)}\). A large number of clinical studies have shown the beneficial effects of n-3 PUFA intake on chronic inflammatory settings including rheumatoid arthritis \(^{(54)}\) and inflammatory bowel diseases \(^{(55)}\). It is proposed that EPA and DHA can incorporate into human inflammatory cells and decrease arachidonic acid (AA, 20:4n-6) levels, and subsequently reduce the production of AA-derived eicosanoids which were considered pro-inflammatory molecules \(^{(56; 57)}\). Since the current pattern of Western diets contains excessive
intake of n-6 PUFA, it is recommended that increasing consumption of n-3 PUFA, which may elicit anti-inflammatory effects, could help reduce the risk of many chronic diseases including CVD.

In general, abundant evidence has proved the great cardioprotective benefits of consumption EPA and DHA. The beneficial effects of consumption of ALA on modulating circulating lipid concentrations, altering membrane structure and function by enhancing the total n-3 fatty acid content of cell membrane phospholipids, and reducing inflammatory reactions by blocking the formation of AA-derived eicosanoids, might be attributed to their conversion to EPA and DHA. Thus, the knowledge gap is which specific n-3 PUFA, EPA, DHA or their precursor, ALA actually plays the key role in cardioprotective effects on preventing CVD risk.

2.1.2.2 n-6 polyunsaturated fatty acids

The most common unsaturated fat type in Western diets is n-6 PUFA. This group of fatty acids is essential for many physiological functions in human system and is well known for their health benefits preventing risk of CVD events. Therefore, the AHA recommends a consumption of at least 5-10% of energy from n-6 PUFA to reduce the risk of CVD. The most common n-6 PUFA in our diets are linoleic acid (LA; 18:2n-6) and AA. LA, the most abundant n-6 PUFA, can be commonly found in vegetable oils such as corn, soybean, safflower and canola oils, while AA is mainly stored in poultry meat and eggs. It is worth mentioning that besides dietary AA intake, AA can also be synthesized by the conversion of LA via desaturation and elongation reactions, although such conversion is extremely low, usually below 0.5%.
One remarkable benefit of consumption of n-6 PUFA rich diets is that they are well known for their cholesterol-lowering effects. The dietary proportion of carbohydrates can be replaced by n-6 PUFA with more favorable effects on blood lipid profiles than other classes of fatty acids. A meta-analysis of 60 controlled trials by Mensink et al. \(^{(21)}\) reported that a replacement of 1% of energy of carbohydrates by SFA induced increased LDL-C levels, whereas the replacement by n-6 PUFA reduced LDL-C levels. When dietary SFA is replaced by n-6 PUFA, decreased LDL-C levels are also observed. Results of a meta-analysis on 72 reports by Clarke et al. \(^{(22)}\) concluded that the isocaloric replacement of SFA by n-6 PUFA within just a few weeks produced a -0.39 mmol/l change in total cholesterol level, whereas replacement of SFA by MUFA led to a change of -0.24 mmol/l. Thus, it is well established that the substitution of n-6 PUFA for proportions of carbohydrates and SFA in the diet can lead to a substantial improvement in blood lipids and may reduce the risk of CVD \(^{(62)}\).

n-6 PUFA is known to play a central role in inflammation response which accounts for an important CVD risk factor. Because n-6 PUFA has long been considered as pro-inflammatory molecules, arguments for reducing n-6 PUFA intakes are always discussed. AA, the main precursor of eicosanoids, is responsible for the synthesis of a wide variety of pro-inflammatory molecules. However, evidence also shows that some of the eicosanoids are also anti-inflammatory compounds. Current investigations on this topic are inconclusive, since the pro- or anti-inflammatory activity is always associated with the levels and the nature of these AA derivatives which can change dramatically depending on the inflammatory response. In general, increased AA consumption can exacerbate symptoms manifest when individuals are injured or with a history of inflammatory diseases \(^{(63)}\); in healthy subjects, higher intake of AA does not appear to elevate the levels of inflammatory biomarkers such as CRP, IL-6 and TNF receptors.
Overall, little direct evidence can draw a conclusion as to whether n-6 PUFA consumption leads to altered inflammatory levels in humans.

Cross-sectional studies on dietary PUFA intakes (both n-3 and n-6 PUFA) have suggested that PUFA consumption is often associated with improvement of blood pressure profile \(^{(64)}\). An early intervention study by Mortensen et al. \(^{(65)}\) compared the beneficial effects of n-3 and n-6 PUFA consumption and their results showed that systolic blood pressure, plasma total lipids, and TG concentrations decreased during the n-3 PUFA phase while no changes were observed during the n-6 PUFA feeding. In an observational study on 4,033 healthy men, Grimsgaard et al. \(^{(66)}\) reported a significant increase in systolic blood pressure after a SFA diet but a significant decrease after a LA rich diet. Another observational study investigating 4,680 men and women ages 40 to 59 years \(^{(67)}\) showed increased LA intake was negatively associated with both systolic and diastolic blood pressure. Results suggested that dietary n-6 PUFA intake may contribute to control of adverse blood pressure levels in general population. Nevertheless, it is suggested that dietary n-3 PUFA intake may significantly improve blood pressure profile in humans, but n-6 PUFA consumption may also be associated with a decrease in blood pressure profile and in favor of reducing CVD risks \(^{(64)}\).

n-6 PUFA intake may contribute to body weight regulation. Given the fact that obesity or overweight is one of the important CVD risk factors, it has been suggested that n-6 PUFA, particularly AA, can play a dominant role on the regulation of lipogenic genes in adipocytes \(^{(68)}\). However, to date most investigations of n-6 PUFA on adipose tissue are restricted to cell and animal levels as the conflicting results are widely observed. Hernandez-Morante et al. \(^{(69)}\) conducted one human study in obese patients with BMI values greater than 40kg/m\(^2\) and reported
a significant negative association between n-6 PUFA intake and blood lipid levels. In addition, while most of the studies looked at the benefits of MUFA intake over SFA, very few studies focused on PUFA versus SFA on body weight changes and energy expenditure. Studies by Jones and Schoeller\(^{(70)}\) and Jones et al.\(^{(71)}\) both showed that the high PUFA: SFA ratio failed to affect diet-induced thermogenesis in a to 7-day or 14-day interventions, although obese subjects tended to experience a higher postprandial fat oxidation following such treatment whereas lean individuals showed no differences. Overall, the role of n-6 PUFA in obesity and body weight maintenance remains unanswered and requires further epidemiological and clinical data to draw the conclusion.

2.1.3 Summary

Dietary fat consumption has been closely associated with increasing obesity rates and higher risk of CVD. Therefore, when excessive fat consumption is avoided as suggested, a balanced dietary fatty acid composition is crucial to maintaining healthy status. Overall, based on current nutrition guidelines and research findings, consumption of the unsaturated fats, MUFA and PUFA, is likely more metabolically beneficial compared to SFA and TFA\(^{(72)}\). However, conflicting results for metabolic responses to different fatty acid intakes have also been observed. Therefore, comprehensive studies looking at long-term MUFA and PUFA dietary interventions become necessary in order to determine the optimal dietary amounts of specific fatty acids for CVD prevention in the future.
2.2 n-3 and n-6 metabolism and effect of genetic variants

2.2.2 n-3 and n-6 metabolism

The debate on the specific role of dietary ALA is ongoing (73; 74). A meta-analysis investigated the associations between intake of ALA in vegetable oils and mortality from fatal coronary heart disease, and also the onset of prostate cancer in several studies. It reported that consumption of ALA helped to reduce heart disease but with a significant increase in risk of developing prostate cancer (75). In 2010, another meta-analysis summarized a weak protective effect of dietary ALA intake as consumption of more than 1.5 g/day of ALA in which the risks of developing prostate cancer were significantly reduced (76).

Overall, abundant evidence proves that the association between dietary ALA consumption and the prevention of heart disease are positive. The consumption of ALA improves cardiovascular health by modulating circulating lipid concentrations, altering membrane structure and function by enhancing the total n-3 fatty acid content of cell membrane phospholipids, and reducing inflammatory reactions (58). However, since dietary EPA and DHA also shows the great efforts on CVD prevention, the lack of understanding is in which specific fatty acids EPA, DHA or their precursor, ALA play the key role in lowering lipid levels and reducing anti-inflammatory effects in CVD.

The metabolic conversion of dietary ALA to EPA and DHA is summarized as Sprecher’s pathway (Figure 2.1) (77). Additionally, the n-6 essential fatty acid, LA, also begins n-6 PUFA metabolism by the same desaturation and elongation pathway (77). It is believed that these n-6 and n-3 PUFA may possibly be competing for the same set of enzymes during metabolism. As a result, consuming high levels of LA may influence the metabolism of n-3 PUFA in the human
body (78). This competition can potentially be monitored by the concentrations of important n-3 or n-6 PUFA (ALA, EPA, DHA, LA, and AA) in plasma or tissues following various levels of LA and ALA dietary consumption in human feeding trials.

Sprecher’s pathway indicates that two groups of enzymes, desaturases and elongases have been involved and influence the conversion rate of dietary ALA to EPA and DHA in complex systems. Pawlosky et al. (79) reported that the conversion from ALA to EPA, EPA to DPA (n-3), and DPA (n-3) to DHA were 0.2%, 65% and 37%, respectively, which indicated that the delta-6-desaturase could be the predominate enzyme during the entire n-3 metabolism. Furthermore, numerous studies have also suggested that increased consumption of ALA does not lead to the elevation of DHA levels in plasma (80; 81; 82; 83). Therefore, to reveal the mechanisms under the dietary ALA intake and the corresponding fatty acid level shifts in human, the ideal design is to include various ALA or LA formula in the human clinical trial.
Figure 2.1 Mechanism of n-6 and n-3 fatty acid pathway \(^{(78)}\).
The most common and ethical way to investigate fatty acid metabolism involves dietary treatments using stable isotope-labeled tracer fatty acids followed by the analysis of plasma fatty acid profiles. This method allows for the avoidance of dangers associated with bio-hazards and radioactive isotopes in human clinical studies in vivo. When stable isotopically-labeled fatty acids pass through the human body, mass spectrometry is able to detect the isotope ratio shifts from human plasma fatty acid levels.

Numerous studies in the past have used [U-\textsuperscript{13}C] enriched ALA as the tracers in dietary treatments for tracking the conversion of ALA to EPA and DHA. This is because the mass ratio shifts on carbons can be easily recognized by mass spectrometry. Both low doses of [U-\textsuperscript{13}C] enriched ALA tracer (below 100 mg) and high doses (above 100 mg) have shown that the conversion to EPA and DHA is less than 8% and less than 0.5%, respectively. [U-\textsuperscript{13}C] enriched ALA is preferred also because both n-6 and n-3 fatty acid metabolism involve \( \beta \)-oxidation by releasing CO\(_2\), which would make it possible to estimate the energy metabolism with the proportion of \( ^{13}\text{CO}_2 \) in breath during human clinical trials.

However, it is very costly to manufacture large amounts of [U-\textsuperscript{13}C] enriched ALA for a large size clinical trial. Instead, deuterium (\( ^2\text{H} \)) enriched ALA has been used in similar human clinical trials as the tracer, showing similar trends in ALA conversion rates as those using [U-\textsuperscript{13}C] with limited conversion to EPA (< 6%) and DHA (< 3.8%). Moreover, the technique of using deuterium water has been proved to be able to detect the shifts of cholesterol levels in the past. Therefore, technically, lipid synthesis can also be measured by using deuterium-enriched water, calculating the number of incorporated deuterium atoms into synthesized fatty acid molecules.
although there are few studies which use regular dietary ALA treatment combined with a dose of deuterium water supplement as a stable isotope tracer in humans in vivo.

Both fatty acid synthesis and metabolism involve the participation of water molecules, thus, measuring the deuterium enriched ALA, LA, EPA, AA and DHA could easily indicate n-3 and n-6 fatty acid metabolism, especially for ALA conversion. Even if deuterium levels in these targeted PUFA’s are not sufficient to be detected, the deuterium water can certainly be used for OA and PA’s syntheses in humans, which still indicates fatty acid metabolism after ALA dietary interventions.

2.2.3 Key factors: enzyme activities and genetic variants

The key enzymes in the biosynthesis of n-6 and n-3 long chain PUFA from 18 carbon unsaturated fatty acids to 20 and 22 carbon fatty acids are the delta-5-desaturase (D5D) and delta-6-desaturase (D6D), which are encoded by the genes fatty acid desaturase 1 and 2 (FADS1 and FADS2, respectively) (91; 92). Following desaturation steps, this pathway is also believed to be regulated by several elongations of very-long-chain fatty acid (ELOVL) enzymes. Beyond the FADS gene clusters, the most related regions in the ELOVL family are the gene clusters ELOVL2 and ELOVL5 which likely transcribe the elongation of C20 to C22, and C18 to C20 compounds during n-3 long chain PUFA synthesis in mammals, respectively (93; 94; 95).

The expression levels of both desaturases and elongases can likely affect the activities of transcription factors such as peroxisome proliferator-activated receptor alpha (PPAR-α) (95) and sterol response element-binding protein 1 (SREBP-1) (96; 97). Many studies have reported that single nucleotide polymorphisms (SNPs) in FADS1 and FADS2 gene clusters are associated with
levels of long chain PUFAs in plasma, tissue and breast milk in humans (98; 99; 100). In humans, the strong association between FADS genotypes and fatty acid levels indicate that the genetic variants in FADS gene clusters influence the concentrations of n-6 and n-3 fatty acids, and in turn affect the essential fatty acid metabolism or even the pathogenesis of multiple diseases (97).

With regard to the elongation steps, knowledge of the regulation of ELOVL2 and ELOVL5 genes in mRNA/protein expression and enzymatic activities remain limited in humans (101; 102) and additionally, no solid conclusions have been reached regarding the association between ELOVL family and the concentrations of n-6 and n-3 fatty acids in Sprecher’s pathway (103). Several genome-wide association studies (GWAS) have shown some trends of certain polymorphisms in ELOVL2 gene are associated with different concentrations of EPA, DPA (n-3), and DHA during n-3 long chain PUFA metabolisms in human plasma (101; 104; 105; 106). One study showed an indirect conclusion that a certain SNP in ELOVL2 was significantly associated with C40 – C42 (107). Thus, it is ideal to involve large participant sizes in GWAS studies which would provide greater statistical power to confirm associations between potential locus and fatty acid concentrations (108).

Thus, clinical trials are needed to recruit a large number of participants and provide more opportunities to examine the dietary factors affecting FADS and ELOVL genetic variants that modulate ALA conversion. The result will also provide more information about dietary recommendations of ALA intake for general population.
2.3 Effects of fatty acid intake on fatty acid ethanolamides and on body fat maintenance

2.3.1 Fatty acids and fatty acid ethanolamides

Fatty acid ethanolamides (FAEs), also known as N-acylethanolamides, are a family of fatty acid derivatives which play a role in molecular signaling associated with physiological actions and multiple diseases \(^{(109)}\). Generally, the biosynthesis of FAEs involves transesterification of fatty acids by N-acyltransferase (NAT) activity from phosphatidylcholine and ethanolamine to N-acylphosphatidylethanolamine (NAPE) \(^{(110)}\). Subsequently, catalysis by the enzyme N-acylphosphatidylethanolamine-phospholipase D (NAPE-PLD) can also generate FAEs \(^{(111)}\). Conversely, FAEs can be degraded by the enzyme, fatty acid amide hydrolase (FAAH), and break down into fatty acids and ethanolamide. Dietary fatty acids can influence FAE levels through a group of enzymes including NAPE-PLD and FAAH \(^{(112)}\). Important FAEs include anandamide (arachidonoyl ethanolamide, AEA), oleyl ethanolamide (OEA), palmitoyl ethanolamide (PEA), linoleoyl ethanolamide (LEA), alpha-linolenoyl ethanolamide (ALEA), and docosahexaenoyl ethanolamide (DHEA).

AEA, the derivative of AA, was first identified in porcine brain \(^{(113)}\) and has been extensively characterized in many tissues such as the placenta, fetal membranes \(^{(114)}\) and bio-fluids such as human plasma and amniotic fluid \(^{(114)}\), and the nervous system \(^{(115)}\). Its biological functions are frequently discussed in terms of activation of the cannabinoid receptor 1 (CB1) \(^{(116)}\) and the AEA isomer, virodhamine (VA), which acts as an antagonist by binding to CB2 \(^{(117)}\). OEA and PEA are not associated with cannabinoid receptors although they are known to be structurally related to AEA \(^{(118)}\). In mammals, OEA is synthesized in the small intestine and its levels are always
positively correlated to the level of food intake. When food intake is high, OEA levels are usually elevated. While PEA plays a role in inflammation and nociception \(^{119}\), both OEA and PEA are shown to be involved in the activation of PPAR-\(\alpha\) \(^{120; 121}\). In addition, FAEs such as PEA and LEA are believed to have biological activities associated with anti-inflammatory properties over a range of concentrations as they can be observed in various tissues in both humans and animals \(^{122; 123; 124}\). However, very little is known about ALEA and DHEA, and their biological importance needs further confirmation.

2.3.2 Fatty acid ethanolamides and food intake

FAEs are a class of endogenous molecules with bioactive functions. The physiological significance was mainly recognized of three compounds: PEA as an anti-inflammatory and analgesic substance; AEA as an endocannabinoid ligand; and OEA as an anorexic substance. Recently, major research has focused on the role of FAEs in the regulation of food intake, energy expenditure and fat mass distribution, in particular OEA.

OEA, the endogenous lipid derived from OA, serves as a lipid messenger linking fat intake and satiety \(^{125}\). Food intake can trigger OEA formation in the small intestine. In rat studies, results showed three possible mechanisms accounting for such effect. First, the production of NAPE, the OEA precursor, is accelerated; second, the activation of NAPE-PLD, which is responsible for OEA synthesis, is enhanced; third, the OEA hydrolysis enzyme, FAAH, is inhibited in the gut \(^{126}\). Interestingly, such response to food intake only happens to the production of OEA and its close analog, LEA, but not to other equally abundant FAEs \(^{127}\). Further investigation suggested that among the major macronutrients, only fat had the potential to stimulate the production of OEA in the gut while sugar and protein had no such influence \(^{125}\). In particular, free OA which
is acquired from diet can stimulate mucosal cells to generate OEA in the lumen of the small intestine. In conclusion, OEA mobilization is selective and can be activated by food intake, especially diet-induced OA.

Another possible key factor regarding OEA production is the regulatory role of the membrane glycoprotein CD36. Studies with genetically modified mice identified a clear role of CD36 during fatty acid metabolism: a biosensor for diet-induced OA (125; 128). Evidence suggested that OA from digestive breakdown of complex dietary lipids might be internalized by mucosal cells in the small intestine through CD36, and further converted into its corresponding NAPE and FAE molecules (125). Although the actual role of CD36 in the entire process is not fully understood, one investigation on human CD36 pointed out that there were significant associations between oral fat intake and the genetic variants in CD36 gene (129). Data showed that people who were genotyped for high expression of the CD36 were 8-fold more sensitive to certain fatty acids, mainly, OA, than people with low CD36 expression. Thus, it is clear that dietary OA can trigger the OEA production in the gut, via a specific channel CD36-related process.

OEA can produce satiety and further regulate food intake and body weight, which makes OEA become a potential therapeutic solution for obesity. Evidence in rodents showed that oral administration of OEA effectively influenced the behavior of those free-feeding rats and mice, and caused a long-lasting inhibition of food intake and decreased meal frequency (120; 130; 131). These study data also ruled out possible factors including induced fear or anxiety, altered corticosterone levels, and taste aversion, indicating that there was a significant relationship between OEA and satiety in the rodent models.
Here, a question has been raised whether the ability of OEA to regulate food intake as an exogenous treatment might exert similar effect when OEA is endogenously produced. Fu et al. \(^{(132)}\) investigated the NAPE-PLD expression using an injected viral vector into the duodenum of rats during the following 12 days and found that the OEA production was increased together with a decreased meal frequency, which is in agreement with OEA having a presumed role in controlling food intake. Recently, Jones et al. \(^{(133)}\) investigated the effects of diets varying fatty acid compositions on plasma FAE levels and their potential associations with energy expenditure via lipid-signaling mechanisms. Data suggested that OEA levels were upregulated by dietary fatty acid profiles and were able to further modulate fat mass distribution in humans.

Regarding the mechanism of induced satiety by elevated OEA levels, it is well acknowledged that the action is activated by the binding of OEA onto PPAR-\(\alpha\) \(^{(120)}\). OEA binding as such stimulates the transcription of various PPAR-\(\alpha\) target genes in the small intestinal mucosa and liver. When the endogenous OEA levels are sufficient after the conversion from induced dietary OA, PPAR-\(\alpha\) is fully activated and is able to trigger the signal to enhance the satiety response via the vagus nerve, although the detailed mechanism is not yet fully understood \(^{(134)}\). It is noted that this action is very specific as only OEA but no other FAEs showed such strong binding; and only PPAR-\(\alpha\) but not other PPAR subtypes were activated by the OEA binding.

Therefore, it is clear that diet-induced OA is the substrate of OEA production, and is up taken in the epithelium of the small intestine via CD36. After OA is converted endogenously into OEA, OEA activates PPAR-\(\alpha\), and acts as a master regulator of lipid metabolism. Finally, PPAR-\(\alpha\) enhances the satiety response and regulates food intake by prolonging the interval between meals.
2.4 Dietary fatty acid intake and gut microbiota

It is believed that the composition of the gastrointestinal microbiota is influenced by both genetic factors and dietary intakes and that the microflora residing in human colon can be affected by dietary intervention \(^{(135; 136)}\). Evidence shows that the population of the gastrointestinal microbiome has a symbiotic link to systemic inflammation, immunity, intestinal function and metabolism \(^{(137; 138)}\). Healthy diets are believed to cause positive shifts in the colonic microbiome in the human gut \(^{(139)}\). The majority of dietary feeding studies focus on modulating intestinal microbial populations through the use of probiotics and prebiotics \(^{(140)}\). Human feces are considered to be the most convenient source and most representative sample of microbial populations for characterization of the gut microbiota profile \(^{(140)}\). Replacement of SFA with MUFA and PUFA rich oils is believed to positively impact on prevention of obesity-related disorders and CVD events. However, there is lack of information about the impacts of the metabolism of MUFA and PUFA in the human gut in these gut microorganisms and/or further on gastrointestinal diseases \(^{(38)}\).

2.4.1 Introduction to gut microbiota

The human gut is the natural habitat for a complex of microorganisms and this bacteria community is also known as gut microbiota. Because the uptake of major nutrients in humans happens along the gastrointestinal tract, the sophisticated community of gut microbiota provides useful functions for humans, including helping digestion of nutrients, stimulating cell growth, creating immune response \(^{(141)}\), and further influencing the whole body metabolism. Therefore, the most important factor that can change the gut microbiota profile is the diet. Studies have indentified some associations between gut microbiota and dietary intakes. This relationship may
be interesting in medical field as long term dietary interventions may allow modulation of an individual’s enterotype to improve health. There are roughly 12 phyla present in the digestive tracts of mammals. It is believed that a healthy balance between two dominant bacterial divisions, Bacteroidetes (20-40%) and Firmicutes (60-80%) should be maintained for intestinal health. Other major phyla in gastrointestinal tract (less than 1%) include Proteobacteria, Actinobacteria, Cyanobacteria, and TM7. Studies show differences in the diversity and abundances of gut microbiome between obese and healthy weight individuals after different dietary intakes both in animals and humans (142).

2.4.2 Association between gut microbiota and dietary intakes

It is not surprising that the composition of the gut microbiota can change dramatically in response to the long-term dietary intake as the nutrients through diets are essential to these bacteria. In order to investigate the linkages between the composition of the gut microbiota and dietary intakes, genetic factors along with immune functions, Hildebrandt et al. completed a 2-phase crossover animal trial, feeding 10 female 14-wk-old C57B/6 mice, 5 wild-type and 5 with RELMβ knockout genes, with a standard chow control or a high-fat diet (143). The RELMβ, a colonic goblet cell-specific gene, whose expression is dependent upon the presence of the gut microorganisms, is considered as an indicator of intestinal immune function. Result of this study showed that RELMβ null mice were relatively resistant to obesity showing a reduced ratio of Bacteroidetes to Firmicutes after consumption of a high-fat diet. Interestingly, similar changes in gut microbiota profiles were observed in both knockout and wild-type mice in response to the high-fat diet. Results suggest that a high-fat diet plays a role in reducing the ratio of
Bacteroidetes to Firmicutes in the composition of the gut microbiota, while feeding a fat-restricted diet increase this ratio.

2.4.3 Action of dietary fatty acids on gut microbiota

The traditional Western diet, commonly rich in SFA, has been associated with increasing risks of CVD, and diet-induced obesity. However, effects of healthy PUFA and MUFA-rich diets on the composition of the gut microbiota have not been well studied. Liu and colleagues designed a mouse study using 45, seven-wk-old *Mus musculus* to investigate the different effects of three diets, including one rich in n-3 PUFAs, one rich in n-6 PUFA, and third one rich in SFA (144). After the 14-wk treatment period, fecal pellets before and after the treatment periods were analyzed by V3 region of the 16S rRNA in order to assess shifts in microbial profile at the phylum and family levels in response to different diets. Significant decreases in the abundance of Bacterodetes were observed across all the three treatment groups over time, while SFA group showed a greater decrease in Bacterodetes by 28% than the other two PUFA groups, by 10% in n-3 PUFAs and 12% in n-6 PUFAs, respectively. Proportions of bacteria at the family level shifted across the three treatments, but no significant effects of diets on body mass change were observed. In conclusion, SFA was found to have more effects than n-3 or n-6 PUFA in shifting gut microbiota composition towards an obese profile in mice, but the shifts in gut microbiota proportions cannot influence body weight changes.

2.4.4 Gut microbiota and obesity

Obesity is commonly associated with a number of chronic events, including inflammation, insulin resistance, type II diabetes, and cardiovascular disease. Although the mechanistic links
between diversity of gut microbes and obesity are not clear, several studies have attempted to examine the relationship between obesity and the gut microbiota. A classical mice model was developed by Ley et al. using obese mice ob/ob, lean ob/+ , and wild-type siblings, and their ob/+ mothers to investigate the impact of genetic obesity on gut microbiota (145). Results from a 16S rRNA gene sequence survey found that ob/ob mice had a decreased abundance of Bacteroidetes and an increased population of Firmicutes. Date indicated that the ratio of Bacteroidetes to Firmicutes dynamically reflected the overall weight condition in the mouse model. A follow-up study to extend these observations to humans showed that the relative proportion of Bacteroidetes was reduced in obese participants compared to the lean controls. When obese individuals lost weight due to either carbohydrate-restricted or fat-restricted diets, the composition of gut microbiota showed the shift towards increasing abundance of Bacteroidetes (139). Another study on 154 volunteers confirmed that obesity is in relation to phylum-level changes in the microbiota, and the total diversity of the gut microbiota decreased in obese individuals with raised food intake (146). Overall, these studies revealed an attractive relationship between obesity and the gut microbiota, which might have potential therapeutic implications.

2.4.5 Conclusions

Numerous studies have demonstrated that the composition of the gut microbiota can shift dramatically in response to the change of dietary intakes. Overall, consumption of high-fat diets rich in SFA shows a significant relationship to the gut microbiota shift with a reduction of Bacteroidetes population and an increased abundance of Firmicutes. In contrast, diets rich in unsaturated fatty acids, generally, may not influence the composition of gut microbiota, however, data are limited. The prospective approaches to the mechanism of development of inflammation
in response to the shifts in the gut microbiota show that diet-induced obesity and insulin resistance may be associated with dietary energy intake. Moreover, it is still difficult to define a causal relationship in humans between diet and microbial composition since many lifestyle factors and genetic variants also have great impacts on altering the composition of gut microbiota. In addition, the knowledge gaps between shifts in the gastrointestinal microbiome in humans and dietary interventions rich in MUFA and PUFA must be addressed. There exists a need to investigate the microbial and metabolic mechanisms underpinning the health benefits associated with consumption of different dietary interventions which involve several formulas rich in MUFA and/or PUFA.

2.5 Summary

Although replacing SFA with unsaturated fats has favorably implications for management of CVD risk, there are confusions surrounding the specific health benefits and the optimal intake levels of different types of unsaturated fatty acids including n-9, n-6 and short- and long-chain n-3 fatty acids in the diets. Therefore, investigating independent health benefits of these common dietary MUFA and PUFA in a low SFA background setting on reducing CVD risk becomes necessary. In addition, this review has identified a number of key gaps in knowledge including diet-gene interactions associated with the whole body lipid metabolism, effects of FAEs on the regulation of food intake and body weight control, and associations between dietary fatty acid intakes and changes in gut microbiota composition. Therefore, further intervention studies of long-term duration can be specifically designed to address these questions, and further provide better suggestions and recommendations to general population who appear to under the risk of CVD.
2.6 References


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Bridge to chapter III

In order to explain the current knowledge gaps discussed in chapters I and II, the Canola Oil Multicenter Intervention Trial (COMIT) was conducted. The following chapter comprises a manuscript which provides the information the comprehensive design of the intervention study. This chapter also introduces the study rationale, objectives, primary and secondary outcomes and various analytical methods within in the entire multicenter study. In addition, as the preliminary results of the massive analyses, the post-treatment fatty acid profiles of all complete participants are presented to reflect the good compliance of the entire study across all participating clinical sites.
Chapter III

Plasma fatty acid changes following consumption of dietary oils containing n-3, n-6 and n-9 fatty acids at different proportions: preliminary findings of the Canola Oil Multicenter Intervention Trial (COMIT)

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This manuscript has been published in Trials. (2014), 15:136
3.1 Abstract

The Canola Oil Multicenter Intervention Trial (COMIT) was a randomized controlled cross-over study designed to evaluate the effects of five diets that provided different oils/oil blends on cardiovascular disease (CVD) risk factors in individuals with abdominal obesity. The present objective is to report preliminary findings on plasma fatty acid profiles in volunteers with abdominal obesity, following consumption of diets enriched with n-3, n-6 and n-9 fatty acids.

COMIT was conducted at three clinical sites, Winnipeg, Québec City, and University Park, PA. Inclusion criteria were waist circumference (≥90 cm for males and ≥84 cm for females), and at least one other criterion: triglycerides ≥1.7 mmol/L, HDL cholesterol <1 mmol/L (males) or <1.3 mmol/L (females), blood pressure ≥130 mmHg (systolic) and/or ≥85 mmHg (diastolic) and glucose ≥5.5 mmol/L. Weight-maintaining diets that included shakes with one of the dietary oil blends were provided during each of the five 30-day dietary phases. Dietary phases were separated by 4-week washout periods. Treatment oils were canola oil, high oleic canola oil, high oleic canola oil enriched with docosahexaenoic acid (DHA), flax oil and safflower oil blend, and corn oil and safflower oil blend. A per protocol approach with a mixed model analysis was decided as appropriate for data analysis. One hundred seventy volunteers were randomized and 130 completed the study with a dropout rate of 23.5%. Mean plasma total DHA concentrations, which were analyzed among all participants as a measure of adherence, increased by more than 100% in the DHA-enriched phase, compared to other phases, demonstrating excellent dietary adherence. Recruitment and retention strategies were effective in achieving a sufficient number of participants who completed the study protocol to enable sufficient statistical power to resolve small differences in outcome measures. It is expected that the study will generate important data.
thereby enhancing our understanding of the effects of n-3, n-6 and n-9 fatty acid-containing oils on CVD risks. Trial registration: ClinicalTrials.gov NCT01351012
3.2 Introduction

It is well established that decreasing dietary saturated fatty acids (SFA) reduces CVD risk \(^{(1)}\). Current dietary recommendations advise that unsaturated fatty acids replace SFA with little guidance provided about precise amounts of each that should be substituted \(^{(2)}\). Epidemiological evidence indicates various fatty acids classes including n-9 monounsaturated (MUFA), n-3 and n-6 polyunsaturated fatty acids (PUFA) as replacements for SFA with more health benefits \(^{(3; 4; 5; 6; 7; 8)}\). Despite the large body of research evaluating the effects of different fatty acid classes, studies that systematically and simultaneously compare multiple fatty acid classes have not been conducted. Moreover, a need exists to evaluate additional biomarkers beyond blood lipids/lipoproteins that better estimate risk of clinical outcomes \(^{(9)}\), and to achieve sufficiently large sample sizes in order to resolve modest differences and high variability in endpoint measurements. Additionally, significant knowledge gaps remain in our understanding of the effects of, and mechanisms underpinning the action of, the various fatty acid classes on risk factors for chronic diseases.

One such controversy is the debate surrounding alpha-linolenic acid (ALA). Whether its effects are dependent on its conversion to longer chain n-3 fatty acids \(^{(10; 11; 12)}\) needs to be better substantiated. The relative efficacy of different classes of PUFA, specifically linoleic acid (LA) \(^{(13)}\), ALA and DHA in modulating inflammatory processes and endothelial function also remains to be elucidated \(^{(14; 15)}\). Reported undesirable effects of LA \(^{(16)}\) are of particular concern. Direct comparison of dietary n-6 with n-3 fatty acids on inflammatory biomarkers and endothelial function would also be helpful in clarifying these issues. Since inflammation directly impacts
endothelial function and the progression of atherosclerosis\textsuperscript{(17; 18)}, endothelial function measurements would serve as useful biomarkers of CVD risk.

Abdominal obesity and insulin resistance are criteria for metabolic syndrome\textsuperscript{(19; 20)}. Dietary fat has been implicated in the pathogenesis of this syndrome via ectopic adipose tissue deposition\textsuperscript{(21; 22)}. Different classes of fatty acids appear to have differential effects on body fat accretion\textsuperscript{(23; 24; 25; 26)}. Consequently, a need also exists to evaluate the effects of various fatty acid classes on body composition and body fat distribution.

Based on the above rationale, a multicenter randomized clinical trial was designed to evaluate the biological effects of conventional canola oil, high oleic canola oil, DHA-enriched high oleic canola oil, a blend of flax oil with safflower oil, and a blend of corn oil and safflower oil. The contrast selected allowed for comparisons of the effects of oils rich in n-9 versus n-3 and n-6 oil blends. Circulating lipids/lipoproteins, inflammatory biomarkers, endothelial function and body composition were evaluated after consumption of each diet. Additionally, mechanistic assessments including reverse cholesterol transport, stable isotope fatty acid conversion, and fatty acid desaturase genetic variation analysis were also carried out. Consequently, the purpose of this study was to comprehensively investigate the effects of major fatty acid classes on biomarkers of chronic disease at a mechanistic level. This paper describes the protocol and subject recruitment experience for the COMIT study and presents plasma fatty acid data.
3.3 Methods/Design

3.3.1 Study design

COMIT was designed as a 5-phase randomized, controlled, double-blind, cross-over clinical trial. One-hundred and seventy subjects (n=95 females) aged 18 years or older were selected with at least one metabolic syndrome clinical criterion. Participants were recruited at two participating centers in Canada including the Richardson Centre for Functional Foods and Nutraceuticals (RCFFN) at the University of Manitoba in Winnipeg (n=69), and the Institute of Nutrition and Functional Foods (INAF) at Laval University in Québec City (n=58), as well as one clinical institution in the U.S. i.e.: Departments of Nutritional Sciences and Biobehavioral Health, the Pennsylvania State University, University Park (n=43). The Risk Factor Modification Centre (RFMC) at St. Michael’s Hospital in Toronto participated in the sample analyses.

3.3.2 Study recruitment and retention

Recruitment was conducted using radio and newspaper advertisements, direct mailings, community meetings and advertised information sessions as depicted in Table 3.1. On first contact, volunteers completed a questionnaire to determine their eligibility for the study. Qualifying participants were instructed to provide a fasting blood sample at their convenience. Eligible participants were selected based on the inclusion criteria and exclusion criteria described below. The flow of participants from initial contact to completion is given in Figure 3.1.

At RCFFN in Winnipeg, a social program for study participants termed “Club Richardson” was initiated for the purpose of fostering interaction and cooperation among participants to enhance their retention and compliance. Nutrition-related forums and outings were conducted, and tickets
to hockey and soccer games were provided. Specific events were conducted to coincide with celebrations such as Christmas. Wine and cheese events were also held during the washout periods. Cards and flowers were gifted to coincide with birthdays. Similar retention strategies were used at the other two clinical centers. Personal interaction was a high priority among the clinical staff and study participants at all centers.

Figure 3.31 Flow of participants in the COMIT study.

The participant flow through each step of the COMIT recruitment, screening and study protocol process.
Table 3.1 Recruitment strategies employed at participating clinical sites.

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<td>Recruitment seminars at community centers at various localities</td>
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<td><strong>Québec City</strong></td>
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</table>
3.3.3 Inclusion criteria

Initially, discussions about the eligibility criteria of the study were based on the following three parameters; 22-32 Kg/m² body mass index (BMI), plasma triglyceride >1.7 mmol/L and waist to hip ratio of >0.85. When the protocol was finalized, it was decided that focus should be placed on waist circumference as the primary inclusion criterion because of the relationship between visceral fat and endothelial function (27). At the beginning of the study, the ATP III metabolic syndrome criteria for waist circumference (>102 cm for men and >88 cm for women) were followed (28). As the trial progressed, the International Diabetes Federation metabolic syndrome criteria for waist circumference (≥94 cm for men and ≥80 cm for women) were adopted (29) to identify individuals in the initial stages of abdominal obesity who might benefit from dietary intervention. This change occurred at the same time in all centers and did not affect the subjects already recruited as the waist circumference cut-off values were lowered. Other inclusion criteria were triglyceride level (TG) ≥1.7 mmol/L, HDL cholesterol level (HDL) <1 mmol/L (males) or <1.3 mmol/L (females), blood pressure ≥130 mmHg (systolic) and/or ≥85 mmHg (diastolic) and glucose level ≥5.5 mmol/L. To be eligible, participants were required to meet at least one of the inclusion criteria.

3.3.4 Exclusion criteria

Individuals with thyroid disease, unless controlled by medication, diabetes mellitus, kidney disease, or liver disease, current smokers or those consuming more than two alcoholic drinks per week, were excluded from the study. Individuals taking medications known to affect lipid metabolism or endothelial function, including aspirin or other non-steroidal anti-inflammatory drugs, cholestyramine, colestipol, niacin, clofibrate, gemfibrozil, probucol, or HMG-CoA
reductase inhibitors were excluded. In addition, individuals were not allowed to participate if they were unwilling to stop taking any supplements at least 2-weeks prior to the study. Creatinine, serum glutamate pyruvate transaminase (SGPT), serum glutamic oxaloacetic transaminase (SGOT), alkaline phosphatase (ALKP), lactate dehydrogenase (LDH), glucose and lipid profiles that included total cholesterol (TC), TG, LDL, and HDL were also analyzed at screening.

3.3.5 Ethical considerations

The study protocols with ethical considerations were reviewed and approved by research ethics boards at each participating institution, including Biomedical Ethics Board at the University of Manitoba, Comité sectoriel d'éthique de la recherche en sciences de la santé de l'Université Laval, and the Institutional Review Board at the Pennsylvania State University. The trial was registered at clinicaltrials.gov under the registration number NCT01351012. Written informed consents were obtained after an initial interview with the participants that included a full description of the study and a discussion of the compliance issues and study expectations.

3.3.6 Randomization

Random permutations of the five treatments were created by the study director at the main site using a random number generator to ensure that all the subjects received treatments in a balanced and random order. Sequences were coded for the purpose of blinding the investigators and the subjects. Coded sequences were kept in sealed envelopes at the Winnipeg center and were assigned to subjects by study coordinators at respective sites as they joined the study across the three centers.
3.3.7 Study diets

Participants consumed a controlled weight-maintenance, full-feeding diet with a fixed macronutrient composition (35% fat, 50% carbohydrate, 15% protein, <200 mg cholesterol, 35-40 g fiber). They were provided with three meals and two snacks a day using a seven day rotating menu for the complete duration of each dietary phase, which lasted 4 weeks. Treatment oils were provided as a shake-styled beverage, divided in two equal doses to be consumed at breakfast and supper. Participants were instructed to consume one of their meals with a treatment beverage under supervision on site. Treatment oils were: 1) conventional canola oil (Canola), 2) high oleic canola oil (CanolaOleic), 3) 85% high oleic canola and 15% DHA oil blend (CanolaDHA), 4) 60% flax oil and 40% safflower oil blend (FlaxSaff), and 5) 25% corn oil and 75% safflower oil blend (CornSaff). The amount of treatment oil/oil blend was determined based on the calculated energy requirements of the participants, with the 3000 Kcal intake targeted to consume 60 g oil per day, and the 1800 and 2400 Kcal groups consume 36 g per day and 48 g per day, respectively. Oils were weighed to the nearest gram and added to the shakes. Meals and shakes were prepared in the metabolic kitchen where kitchen staff and clinical coordinators were blinded to the treatments. Meals and shakes were provided in cold packs for meals consumed offsite. These take-out meals were provided to participants at each center during their daily visit. Weekend meals and shakes were delivered to the participants’ place of residence or were dispensed at the feeding site on Fridays.

A subgroup of participants in Winnipeg prepared their own meals under the supervision of a trained individual. This was possible due to personal cultural practices using a common kitchen
to prepare community meals. Treatment shakes were prepared at the RCFFN and delivered to the community every 2-3 days.

Subjects were instructed to consume only the prepared meals and to limit their consumption of alcohol or caffeinated beverages. Diets were planned for every subject according to their energy requirements and were nutritionally adequate. Energy requirement was calculated using the Mifflin-St Jeor equation \(^{(30)}\). During each study period, body weights were monitored, and if subjects gained or lost weight, energy adjustments were made. Subjects were given a diet that met the Canadian and U.S. recommended nutrient intakes. The nutrient content of the diet was analyzed using FOOD PROCESSOR (ESHA Research, Salem, OR) to verify macronutrient composition and assure micronutrient adequacy.
Table 3.2 Fatty acid composition of dietary oils \(^a\).

<table>
<thead>
<tr>
<th>Fatty Acids</th>
<th>Canola</th>
<th>CanolaOleic ((85:15))</th>
<th>CanolaDHA ((60:40))</th>
<th>FlaxSaff ((60:40))</th>
<th>CornSaff ((25:75))</th>
</tr>
</thead>
<tbody>
<tr>
<td>C4:0</td>
<td>0.00</td>
<td>0.00</td>
<td>0.00</td>
<td>0.00</td>
<td>0.00</td>
</tr>
<tr>
<td>C6:0</td>
<td>0.00</td>
<td>0.00</td>
<td>0.00</td>
<td>0.00</td>
<td>0.00</td>
</tr>
<tr>
<td>C8:0</td>
<td>0.00</td>
<td>0.00</td>
<td>0.00</td>
<td>0.00</td>
<td>0.00</td>
</tr>
<tr>
<td>C10:0</td>
<td>0.00</td>
<td>0.00</td>
<td>0.00</td>
<td>0.00</td>
<td>0.00</td>
</tr>
<tr>
<td>C12:0</td>
<td>0.05</td>
<td>0.04</td>
<td>0.04</td>
<td>0.00</td>
<td>0.00</td>
</tr>
<tr>
<td>C14:0</td>
<td>0.04</td>
<td>0.04</td>
<td>0.47</td>
<td>0.00</td>
<td>0.00</td>
</tr>
<tr>
<td>C16:0</td>
<td>2.44</td>
<td>2.20</td>
<td>3.15</td>
<td>2.94</td>
<td>3.52</td>
</tr>
<tr>
<td>C17:0</td>
<td>0.04</td>
<td>0.05</td>
<td>0.10</td>
<td>0.00</td>
<td>0.01</td>
</tr>
<tr>
<td>C18:0</td>
<td>1.10</td>
<td>1.10</td>
<td>1.02</td>
<td>1.90</td>
<td>1.14</td>
</tr>
<tr>
<td>C20:0</td>
<td>0.39</td>
<td>0.39</td>
<td>0.34</td>
<td>0.00</td>
<td>0.06</td>
</tr>
<tr>
<td>C22:0</td>
<td>0.19</td>
<td>0.19</td>
<td>0.18</td>
<td>0.00</td>
<td>0.00</td>
</tr>
<tr>
<td>C24:0</td>
<td>0.11</td>
<td>0.11</td>
<td>0.09</td>
<td>0.00</td>
<td>0.00</td>
</tr>
<tr>
<td>Total saturated</td>
<td>4.33</td>
<td>3.91</td>
<td>5.19</td>
<td>4.87</td>
<td>4.73</td>
</tr>
<tr>
<td>C14:1</td>
<td>0.00</td>
<td>0.00</td>
<td>0.01</td>
<td>0.00</td>
<td>0.00</td>
</tr>
<tr>
<td>C16:1</td>
<td>0.15</td>
<td>0.13</td>
<td>0.12</td>
<td>0.00</td>
<td>0.02</td>
</tr>
<tr>
<td>C17:1</td>
<td>0.07</td>
<td>0.12</td>
<td>0.10</td>
<td>0.00</td>
<td>0.00</td>
</tr>
<tr>
<td>C18:1</td>
<td>35.2</td>
<td>42.9</td>
<td>38.0</td>
<td>10.7</td>
<td>10.6</td>
</tr>
<tr>
<td>C20:1</td>
<td>0.73</td>
<td>0.72</td>
<td>0.62</td>
<td>0.00</td>
<td>0.02</td>
</tr>
<tr>
<td>C22:1</td>
<td>0.03</td>
<td>0.04</td>
<td>0.04</td>
<td>0.00</td>
<td>0.00</td>
</tr>
<tr>
<td></td>
<td>Canola</td>
<td>CanolaOleic</td>
<td>CanolaDHA</td>
<td>FlaxSaff</td>
<td>CornSaff</td>
</tr>
<tr>
<td>----------------</td>
<td>---------------</td>
<td>-------------</td>
<td>-----------</td>
<td>----------</td>
<td>----------</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td>37.7</td>
<td>43.2</td>
<td>38.3</td>
<td>10.7</td>
<td>10.6</td>
</tr>
<tr>
<td><strong>Monounsaturated</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C18:2</td>
<td>11.7</td>
<td>8.84</td>
<td>7.7</td>
<td>22.5</td>
<td>41.6</td>
</tr>
<tr>
<td>C18:3</td>
<td>5.86</td>
<td>1.38</td>
<td>1.18</td>
<td>19.19</td>
<td>0.17</td>
</tr>
<tr>
<td>C18:4</td>
<td>0.00</td>
<td>0.00</td>
<td>0.00</td>
<td>0.00</td>
<td>0.00</td>
</tr>
<tr>
<td>C20:4</td>
<td>0.00</td>
<td>0.00</td>
<td>0.04</td>
<td>0.00</td>
<td>0.00</td>
</tr>
<tr>
<td>C20:5</td>
<td>0.00</td>
<td>0.00</td>
<td>0.09</td>
<td>0.00</td>
<td>0.00</td>
</tr>
<tr>
<td>C22:5</td>
<td>0.00</td>
<td>0.00</td>
<td>1.42</td>
<td>0.00</td>
<td>0.00</td>
</tr>
<tr>
<td>C22:6,</td>
<td>0.00</td>
<td>0.00</td>
<td>3.48</td>
<td>0.00</td>
<td>0.00</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td>17.6</td>
<td>10.3</td>
<td>14.0</td>
<td>41.7</td>
<td>41.8</td>
</tr>
<tr>
<td><strong>Polyunsaturated</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

aData are given per 60 g oil. Canola: Conventional canola oil, CanolaOleic: High oleic canola, CanolaDHA: High oleic canola and DHA blend, FlaxSaff: Flax and Safflower blend, CornSaff: Corn and Safflower blend.
3.3.8 Compliance

Compliance during the trial was determined based on the remaining amounts of the treatment shakes in pre-packaged food packs intended for off-site consumption and by a weekly dietary questionnaire on dietary deviations which ensured that macro- and micronutrient intakes remained unchanged. Subjects consumed at least one meal on site further ensuring the compliance. These observations were further confirmed by expected changes in plasma fatty acid profiles at the end of each dietary period. For example, a greater than 2-fold baseline to endpoint increase in serum DHA was expected during the CanolaDHA dietary period.

3.3.9 Intervention

Four oils with different n-9: n-6: n-3 ratios were selected as the intervention: conventional canola oil (6:2:1), high oleic canola oil (31:6:1), DHA-enriched high oleic canola oil (6:1:1), and a flax oil and safflower oil blend (1:1:1). The fatty acid composition varied appreciably among treatment oils (Table 3.2), allowing comparison of the effects of n-3 fatty acids, ALA and DHA, n-6 fatty acid, LA, and n-9 fatty acid, oleic acid (Table 3.3).
Table 3.3 Percentage of calories from different fatty acid classes in each diet.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>SFA (%)</th>
<th>MUFA (%)</th>
<th>PUFA (%)</th>
<th>Omega-3 (%)</th>
<th>Omega-6 (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>ALA</td>
<td>DHA</td>
</tr>
<tr>
<td>Canola</td>
<td>7.20</td>
<td>62.8</td>
<td>29.5</td>
<td>9.80</td>
<td>0.00</td>
</tr>
<tr>
<td>CanolaOleic</td>
<td>6.50</td>
<td>72.0</td>
<td>17.0</td>
<td>2.30</td>
<td>0.00</td>
</tr>
<tr>
<td>CanolaDHA</td>
<td>8.60</td>
<td>63.8</td>
<td>23.3</td>
<td>2.00</td>
<td>5.8</td>
</tr>
<tr>
<td>CornSaff</td>
<td>7.90</td>
<td>17.7</td>
<td>69.6</td>
<td>&lt;1.00</td>
<td>0.00</td>
</tr>
<tr>
<td>FlaxSaff</td>
<td>8.10</td>
<td>17.9</td>
<td>69.4</td>
<td>32.0</td>
<td>0.00</td>
</tr>
</tbody>
</table>
3.3.10 Comparison dietary fat blend

An oil blend of corn (25%) and safflower (75%) with n-9: n-6: n-3 ratio of 6:25:0.1 was used as the comparison instead of a high saturated fat blend. Since the effects of saturated fat have been well established, it was deemed unnecessary to include it as a control. Moreover, we previously examined the effects of high oleic canola and flax oil compared to a diet high in saturated fat \(^{23, 31}\). Additionally, n-6 fatty acids, which are abundant in the comparison oil blend, are considered essential and healthy, however, the concept of the n-6 to n-3 ratio and the effects on inflammation merits re-examination \(^{13, 16}\). Use of n-6 rich oil enabled us to investigate these concepts.

3.3.11 Outcome measures

Endothelial function was identified as the primary endpoint based on our rationale of evaluating emerging CVD risk factors beyond traditional lipid biomarkers for CVD. Secondary endpoints and laboratory analyses are presented in Table 3.4. All centers followed identical procedures on all the measurements to minimize the variation. The Peripheral Arterial Tonometry (PAT) technique was utilized to measure endothelial function. By using an Endo-PAT device (Itamar Medical, Israel), this technique measures the reactive hyperemia which is significantly correlated with endothelium-dependent vasodilatation \(^{32}\). Endothelial function testing was carried out after a 12 h fast in the morning before the breakfast at beginning and end of each phase. Scheduling pre-menopausal women for testing within the first seven days of their cycle was attempted to avoid the impact of hormonal fluctuations on the endothelial function test. Dual energy x-ray absorptiometry (DEXA) scanning and blood pressure recording using a digital blood pressure monitor, were completed at beginning and end of each treatment. Blood pressure was recorded
three times and the mean value used for data analysis. Waist circumference also was taken at the
beginning and end of each phase. Twelve-hour fasting blood samples were collected on days 1, 2,
29 and 30 in both EDTA or heparin coated tubes. Blood samples obtained on days 1 and 2 were
used to determine baseline values for study endpoints, whereas blood samples obtained on the
two last days were used for the endpoint values. Blood was centrifuged for 20 min at 520 × g to
separate either serum or plasma depending on the requirement of the analysis. Aliquots of
plasma and sera were stored at -80°C until analysis. Deuterium oxide water was administered on
day 29 (0.7 g × kg body water) to track ALA interconversion to longer chain n-3 PUFA.

Aliquots of one milliliter plasma EDTA samples obtained on days 29 and 30 of each phase from
all participants were assembled at RCFFN for total plasma fatty acid profile analyses. Given the
double-blinded cross-over design, compliance was assessed by total plasma fatty acid
concentrations across the three clinical sites and all five phases. Plasma fatty acids were
extracted by the classic Folch method using chloroform-methanol (2:1, v/v) containing 0.01%
butylated hydroxytoluene (Sigma-Aldrich, Oakville, ON, Canada), followed by methylation with
methanolic HCl. Fatty acid methyl esters were then analyzed using an Agilent 6890N gas
chromatograph equipped with a flame ionization detector (Agilent Technologies, Mississauga,
ON, Canada). During the extraction and methylation, heptadecanoic acid (C17:0) as an internal
standard (Sigma-Aldrich, Oakville, ON, Canada). Known fatty acid standards (NuChek Prep, Inc.
Elysian, MN, USA) were used to identify the individual fatty acids in plasma samples. The level
of each fatty acid was then calculated according to the corresponding peak area relative to the
total area of total interested fatty acids and considered as percentage of total fatty acids.
Table 3.4 Secondary endpoints analyzed in the COMIT study.

<table>
<thead>
<tr>
<th>Secondary endpoints</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cholesterol</td>
</tr>
<tr>
<td>LDL</td>
</tr>
<tr>
<td>HDL</td>
</tr>
<tr>
<td>Triglycerides</td>
</tr>
<tr>
<td>Blood glucose</td>
</tr>
<tr>
<td>Apolipoprotein AI</td>
</tr>
<tr>
<td>Apolipoprotein B</td>
</tr>
<tr>
<td>CRP</td>
</tr>
<tr>
<td>IL-6</td>
</tr>
<tr>
<td>E-selectin</td>
</tr>
<tr>
<td>ICAM</td>
</tr>
<tr>
<td>VCAM</td>
</tr>
<tr>
<td>Body fat %</td>
</tr>
<tr>
<td>Lean mass</td>
</tr>
<tr>
<td>Fat mass</td>
</tr>
<tr>
<td>Android to gynoid ratio</td>
</tr>
<tr>
<td>Conversion of ALA to EPA and DHA</td>
</tr>
<tr>
<td>Fatty acid ethanolamide metabolites of oleic acid</td>
</tr>
</tbody>
</table>
**FADS and ELOVL genetic variant (SNP) analyses**

<table>
<thead>
<tr>
<th>Lipoprotein particle size</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lipoprotein cholesterol ester composition</td>
</tr>
<tr>
<td>Body weight</td>
</tr>
<tr>
<td>Waist circumference</td>
</tr>
<tr>
<td>Blood pressure</td>
</tr>
</tbody>
</table>
3.3.12 Data management and statistical analysis

Initially, a sample size of 40 subjects per each of the three centers (120 in total) was determined based on endothelial function as assessed by flow mediated dilatation (FMD), as the primary outcome with an anticipated 20% difference in means and a standard deviation of 1.35 \(^{(34)}\). The alpha and power were 0.05 and 0.8, respectively. Endothelial function is a continuous variable which measures endothelium-dependent vasodilatation. Just before the beginning of the trial, it was decided to use the Endo-PAT technique for the measurement of the endothelial function and a new sample size calculation was not made since Reactive hyperemia index (RHI) measured by the Endo-PAT technique is significantly correlated with FMD \(^{(35)}\) and has a similar variability \(^{(36)}\). We hypothesized that the favorable lipid changes associated with our proposed dietary interventions would improve endothelial function short-term. One hundred and seventy subjects were recruited with the anticipation of approximately 20% dropout rate, based on our prior experience and considering the long-term commitment needed for the trial. Data from all centers were uploaded to a secure file storage system and specific centers were selected for analysis based on their expertise. Data were manually checked for accuracy. A per protocol approach with a mixed model analysis was decided as appropriate for data analysis utilizing SAS version 9.2. Kolmogorov-Smirnov or Cramer-von Mises tests and histograms were used to test the Gaussian nature of the variables and log transformation was used if necessary.

3.4 Results

3.4.1 Recruitment, screening, enrollment and retention

From the methodologies used to recruit participants (Table 3.1), the most successful recruitment strategy at the Winnipeg center was direct mailings in selected neighbourhoods. Other centers
used University email list-serves to recruit participants. Figure 3.1 presents the flow of participants through the study. After the initial contact, 63% of volunteers were screened for clinical assessment. Only 24% of the volunteers who underwent clinical assessment were eligible for the study. A total of 170 men and women were randomized, which represents 15% of the total number who underwent primary screening. The enrollment rate was 17%, 18% and 12% for the Winnipeg, Québec City and University Park centers, respectively.

As shown in Figure 3.1, Winnipeg had the highest number of participants (n=69), while University Park had the fewest (n=43). The dropout rates in the centers were 22%, 21%, and 30% for Winnipeg, Québec City and University Park, respectively. The overall dropout rate was 23.5%. The effect of the dropouts on the study was minimal because 77.5% of the dropouts occurred within the first two phases of the study. Moreover, baseline characteristics of the dropouts and the completers did not differ significantly (data not shown). Challenges in retaining participants were expected given the 9-month duration of the trial, and participant study requirements, i.e., frequent visits to the centers, as well as the full-feeding protocol. As presented in Figure 3.1, health issues, moving out of town, level of commitment and job issues were the major factors behind cessation in the Winnipeg and Québec City centers. Food issues and academic/work issues were the major reasons for the University Park center.

3.4.2 Participant characteristics

Participants were randomized to dietary treatments from September, 2010 through March, 2012. Participants were classified as having metabolic syndrome as defined by IDF (Table 3.5). Winnipeg had the highest percentage of participants with metabolic syndrome (56%), followed by University Park (43%) and Québec City (39%). Overall, 47% of participants were
characterized as having metabolic syndrome. Although almost 50% of the participants had metabolic syndrome, their 10-year coronary heart disease risk score was less than 4% as determined by Framingham risk score calculation (data not shown). Baseline characteristics of participants from the different centers and the entire cohort are presented in Table 3.6.
Table 3.5 The number of metabolic syndrome criteria met by the participants in the COMIT study at each participating center $^a$.

<table>
<thead>
<tr>
<th></th>
<th>Winnipeg</th>
<th>Québec city</th>
<th>University Park</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>One</td>
<td>13.0%</td>
<td>6.5%</td>
<td>0%</td>
<td>7.7%</td>
</tr>
<tr>
<td>Two</td>
<td>31.5%</td>
<td>54.4%</td>
<td>56.7%</td>
<td>45.4%</td>
</tr>
<tr>
<td>Three</td>
<td>16.7%</td>
<td>26.1%</td>
<td>30.0%</td>
<td>23.1%</td>
</tr>
<tr>
<td>Four</td>
<td>33.3%</td>
<td>10.9%</td>
<td>13.3%</td>
<td>20.8%</td>
</tr>
<tr>
<td>Five</td>
<td>5.6%</td>
<td>2.2%</td>
<td>0%</td>
<td>3.1%</td>
</tr>
</tbody>
</table>

$^a$ Based on International Diabetic Federation defined criteria for metabolic syndrome; waist circumference ($\geq$94 cm for men and $\geq$80 cm for women), triglycerides (TG) $\geq$1.7 mmol/L, HDL cholesterol (HDL) $<$1 mmol/L (males) or $<$1.3 mmol/L (females), blood pressure $\geq$130 mmHg (systolic) and/or $\geq$85 mmHg (diastolic) and glucose $\geq$ 5.5 mmol/L.
3.4.3 Post-treatment fatty acid profiles

Total plasma fatty acid profiles of the completers of all five phases are summarized in Table 3.7. All fatty acid concentrations were calculated as percentage values of total identified fatty acids measured. As DHA was part of the dietary treatment, post-treatment plasma DHA values were used to evaluate the overall compliance of the intervention study. The observed significant increases in DHA concentration only following the CanolaDHA treatment indicated good compliance in all centers. Besides the shift in DHA levels, other changes also successfully reflected their dietary intake after the 28-d treatment phases. Briefly, Canola and CanolaOleic showed highest level of MUFA contents (p<0.05), since their oleic acid levels were significantly higher than other treatments. Two PUFA-rich diets significantly elevated PUFA contents compared to the other three canola-based MUFA diets (p<0.05), due to their higher levels of linoleic acid. FlaxSaff provided the lowest level of arachidonic acid level (p<0.05), indicating the competition between n-3 and n-6 synthesis as the FlaxSaff oil treatment provided the highest level of dietary ALA. CanolaDHA and FlaxSaff also showed higher levels of EPA compared to other three treatments (p<0.05). In general, the ratio of n-6 to n-3 also successfully explained the corresponding response to n-3 rich and n-6 rich dietary treatments, indicating good compliance across the entire study among participants.
Table 3.6 Baseline characteristics of participants at each participating center.

<table>
<thead>
<tr>
<th></th>
<th>Winnipeg</th>
<th>Québec city</th>
<th>University Park</th>
<th>Total</th>
<th>P value&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Male</td>
<td>16</td>
<td>29</td>
<td>15</td>
<td>60</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Female</td>
<td>38</td>
<td>17</td>
<td>15</td>
<td>70</td>
<td></td>
</tr>
<tr>
<td>BMI (Kg/m&lt;sup&gt;2&lt;/sup&gt;)</td>
<td>29.7±5.00</td>
<td>30.0± 4.30</td>
<td>29.7± 3.30</td>
<td>29.8±4.40</td>
<td>0.9277</td>
</tr>
<tr>
<td>Age (years)</td>
<td>43.9±15.8</td>
<td>49.9± 14.2</td>
<td>45.9± 9.60</td>
<td>46.5±14.2</td>
<td>0.1045</td>
</tr>
<tr>
<td>Total cholesterol (mmol/L)</td>
<td>5.40±1.10</td>
<td>5.40± 1.00</td>
<td>5.10± 0.90</td>
<td>5.30±1.10</td>
<td>0.3705</td>
</tr>
<tr>
<td>HDL-cholesterol (mmol/L)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>female</td>
<td>1.30±0.04</td>
<td>1.40± 0.10</td>
<td>1.30± 0.10</td>
<td>1.30±0.04</td>
<td>0.4130</td>
</tr>
<tr>
<td>male</td>
<td>1.10±0.10</td>
<td>1.10± 0.04</td>
<td>1.10± 0.10</td>
<td>1.10±0.03</td>
<td>0.9060</td>
</tr>
<tr>
<td>LDL-cholesterol (mmol/L)</td>
<td>3.40±1.00</td>
<td>3.40± 0.90</td>
<td>3.20± 0.90</td>
<td>3.40±0.90</td>
<td>0.5724</td>
</tr>
<tr>
<td>Triglycerides (mmol/L)</td>
<td>1.60±0.90</td>
<td>1.80± 0.80</td>
<td>1.50± 0.90</td>
<td>1.70±0.90</td>
<td>0.3380</td>
</tr>
<tr>
<td>Glucose (mmol/l)</td>
<td>5.40±1.70</td>
<td>5.30±0.60</td>
<td>5.30±0.50</td>
<td>5.40±1.10</td>
<td>0.7884</td>
</tr>
<tr>
<td>Body weight (kg)</td>
<td>80.7±15.9</td>
<td>85.8±15.3</td>
<td>89.9±15.7</td>
<td>84.7±15.9</td>
<td>0.0424</td>
</tr>
<tr>
<td>Waist (cm) Circumference</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>female</td>
<td>90.6±11.9</td>
<td>97.4±10.3</td>
<td>98.5±9.90</td>
<td>93.4±11.6</td>
<td>0.0331</td>
</tr>
<tr>
<td>male</td>
<td>103±13.8</td>
<td>107±8.70</td>
<td>107±6.50</td>
<td>106±9.90</td>
<td>0.5082</td>
</tr>
<tr>
<td>SBP (mmHg)</td>
<td>122±20.3</td>
<td>120±14.2</td>
<td>119±12.4</td>
<td>121±16.7</td>
<td>0.5477</td>
</tr>
<tr>
<td>DBP (mmHg)</td>
<td>81.2±12.4</td>
<td>70.5±10.5</td>
<td>79.7±7.40</td>
<td>77.0±11.8</td>
<td>&lt;0.0001</td>
</tr>
</tbody>
</table>

<sup>a</sup> P values were calculated using ANOVA.
Analysis of variance was used to analyze between-group differences in continuous variables. For categorical variables, Fisher's exact test or chi square test was used. Abbreviations: BMI: Body Mass Index; SBP: Systolic Blood Pressure; DBP: Diastolic Blood Pressure.
Table 3.7 Plasma fatty acid profile of participants at the end of each dietary phase in 130 participants (g/100g).

<table>
<thead>
<tr>
<th></th>
<th>Canola</th>
<th>CanolaOleic</th>
<th>CanolaDHA</th>
<th>FlaxSaff</th>
<th>CornSaff</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>14:0</strong></td>
<td>0.71±0.04</td>
<td>0.74±0.04</td>
<td>0.71±0.04</td>
<td>0.72±0.04</td>
<td>0.68±0.04</td>
</tr>
<tr>
<td><strong>c14:1</strong></td>
<td>0.12±0.02</td>
<td>0.08±0.02</td>
<td>0.09±0.02</td>
<td>0.08±0.02</td>
<td>0.07±0.02</td>
</tr>
<tr>
<td><strong>c16:0</strong></td>
<td>27.04±0.20&lt;sup&gt;a&lt;/sup&gt;</td>
<td>27.40±0.20&lt;sup&gt;a&lt;/sup&gt;</td>
<td>28.10±0.20&lt;sup&gt;b&lt;/sup&gt;</td>
<td>27.41±0.20&lt;sup&gt;a&lt;/sup&gt;</td>
<td>27.35±0.20&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td><strong>c16:1</strong></td>
<td>1.14±0.08</td>
<td>1.10±0.08</td>
<td>0.94±0.08</td>
<td>1.30±0.08</td>
<td>1.13±0.75</td>
</tr>
<tr>
<td><strong>c18:0</strong></td>
<td>11.83±0.10&lt;sup&gt;a&lt;/sup&gt;</td>
<td>11.79±0.10&lt;sup&gt;a&lt;/sup&gt;</td>
<td>12.28±0.10&lt;sup&gt;b&lt;/sup&gt;</td>
<td>12.51±0.10&lt;sup&gt;c&lt;/sup&gt;</td>
<td>12.34±0.10&lt;sup&gt;bc&lt;/sup&gt;</td>
</tr>
<tr>
<td><strong>c18:1 n-9</strong></td>
<td>14.90±0.19&lt;sup&gt;a&lt;/sup&gt;</td>
<td>15.52±0.19&lt;sup&gt;b&lt;/sup&gt;</td>
<td>13.36±0.19&lt;sup&gt;c&lt;/sup&gt;</td>
<td>12.10±0.18&lt;sup&gt;d&lt;/sup&gt;</td>
<td>11.62±0.18&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
<tr>
<td><strong>c18:2 n-6</strong></td>
<td>22.00±0.23&lt;sup&gt;a&lt;/sup&gt;</td>
<td>21.52±0.23&lt;sup&gt;a&lt;/sup&gt;</td>
<td>18.68±0.23&lt;sup&gt;b&lt;/sup&gt;</td>
<td>25.13±0.23&lt;sup&gt;c&lt;/sup&gt;</td>
<td>25.93±0.23&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
<tr>
<td><strong>c18:3 n-6</strong></td>
<td>0.17±0.07</td>
<td>0.18±0.07</td>
<td>0.23±0.07</td>
<td>0.12±0.07</td>
<td>0.17±0.07</td>
</tr>
<tr>
<td><strong>c18:3 n-3</strong></td>
<td>0.79±0.03&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.63±0.03&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.57±0.03&lt;sup&gt;bc&lt;/sup&gt;</td>
<td>1.61±0.03&lt;sup&gt;d&lt;/sup&gt;</td>
<td>0.49±0.03&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td><strong>c20:0</strong></td>
<td>0.50±0.02&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.48±0.02&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>0.49±0.02&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.41±0.02&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.46±0.02&lt;sup&gt;ab&lt;/sup&gt;</td>
</tr>
<tr>
<td><strong>c20:1 n-9</strong></td>
<td>0.32±0.02&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.32±0.02&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.27±0.02&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>0.18±0.02&lt;sup&gt;c&lt;/sup&gt;</td>
<td>0.23±0.02&lt;sup&gt;bc&lt;/sup&gt;</td>
</tr>
<tr>
<td><strong>c20:2 n-6</strong></td>
<td>0.35±0.03&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.31±0.03&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>0.24±0.03&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.32±0.03&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>0.37±0.03&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td><strong>c20:3 n-6</strong></td>
<td>2.45±0.07&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>2.53±0.07&lt;sup&gt;b&lt;/sup&gt;</td>
<td>1.79±0.07&lt;sup&gt;c&lt;/sup&gt;</td>
<td>1.86±0.07&lt;sup&gt;c&lt;/sup&gt;</td>
<td>2.28±0.07&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td><strong>c20:4 n-6</strong></td>
<td>9.28±0.15&lt;sup&gt;a&lt;/sup&gt;</td>
<td>9.67±0.15&lt;sup&gt;b&lt;/sup&gt;</td>
<td>9.70±0.15&lt;sup&gt;b&lt;/sup&gt;</td>
<td>8.27±0.15&lt;sup&gt;c&lt;/sup&gt;</td>
<td>9.59±0.15&lt;sup&gt;ab&lt;/sup&gt;</td>
</tr>
<tr>
<td><strong>c20:5 n-3</strong></td>
<td>1.09±0.04&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.86±0.04&lt;sup&gt;b&lt;/sup&gt;</td>
<td>1.53±0.04&lt;sup&gt;c&lt;/sup&gt;</td>
<td>1.45±0.04&lt;sup&gt;c&lt;/sup&gt;</td>
<td>0.49±0.04&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
<tr>
<td><strong>c22:0</strong></td>
<td>0.94±0.03&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>0.90±0.03&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>1.00±0.03&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.90±0.03&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.93±0.03&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td><strong>c22:4 n-6</strong></td>
<td>0.43±0.08</td>
<td>0.29±0.08</td>
<td>0.25±0.08</td>
<td>0.22±0.08</td>
<td>0.39±0.08</td>
</tr>
<tr>
<td><strong>c22:5 n-3</strong></td>
<td>0.81±0.03&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.72±0.03&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.34±0.03&lt;sup&gt;c&lt;/sup&gt;</td>
<td>0.97±0.03&lt;sup&gt;d&lt;/sup&gt;</td>
<td>0.62±0.03&lt;sup&gt;e&lt;/sup&gt;</td>
</tr>
<tr>
<td><strong>c22:6 n-3</strong></td>
<td>2.84±0.09&lt;sup&gt;a&lt;/sup&gt;</td>
<td>2.79±0.09&lt;sup&gt;a&lt;/sup&gt;</td>
<td>7.21±0.10&lt;sup&gt;b&lt;/sup&gt;</td>
<td>2.59±0.09&lt;sup&gt;a&lt;/sup&gt;</td>
<td>2.66±0.09&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>c24:0</td>
<td>c24:1 n-9</td>
<td>Total SFA</td>
<td>Total MUFA</td>
<td>Total PUFA</td>
</tr>
<tr>
<td>----------------</td>
<td>--------</td>
<td>-----------</td>
<td>-----------</td>
<td>------------</td>
<td>------------</td>
</tr>
<tr>
<td></td>
<td>0.72±0.03&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.65±0.03&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.74±0.03&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.74±0.03&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.73±0.03&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

The values are % abundance of each fatty acid to total fatty acids given as least squares mean ± SE for 130 individuals. Total fatty acid values and ratios are calculated. Mixed-effects repeated-measures analysis of variance with treatment, age, gender and the dietary phase as fixed effect, the last diet (sequence effect) as a random effect, and the measures for each subject by phases were used for the data analysis. Canola: Conventional canola oil, CanolaOleic: High oleic canola, CanolaDHA: High oleic canola and DHA blend, FlaxSaff: Flax and Safflower blend, CornSaff: Corn and Safflower blend. <sup>abcde</sup> Mean values with different superscript letters across rows denote significant differences at p<0.05.
3.4.4 Primary and secondary outcome measures

Data analyses of endothelial function and secondary outcome measures given in Table 3.4 are presented elsewhere.

3.4.5 Adverse effects

Adverse effects were monitored using a questionnaire distributed by study staff on every Friday of each dietary phase. Additionally, the clinical study staff assessed the tolerability of the treatment each day when they interacted with the participants. No adverse effects of any treatment diets were reported.

3.5 Discussion

Recruitment and retention strategies employed resulted in successful completion of the required number of participants. Retention was high also because of the dedication and commitment of the study volunteers and clinical coordinators, as well as the success of the social retention enhancement program. Differences across centers in demographic characteristics and baseline parameters were limited to the male to female ratio, total cholesterol level and diastolic blood pressure. The success rate of participant completion was numerically close between Winnipeg and Québec City, with the University Park center having a lower rate. The major reason for dropouts in Winnipeg and Québec City were health reasons, while at University Park it was related to experimental diet acceptability.

Predictable elevations of specific plasma fatty acids representing the major dietary fatty acids in the intervention oils were observed as expected. For example, the three-fold elevation of plasma ALA following FlaxSaff dietary phase, compared to CornSaff, demonstrated the ability of flax
oil enriched shakes to raise serum ALA levels. Similarly, the 1.3 fold elevation of oleic acid after the CanolaOleic treatment, compared to CornSaff treatment, is consistent with its high oleic acid content. Significantly higher eicosapentaenoic acid (EPA) levels in Canola and FlaxSaff, compared to other treatments, are evidence for the metabolic conversion of ALA to EPA. Significant elevation of EPA in CanolaDHA, compared to other treatments except FlaxSaff, could probably be due to the higher EPA content in algal sourced DHA that was used here. Or, DHA might convert back to EPA in CanolaDHA, which can be further confirmed by the stable isotope trafficking assays in this study \(37, 38\). Similarly, the more than two-fold elevation of DHA after the CanolaDHA treatment, compared to other treatments, is not surprising and is in line with previous reports \(39, 40, 41\). Lack of a DHA raising effect of FlaxSaff treatment agrees with prior work demonstrating low efficiency of conversion of ALA to DHA \(31\). Remarkable consistency was observed in compliance among participants as depicted here by predictable shifts in their fatty acid profile at the end of each dietary period, highlighting the successful completion of this long-term full-feeding clinical trial.

Multicenter, randomized, controlled clinical trials exist as the gold standard for evidence-based research \(42\). The multicenter nature of the trial ensured the robustness of results across different centers. The iso-caloric, weight-maintaining, full-feeding regimen eliminated the effect of variable habitual, background diets on study outcomes. Caloric excess or restriction can potentially affect blood lipids, and even body composition and body weight loss can affect endothelial function \(27\), hence requiring standardized study conditions to prevent weight change.

Study treatments oils were blended at the RCFFN and shipped to participating centers, removing an important source of variation. Sample analysis for specific endpoints was completed for all samples at the center designated for a particular analysis, in order to remove the effect of inter-
laboratory variation. Clinical measurement protocols, particularly for endothelial function testing, were harmonized across centers by training personnel from the University Park center staff. The cross-over design eliminated the effect of individual differences on response to treatments and reduced the required sample size. Endothelial function was found to be significantly changed in studies with a crossover or a parallel design over a period of 4 wks following dietary intervention indicating that the study design and the duration of the current study is appropriate for detecting changes in endothelial function (43; 44). Moreover, an acute study that measured endothelial function by FMD 4 hours after a high-fat meal supplemented with either 25 g olive oil or 40 g walnut in hypercholesterolemic subjects (34) also showed significant changes. FMD (45) and Endo-PAT techniques (46) are both dependent on nitric oxide mediated vasodilatation which is affected by both short- and long-term changes in endothelial function. Even though the measure of percent change in endothelial function has no clinical relevance as yet, a lower range of endothelial function (lower two tertiles) as measured by FMD, is associated with enhanced risk of future cardiovascular events (47).

The final sample size of 130 allowed us to detect a difference of 9.4% or greater in the RHI, which is a measure of the endothelial function, or a 10% difference of LDL-C with a power of 0.8 at a significance of 0.05.

Diets were designed to address contemporary nutrition questions. Although MUFA generally decreases TC and LDL-C, the efficacy is considered lesser than PUFA (48; 49). However, the relative benefits of individual unsaturated fatty acid classes are increasingly being challenged; hence this study was designed to resolve some of these issues.

The primary and ancillary measurements in our study will link the changes in surrogate cardiovascular biomarkers such as blood lipids and glucose, inflammatory biomarkers and
hormones to clinical endpoints such as endothelial function and body fat composition. The mechanistic studies, including lipid trafficking, apolipoproteins, lipoprotein particle size, and reverse cholesterol transport, will assist in clarifying the relationship between the biomarkers and clinical endpoints. Finally, results will be evaluated from the perspective of genetic variation, assessed through single nucleotide polymorphisms among individuals to assess why biomarker responses vary as a function of diet treatments.

The study possesses certain limitations. The disparity in male: female ratio and the baseline differences in cholesterol across centers can be considered as confounding treatment effects. However, use of a mixed model approach in statistical analyses is expected to minimize this effect. The effect of the preceding diet or the sequence of treatment is another confounding factor that was anticipated to be minimized by the mixed model approach. Moreover, differences in bioactives other than fatty acid composition with in the treatment oils might affect clinical measurements such as endothelial function. The intra-individual and inter-operator variability in endothelial function testing might likewise obscure a treatment effect. Thus, standardization of the test procedure represents an important factor in maintaining the robustness of endothelial function data. Steps were taken to limit this variability by controlling the environment and also by minimizing any additional factors such as the stage in the menstrual cycle of female participants, medication, and tea or coffee drinking. Additionally, use of the corn and safflower oil blend as the alternate treatment would be expected to improve blood lipids and other parameters. Therefore, it may not be possible to see an improvement in blood lipids in certain treatments compared to the corn and safflower oil blend treatment.
3.6 Conclusions

The successful completion of 130 participants through the study protocol of this 9-month long, full-feeding, multicenter study represents a significant achievement. Recruitment and retention strategies used in this study were instrumental in achieving this goal. Standardized protocols and efforts to minimize variation are expected to lead to findings that help fill the gaps in our knowledge of the metabolism, lipid trafficking, and clinical efficacy of the major classes of dietary fatty acids.

3.7 Acknowledgements

Canola Council of Canada, Flax Council of Canada, Agriculture and Agri-Food Canada and Dow Agrosciences collectively funded this study through the Growing Forward program of Agriculture and Agri-Food Canada. Dr. Rasheda Rabbani, Biostatistician at University of Manitoba is kindly acknowledged for statistical analysis of data. Funders did not participate in the design, conduct, management, data collection and analysis or preparation and review of the manuscript.
3.8 References


The following chapter comprises a manuscript which presents one of the primary outcomes of the dietary intervention trial, blood lipids. The data in this chapter show that all dietary oil treatments varying in unsaturated fatty acid composition reduced total cholesterol levels and LDL-C levels from baseline to treatment endpoint. In particular, current data show that the novel DHA-rich high oleic canola oil enhanced HDL-C levels, reduced triglyceride levels and decreased both systolic and diastolic blood pressure profiles, which led to a greater improvement on the Framingham coronary heart disease risk scores compared with other dietary oil treatments.
Chapter IV

DHA-enriched high oleic canola oil improves lipid profile and lowers predicted cardiovascular risk in the canola oil multicenter randomized controlled trial

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This manuscript has been published in The American Journal of Nutrition. (2014), 100(1):88-97
4.1 Abstract

It is well recognized that levels of trans and saturated fats should be minimized in Western diets, however, considerable debate remains regarding the optimal amounts of dietary n-9, n-6 and n-3 fatty acids. The objective was to examine the effects of varying n-9, n-6 and longer-chain n-3 fatty acid composition on markers of coronary heart disease (CHD) risk. A randomized, double-blind, five-period, crossover design was employed. Each 4-week treatment period was separated by 4-week washout intervals. Volunteers with abdominal obesity consumed each of 5 identical weight-maintaining, fixed-composition diets with one of the following treatment oils (60 g/3000 Kcal) in beverages: 1) conventional canola oil (Canola) (n-9 rich); 2) DHA-enriched high oleic canola oil (CanolaDHA) (n-9 and n-3 rich); 3) a blend of corn/safflower oil (25:75) (CornSaff) (n-6 rich); 4) a blend of flax/safflower oil (60:40) (FlaxSaff) (n-6 and short-chain n-3 rich); or 5) high oleic canola oil (CanolaOleic) (highest in n-9). One hundred and thirty individuals completed the trial. At endpoint, total cholesterol (TC) was lowest after the FlaxSaff phase (p<0.05 vs Canola and CanolaDHA) and highest after the CanolaDHA phase (p<0.05 vs CornSaff, FlaxSaff and CanolaOleic). LDL-cholesterol (LDL-C) and HDL-cholesterol (HDL-C) were highest, and triglyceride (TG) was lowest after CanolaDHA (p<0.05 vs other diets). All diets reduced TC and LDL-C from baseline to treatment endpoint (p<0.05). CanolaDHA was the only diet that raised HDL-C from baseline (3.5±1.8%, p<0.05) and produced the greatest reduction in TG (-20.7±3.8%, p<0.001), and systolic blood pressure (-3.3±0.8%, p<0.001) compared to other diets (p<0.05). Percent reductions in Framingham 10-year CHD risk scores (FRS) from baseline were greatest following CanolaDHA (-19.0±3.1%, p<0.001) compared to other treatments (p<0.05).
Conclusion: Consumption of CanolaDHA, a novel DHA-rich canola oil, improves HDL-C, TG and blood pressure, thereby reducing FRS compared with other oils varying in unsaturated fatty acid composition.
4.2 Introduction

Current dietary recommendations advise reduction of saturated and trans fat, but fall short of providing specific recommendations for optimal amounts of omega-9 (n-9), omega-6 (n-6) as well as short and longer chain omega-3 (n-3) fatty acid\(^1\). Although earlier research supported the use of n-6 polyunsaturated fatty acids (PUFA)\(^2\), newer data attest to the health benefits of consumption of n-9 monounsaturated fatty acid (MUFA)\(^3\) and n-3 PUFA\(^4\) rich fats. However, the preferred proportions of these fatty acids in Western diets remain to be ascertained.

The increasing incidence of metabolic syndrome and prevalence of coronary heart disease (CHD)\(^5\) underscore the need for dietary modification as a critical adjunct to physical activity and pharmacological intervention for prevention and treatment of CHD. The Adult Treatment Panel III dietary guidelines recommend up to 20% calories from MUFA and up to 10% from n-3/n-6 PUFA\(^6\). Novel modified oils, as sources of blended MUFA and n-3/n-6 PUFA, provide increased opportunities for optimizing the fatty acid profiles of foods to improve health. What remains to be evaluated are the effects of various combinations of n-3, n-6 and n-9 fatty acids on circulating lipids and other CHD risk factors.

The objectives of this study were, therefore, to investigate the relative effects of oil mixtures including conventional canola oil (Canola) (6:2:1, n-9:n-6:n-3), a DHA-enriched high oleic canola oil (CanolaDHA) (6:1:1, n-9:n-6:n-3), blended corn oil and safflower oil (25:75, CornSaff) (6:25:0.1, n-9:n-6:n-3), blended flax oil and safflower oil (60:40, FlaxSaff) (1:1:1, n-9:n-6:n-3), and high oleic canola oil (CanolaOleic) (31:6:1, n-9:n-6:n-3) on lipid, lipoprotein and apolipoprotein levels in individuals with central adiposity and CHD risk factors. It was hypothesized that these 5 dietary treatments would elicit differential effects that reflect their n-9, n-6 and n-3 fatty acid profiles.
4.3 Subjects and Methods

4.3.1 Study design

The Canola Oil Multicenter Intervention Trial (COMIT) was conducted as a double blind, randomized crossover trial at the Richardson Centre for Functional Foods and Nutraceuticals (RCFFN) (University of Manitoba), the Institute of Nutrition and Functional Foods (INAF) (Laval University), and the Department of Nutritional Sciences (Pennsylvania State University) (PSU). Institutional ethics boards in participating universities reviewed and approved the study protocol. Written informed consent was obtained from participants as prescribed by Research Ethics Boards at the respective institutions. The study was registered with clinicaltrials.gov (NCT01351012). The methodology of the COMIT study design has been published elsewhere (7).

4.3.2 Eligibility criteria and subject selection

Participants (18 years or older) were recruited from the metropolitan areas of Winnipeg, Quebec City and State College using radio advertisements, direct mailings, email newsletters, seminars and from outreach activities. Adult men and women with at least one of the following cardiovascular risk factors were recruited to the present study: waist circumference >94 cm for men and >80 cm for women; triglyceride (TG) >1.7 mmol/L; HDL cholesterol (HDL-C): <1 mmol/L (men) or <1.3 mmol/L (women); blood pressure ≥130 mmHg (systolic, SBP) and/or ≥85 mmHg (diastolic, DBP); and glucose ≥5.5 mmol/L. Volunteers underwent a medical history in the previous six months to exclude those with thyroid disease, diabetes mellitus, kidney disease, liver disease, current smokers or people consuming more than 2 alcoholic drinks per week. Participants were not taking any medication known to affect lipid metabolism or endothelial function. Participants who were unwilling to stop taking supplements of any kind at least two
weeks in advance were excluded. Eligible individuals were selected based on the inclusion and exclusion criteria described above. The flow of participants through the trial is presented in Figure 4.1.

Figure 4.1 Flow of participants throughout the study.
4.3.3 Randomization

Random permutations of treatments were created using the random number generator at randomization.com (version 07/16/2008) to ensure that all the subjects received all treatments in a random order. The sequences were kept in sealed envelopes and were later allocated to subjects as they joined the study. Random allocation sequences were generated at the lead site (RCFFN), with study coordinators at other sites contacting RCFFN to obtain the allocation sequences to assign the participants to treatment sequences.

4.3.4 Study diets

A randomized, double-blind, five-period, cross-over controlled-feeding study design was employed wherein subjects consumed a consistent, weight-maintaining diet of fixed macronutrient composition, providing 15%, 50% and 35% energy from protein, carbohydrate, and fat, respectively (Table 4.1). Each of 5 treatment periods extended 4 weeks and was separated by a 4-week washout interval, although 10 participants underwent a shortened washout period of 2 to 4 weeks at PSU. Study diets were prepared in kitchen facilities at three clinical sites and one community kitchen due to personal cultural practices (7). Diets were supplemented with beverages in which treatment oils were provided at breakfast and supper as two divided doses in a blinded manner. Both participants and study coordinators were blinded to the treatments. Participants were free-living and instructed to consume at least one meal under the supervision of the study coordinators on weekdays. Compliance was assessed by study coordinators by completion of meals provided under supervision as well as by the presence of food not consumed in their returned meal bags packed for off-site consumption. Body weight was monitored regularly to ensure adequate calories for weight maintenance.
Table 4.1 Nutrient profile of the test diets (based on 3000 kcal).\(^1\)

<table>
<thead>
<tr>
<th>Nutrient</th>
<th>Treatments(^2)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Canola</td>
</tr>
<tr>
<td>Gram (energy %)</td>
<td></td>
</tr>
<tr>
<td>Protein</td>
<td>117 (15%)</td>
</tr>
<tr>
<td>Carbohydrate</td>
<td>380 (50%)</td>
</tr>
<tr>
<td>Total</td>
<td>118 (35%)</td>
</tr>
<tr>
<td>SFA</td>
<td>22 (6.6%)</td>
</tr>
<tr>
<td>MUFA</td>
<td>59 (17.6%)</td>
</tr>
<tr>
<td>PUFA</td>
<td>30 (9.1%)</td>
</tr>
</tbody>
</table>

\(^1\)The energy and macronutrient profile of the three treatment meals is based on a 3000 kcal/d projected diet and was estimated by using FOOD PROCESSOR software.

\(^2\)Treatment oils used in the study Canola: conventional canola oil (Richardson Oilseed Ltd.); CanolaDHA: high oleic canola oil (Richardson Oilseed Ltd.) with DHA (Martek Biosciences Corporation); CornSaff: corn oil (Loblaws Inc.) and safflower oil (Loblaws Inc.) blend; FlaxSaff: flax oil (Shape Foods Inc) and safflower oil blend; CanolaOleic: high oleic canola oil. SFA: saturated fatty acid; MUFA: monounsaturated fatty acid; PUFA: polyunsaturated fatty acid.
Diets were provided at caloric levels matching individual volunteer’s daily requirements calculated using the Mifflin-St Jeor equation (8). Consequently, treatment oils and fatty acid intakes per 3000 kcal diet were 1) Canola: 59% oleic acid (OA), 20% linoleic acid (LA), 10% α-linolenic acid (ALA); 2) CanolaDHA: 63% OA, 6% DHA; 3) CornSaff: 18% OA, 69% LA; 4) FlaxSaff: 18% OA, 38% LA, 32% ALA; and 5) CanolaOleic: 72% OA, 15% LA (Table 4.2). The three canola based oil treatments were all high in OA, with Canola representing conventional canola oil. CanolaDHA was designed with a similar OA content as CanolaOleic, with additional DHA provided by an algal oil. The two safflower oil blends were both high in PUFA but low in MUFA, where CornSaff was rich in LA while the FlaxSaff was enriched with both LA (50% of the LA content of CornSaff) and ALA. CanolaOleic was formulated by replacing ALA with additional OA in Canola. Given the different fatty acid profiles among the five treatment oils, we were able to compare the benefits of long chain n-3 (DHA) and short chain n-3 (ALA) blended with n-9 (OA) or n-6 (LA) rich oils in the prevention of CHD risk.

Amounts of treatment oils provided to subjects were proportionately adjusted to individualized pre-calculated energy intakes of each subject, as established using algorithms based on gender, weight, height and age (8). The nutrient content of the diet was estimated using FOOD PROCESSOR software (ESHA Research, Salem, OR) to ensure the adequacy of macro- and micronutrients.
<table>
<thead>
<tr>
<th></th>
<th>Canola</th>
<th>CanolaDHA</th>
<th>CornSaff</th>
<th>FlaxSaff</th>
<th>CanolaOleic</th>
</tr>
</thead>
<tbody>
<tr>
<td>C16:0 palmitic acid</td>
<td>4.1</td>
<td>5.3</td>
<td>5.9</td>
<td>4.9</td>
<td>3.7</td>
</tr>
<tr>
<td>C18:0 stearic acid</td>
<td>1.8</td>
<td>1.7</td>
<td>1.9</td>
<td>3.2</td>
<td>1.8</td>
</tr>
<tr>
<td>C18:1 oleic acid</td>
<td>58.6</td>
<td>63.2</td>
<td>17.6</td>
<td>17.9</td>
<td>71.5</td>
</tr>
<tr>
<td>C18:2 linoleic acid</td>
<td>19.5</td>
<td>12.7</td>
<td>69.3</td>
<td>37.5</td>
<td>14.7</td>
</tr>
<tr>
<td>C18:3 α-linolenic acid</td>
<td>9.8</td>
<td>2.0</td>
<td>0.3</td>
<td>32.0</td>
<td>2.3</td>
</tr>
<tr>
<td>C20:4 arachidonic acid</td>
<td>0</td>
<td>0.1</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>C20:5 EPA</td>
<td>0</td>
<td>0.2</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>C22:5 DPA</td>
<td>0</td>
<td>2.4</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>C22:6 DHA</td>
<td>0</td>
<td>5.8</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Total saturated</td>
<td>7.3</td>
<td>9.0</td>
<td>7.9</td>
<td>8.1</td>
<td>6.9</td>
</tr>
<tr>
<td>Total monounsaturated</td>
<td>60.3</td>
<td>64.7</td>
<td>17.7</td>
<td>17.9</td>
<td>73.2</td>
</tr>
<tr>
<td>Total polyunsaturated</td>
<td>29.3</td>
<td>23.1</td>
<td>69.6</td>
<td>69.5</td>
<td>17.0</td>
</tr>
</tbody>
</table>

The values are % of total fatty acids. Based on a 3000 kcal diet, 60 g of treatment oil contributed 18% (540 kcal) of total energy. The amount of treatment oil was adjusted to contribute 18% of energy for each calorie level. Canola: conventional canola oil; CanolaDHA: high oleic canola oil with DHA; CornSaff: corn oil and safflower oil blend; FlaxSaff: flax oil and safflower oil blend; CanolaOleic: high oleic canola oil; EPA: eicosapentaenoic acid; DPA: docosapentaenoic acid; DHA: docosahexaenoic acid.
4.3.5 Sample collection and analysis

Fasting blood samples were collected on days 1 and 2 (baseline) as well as 29 and 30 (endpoint) of all phases. Samples from all clinical sites were shipped frozen to and analyzed in a central laboratory at St. Michaels Hospital, University of Toronto. Baseline and endpoint values were separately assayed and determined as mean values of day 1 and day 2, and day 29 and day 30 measures, respectively. Main outcome measures were serum lipid parameters, including total cholesterol (TC), LDL-C, HDL-C, TG, apolipoprotein A-1 (apo A-1), and apolipoprotein B (apo B) levels. The following ratios were also calculated from original values; apo B/apo A-1, TC/HDL-C and TG/HDL-C. Serum lipids were analyzed using a Roche Cobas 6000 c501 System (Roche Diagnostics, Indianapolis, IN). LDL-C was calculated using the Friedewald formula \(^9\). Serum lipid measures were standardized with the Centers for Disease Control (CDC, Atlanta, GA) Lipid Standardization Program for external quality control. Apo A-1 and apo B were measured by nephelometry using the BN Prospec (Siemens, Mississauga, ON). The coefficients of variation of measurement of TC, HDL-C, TG, apo A-1 and apo B were 0.8%, 0.9%, 1.2%, 1.4% and 2.1%, respectively. In addition, at the beginning and end of each phase, blood pressure was monitored at each clinical site by using a digital blood pressure monitor and measured in triplicate after a 5-min rest in a secluded area. Both SBP and DBP were recorded in the morning before meals while subjects were in a seated position, with the cuff placed at the level of the heart in the right arm. Using baseline and endpoint data from each treatment, we entered age, gender, SBP, TC, and HDL for each participant into the Framingham 10-year CHD risk score (FRS) calculator \(^10\).
4.3.6 Statistical analysis

A sample size of 40 subjects at each of the three clinical sites (total of 120) was determined based on endothelial function as the primary outcome with an anticipated 20% difference in the mean and a standard deviation of 1.35 whereas the alpha and power were 0.05 and 0.8, respectively \(^{(11)}\). This estimated sample size of 120 allowed us to detect a 10% difference in LDL-C with a power of 0.8 at a significance of 0.05.

Only participants who completed the entire trial protocol (i.e. all the five dietary phases) were included in the statistical analyses, with analyses conducted according to originally assigned treatment orders. Therefore, per protocol approach was used for data analysis to avoid the need for multiple imputations for missing data. The mixed model procedure in SAS (version 9.2, Cary, NC) was utilized with treatment, age, and gender used as fixed effects, repeated for subjects over treatment periods, while center was added as a random effect in order to adjust for potential confounders including the differences in baseline characteristics and carry-over effects across all centers. In addition, diet effects were estimated while controlling for the dietary treatment immediately preceding. “Last treatment (the previous diet)” was included as another random effect in order to control the sequence of the dietary treatments. When the current diet was the first treatment in the trial, a code “9” was given. Separate statistical comparisons were conducted for 1) mean values at the end of each treatment, and 2) within treatment percent changes. Percent changes from the baseline to endpoint were calculated as follows: \([(\text{post-treatment value} - \text{pre-treatment baseline})/\text{pre-treatment baseline} \times 100]\) for each phase. Kolmogorov and Cramér–von Mises tests and histograms were used to test the Gaussian nature of the variables. Tukey adjusted p-values were used to examine differences between treatments. Least squares means with standard errors are presented in the text and in figures unless stated otherwise.
4.4 Results

4.4.1 Baseline characteristics and treatment differences between study centers

One hundred and seventy participants were randomized to the treatments from September, 2010 through March, 2012. No statistically significant differences were observed between centers for subjects’ age BMI, LDL-C, HDL-C, or TG concentrations at baseline. However, the ratio of males to females, body weight, female waist circumference and DBP differed across centers (Table 4.3). One hundred and thirty of the total randomized subjects (76.5%) completed all five treatment periods (Figure 4.1). In addition to monitoring food not consumed, compliance was also verified and confirmed by predicted shifts in the total plasma fatty acid profile at the end of each intervention period, as previously published (7). No differences were observed in body weight at the end of the five treatments (Table 4.4). From the beginning to the end of the phase, average body weight changes from baseline fluctuated between -0.39 and +0.12 kg. Including the body weight fluctuation in the statistical model did not affect the results.
Table 4.3 Baseline characteristics of participants at each participating center.

<table>
<thead>
<tr>
<th></th>
<th>RCFFN</th>
<th>INAF</th>
<th>PSU</th>
<th>Total</th>
<th>P value&lt;sup&gt;1&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Male, N</td>
<td>16</td>
<td>29</td>
<td>15</td>
<td>60</td>
<td>0.003</td>
</tr>
<tr>
<td>Female, N</td>
<td>38</td>
<td>17</td>
<td>15</td>
<td>70</td>
<td></td>
</tr>
<tr>
<td>BMI (Kg/m&lt;sup&gt;2&lt;/sup&gt;)</td>
<td>29.68±5.03</td>
<td>30.00±4.26</td>
<td>29.70±3.25</td>
<td>29.80±4.37</td>
<td>0.928</td>
</tr>
<tr>
<td>Age (years)</td>
<td>43.87±15.81</td>
<td>49.87±14.22</td>
<td>45.90±9.61</td>
<td>46.46±14.18</td>
<td>0.105</td>
</tr>
<tr>
<td>TC (mmmol/L)</td>
<td>5.39±1.13</td>
<td>5.40±1.02</td>
<td>5.08±0.94</td>
<td>5.32±1.05</td>
<td>0.371</td>
</tr>
<tr>
<td>HDL-C (mmol/L)</td>
<td>1.25±0.28</td>
<td>1.20±0.33</td>
<td>1.20±0.24</td>
<td>1.22±0.29</td>
<td>0.635</td>
</tr>
<tr>
<td>LDL-C (mmol/L)</td>
<td>3.41±1.01</td>
<td>3.38±0.88</td>
<td>3.19±0.87</td>
<td>3.35±0.93</td>
<td>0.572</td>
</tr>
<tr>
<td>TG (mmol/L)</td>
<td>1.61±0.92</td>
<td>1.81±0.80</td>
<td>1.53±0.92</td>
<td>1.67±0.88</td>
<td>0.338</td>
</tr>
<tr>
<td>Glucose (mmol/L)</td>
<td>5.43±1.65</td>
<td>5.29±0.60</td>
<td>5.29±0.45</td>
<td>5.35±1.14</td>
<td>0.788</td>
</tr>
<tr>
<td>Body weight (kg)</td>
<td>80.69±15.93</td>
<td>85.80±15.25</td>
<td>89.86±15.74</td>
<td>84.67±15.93</td>
<td>0.042</td>
</tr>
<tr>
<td>Waist female (cm)</td>
<td>90.64±11.92</td>
<td>97.36±10.33</td>
<td>98.52±9.87</td>
<td>93.94±11.58</td>
<td>0.033</td>
</tr>
<tr>
<td>Waist male (cm)</td>
<td>103.37±13.76</td>
<td>106.88±8.74</td>
<td>106.53±6.53</td>
<td>105.84±9.89</td>
<td>0.508</td>
</tr>
<tr>
<td>SBP (mmHg)</td>
<td>122.44±20.33</td>
<td>119.76±14.19</td>
<td>118.50±12.36</td>
<td>120.62±16.70</td>
<td>0.548</td>
</tr>
<tr>
<td>DBP (mmHg)</td>
<td>81.24±12.41</td>
<td>70.50±10.47</td>
<td>79.67±7.39</td>
<td>77.04±11.80</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

<sup>1</sup>Analysis of variance was used to analyze between-treatment differences in continuous variables. Chi-square was used to determine distribution of gender across centers. P<0.05 was considered significant. TC: total cholesterol; LDL-C: low density lipoprotein cholesterol; HDL-C: high density lipoprotein cholesterol; TG: triglycerides; SBP: systolic blood pressure; DBP: diastolic blood pressure; RCFFN: Richardson Centre for Functional Foods and Nutraceuticals, Winnipeg, MB Canada; INAF: Institute of Nutraceuticals and Functional Foods, Quebec City, QC, Canada; PSU: Pennsylvania State University, State College, PA, USA.
Table 4.4 Between treatment comparisons of blood lipid parameters, blood pressure levels and body weight.\textsuperscript{1, 2}

<table>
<thead>
<tr>
<th></th>
<th>Canola</th>
<th>CanolaDHA</th>
<th>CornSaff</th>
<th>FlaxSaff</th>
<th>CanolaOleic</th>
</tr>
</thead>
<tbody>
<tr>
<td>TC (mmol/L)</td>
<td>4.81±0.14\textsuperscript{ab}</td>
<td>4.87±0.14\textsuperscript{a}</td>
<td>4.74±0.14\textsuperscript{bc}</td>
<td>4.69±0.14\textsuperscript{c}</td>
<td>4.77±0.14\textsuperscript{bc}</td>
</tr>
<tr>
<td>LDL-C (mmol/L)</td>
<td>2.91±0.08\textsuperscript{b}</td>
<td>3.02±0.08\textsuperscript{a}</td>
<td>2.85±0.08\textsuperscript{b}</td>
<td>2.84±0.08\textsuperscript{b}</td>
<td>2.86±0.08\textsuperscript{b}</td>
</tr>
<tr>
<td>HDL-C (mmol/L)</td>
<td>1.20±0.02\textsuperscript{b}</td>
<td>1.30±0.02\textsuperscript{a}</td>
<td>1.20±0.02\textsuperscript{b}</td>
<td>1.17±0.02\textsuperscript{b}</td>
<td>1.18±0.02\textsuperscript{b}</td>
</tr>
<tr>
<td>TG (mmol/L)</td>
<td>1.60±0.10\textsuperscript{a}</td>
<td>1.25±0.10\textsuperscript{b}</td>
<td>1.56±0.10\textsuperscript{a}</td>
<td>1.56±0.10\textsuperscript{a}</td>
<td>1.64±0.10\textsuperscript{a}</td>
</tr>
<tr>
<td>TC/HDL ratio</td>
<td>4.29±0.12\textsuperscript{a}</td>
<td>4.01±0.12\textsuperscript{b}</td>
<td>4.24±0.12\textsuperscript{a}</td>
<td>4.28±0.12\textsuperscript{a}</td>
<td>4.29±0.12\textsuperscript{a}</td>
</tr>
<tr>
<td>Apo A-1 (mmol/L)</td>
<td>1.42±0.03\textsuperscript{b}</td>
<td>1.46±0.03\textsuperscript{a}</td>
<td>1.41±0.03\textsuperscript{bc}</td>
<td>1.38±0.03\textsuperscript{c}</td>
<td>1.43±0.03\textsuperscript{ab}</td>
</tr>
<tr>
<td>Apo B (mmol/L)</td>
<td>0.94±0.03\textsuperscript{ab}</td>
<td>0.95±0.03\textsuperscript{a}</td>
<td>0.91±0.03\textsuperscript{b}</td>
<td>0.91±0.03\textsuperscript{b}</td>
<td>0.94±0.03\textsuperscript{ab}</td>
</tr>
<tr>
<td>Apo B/apo A-1</td>
<td>0.67±0.02</td>
<td>0.67±0.02</td>
<td>0.67±0.02</td>
<td>0.68±0.02</td>
<td>0.67±0.02</td>
</tr>
<tr>
<td>SBP (mmHg)</td>
<td>119.32±2.91\textsuperscript{ab}</td>
<td>117.43±2.91\textsuperscript{b}</td>
<td>120.39±2.91\textsuperscript{a}</td>
<td>120.47±2.91\textsuperscript{a}</td>
<td>119.39±2.91\textsuperscript{ab}</td>
</tr>
<tr>
<td>DBP (mmHg)</td>
<td>77.07±4.04\textsuperscript{a}</td>
<td>74.60±4.04\textsuperscript{b}</td>
<td>77.06±4.04\textsuperscript{a}</td>
<td>77.60±4.04\textsuperscript{a}</td>
<td>76.75±4.04\textsuperscript{a}</td>
</tr>
<tr>
<td>Total mass (kg)\textsuperscript{3}</td>
<td>83.88±1.40</td>
<td>84.18±1.39</td>
<td>84.03±1.39</td>
<td>84.31±1.41</td>
<td>83.91±1.41</td>
</tr>
</tbody>
</table>

\textsuperscript{1} Values are least square means ± standard error for n=130. Mean values within a row with different superscript letters are significantly different between treatments at p<0.05 (mixed-model ANOVA and post hoc Tukey-test). \textsuperscript{2} Dependent variables used for data analysis were the mean of the day 29 and day 30 values at the end of each dietary phase. Values are unadjusted. \textsuperscript{3} Data of total body mass at endpoints was based on the DEXA measures at the end of each dietary phase with a total available number of 118 subjects. TC: total cholesterol; LDL-C: low density lipoprotein cholesterol; HDL-C: high density lipoprotein cholesterol; TG: triglycerides; apo A-1: apolipoprotein A-1; apo B: apolipoprotein B; SBP: systolic blood pressure; DBP:
diastolic blood pressure; Canola: conventional canola oil; CanolaDHA: high oleic canola oil with DHA; CornSaff: corn oil and safflower oil blend; FlaxSaff: flax oil and safflower oil blend; CanolaOleic: high oleic canola oil.
4.4.2 Endpoint differences between treatments

Endpoint lipid results for the five dietary interventions are summarized in Table 4.4. Consumption of the FlaxSaff diet resulted in the numerically lowest mean TC (p<0.05 vs Canola and CanolaDHA) while the CanolaDHA diet produced the numerically highest TC level (p<0.05 vs CornSaff, FlaxSaff and CanolaOleic). However, endpoint TC levels did not differ significantly between Canola and CanolaDHA treatments. CanolaDHA feeding resulted in the highest endpoint LDL-C (p<0.05) and HDL-C values (p<0.05) relative to all other treatments. Conversely, CanolaDHA feeding yielded the lowest endpoint TG values (p<0.05) which differed from all other treatments. The mean apo A-1 value was numerically highest after the CanolaDHA diet (p<0.05 vs Canola, CornSaff and FlaxSaff) although it was not significantly different from CanolaOleic, and was the numerically lowest after the FlaxSaff diet (p<0.05 vs Canola, CanolaDHA and CanolaOleic). CornSaff and FlaxSaff diets resulted in the numerically lowest mean apo B levels (p<0.05 vs CanolaDHA). Across treatments, CanolaDHA also elicited a lower SBP compared to CornSaff and FlaxSaff (p<0.05), and the lowest DBP across all diets (p<0.05).

4.4.3 Changes from baseline to endpoint within treatments

All diets significantly reduced TC (p<0.01) and LDL-C levels (p<0.05) from pretreatment baseline levels (Figure 4.2). Percent changes in TC after Canola, CanolaDHA, CornSaff, FlaxSaff and CanolaOleic diets were -8.5±2.5, -7.4±2.5, -11.7±2.5, -11.4±2.5 and -9.4±2.5, respectively. Percent changes in TC were greatest after the CornSaff and FlaxSaff treatments, while Canola and CanolaDHA feeding produced the smallest reductions in TC (p<0.05 vs CornSaff and FlaxSaff). LDL-C percent changes for Canola, CanolaDHA, CornSaff, FlaxSaff
and CanolaOleic diets were -11.3±2.8, -7.0±2.8, -15.4±2.8, -12.9±2.8 and -12.5±2.8, respectively (all p<0.05). The percent LDL-C reduction was lowest (p<0.05) after CanolaDHA compared to CornSaff, FlaxSaff and CanolaOleic. None of the other paired comparisons were statistically significant. The Canola, CornSaff, FlaxSaff and CanolaOleic treatments reduced HDL-C concentrations (p<0.05) from baseline by -4.2±1.8%, -4.4±1.8%, -6.8±1.8%, and -5.0±1.8%, respectively. In contrast, CanolaDHA increased HDL-C from baseline by 3.5±1.8% (p<0.05). The CanolaDHA diet reduced TG by -20.7±3.8% (p<0.001) which was greater than the TG changes produced by all other treatments (p<0.001). None of the other diets significantly affected TG.
Figure 4.2 Percent change in serum lipids from treatment specific baseline in response to five treatment diets.

Percent change was calculated from baseline value of the each dietary phase. Bars represent least square means for n=130. Error bars reflect the standard errors of the means. The treatment effect was analyzed by using mixed-model with repeated measures adjusted by Tukey’s test for multiple comparisons. Mean values with unlike letters are significantly different. P<0.05 was considered significant. TC, total cholesterol; LDL-C: low density lipoprotein cholesterol; HDL-C: high density lipoprotein cholesterol; TG, triglycerides; Canola: conventional canola oil; CanolaDHA: high oleic canola oil with DHA; CornSaff: corn oil and safflower oil blend; FlaxSaff: flax and safflower oil blend; CanolaOleic: high oleic canola oil.
4.4.4 Calculated lipid parameters, blood pressure and Framingham 10-year CHD risk scores

The apo B/apo-A1 ratio did not vary between treatments (Table 4.4). All diets decreased the TC/HDL-C ratio (p<0.01) with the greatest percent reduction for the CanolaDHA diet (-10.1±1.1%), which was different compared to Canola, FlaxSaff, and CanolaOleic (p<0.001) but not CornSaff diet (Figure 4.2). The CanolaDHA treatment also resulted in a substantial decrease in the TG/HDL-C ratio (-22.4±2.8%, p<0.001), which differed from all other treatments (p<0.001). In addition, only CanolaDHA significantly reduced SBP by -3.3±0.8% (p<0.001), and DBP by -4.1±1.0% (p<0.001); the other four diets failed to affect blood pressure (data not shown). Nevertheless, reductions in calculated FRS were observed for all treatments (p<0.05) except for FlaxSaff, when calculated as percent change from baseline (Figure 4.3). Changes in the scores from baseline were -8.2±3.1% for Canola, -19.0±3.1% for CanolaDHA, -10.0±3.0% for CornSaff, and -6.8±3.1% for CanolaOleic. CanolaDHA resulted in the greatest reduction in FRS (p<0.05) compared to all other four treatments.
Figure 4.3 Comparison of percent change of Framingham 10-year CHD risk scores after five treatment diets.

Percent changes were calculated from baseline Framingham 10-year CHD risk score of the each dietary phase. Bars reflect least square means (n=130) with error bars representing standard errors. P values for the treatment effects were analyzed by using mixed-model with repeated measures adjusted by Tukey. P<0.05 was considered significant. Unlike letters are significantly different between treatments. Canola: conventional canola oil; CanolaDHA: high oleic canola oil with DHA; CornSaff: corn oil and safflower oil blend; FlaxSaff: flax oil and safflower oil blend; CanolaOleic: high oleic canola oil.
4.5 Discussion

The present study demonstrates that consumption of a blend of high oleic canola oil and DHA oil improved HDL and TG profiles, as well as blood pressure and FRS compared with the other oil blends studied. Notably, the diet with DHA-rich high oleic canola oil improved HDL-C and TG, but resulted in significantly higher LDL-C concentrations compared with the other dietary treatments. Compared to CanolaDHA, the two PUFA-rich diets, CornSaff and FlaxSaff elicited lower TC, LDL-C and apo B levels. LDL-C, HDL-C and TG values were similar among all treatments except CanolaDHA.

Despite the efficacy of n-3 fatty acids in favorably modulating several cardiovascular risk factors, the tendency of LDL-C concentrations to increase following DHA supplementation has been a concern \(^{(12)}\), because LDL-C is the primary therapeutic target for CHD prevention. The present work shows a statistically significant reduction in LDL-C levels from baseline following the DHA-enriched canola treatment, albeit with higher endpoint LDL-C concentrations versus conventional canola. This suggests a blunting of the LDL-C raising effect of DHA through blending with conventional canola oil. In contrast, Eslick et al demonstrated in a meta-analysis that fish oil containing eicosapentaenoic acid (EPA) and DHA failed to confer a benefit on fasting LDL-C levels \(^{(13)}\). In another study, LDL-C concentrations were 7.1% higher after DHA (0.7 g/day) treatment, compared with the placebo \(^{(12)}\). Moreover, in a 12-week free-living parallel study where subjects were given capsules with 0.52, 1.04 and 1.56 g/day DHA or placebo, every 1 g/day increase in DHA intake was associated with a 7.1% increase in LDL-C levels \(^{(14)}\). In a recent meta-analysis, Wei and Jacobson also evaluated the effects of DHA mono-therapy on plasma lipids and found that compared to placebo, DHA raised LDL-C by 0.19 mmol/L \(^{(15)}\). In addition, Hartweg et al., in a meta-analysis, reviewed the effects of n-3 fatty acids including EPA
and DHA, or fish oil and concluded that n-3 supplementation lowered TG by -0.45 mmol/L in patients with diabetes, but raised LDL-C concentrations by 0.11 mmol/L versus placebo (16). In contrast to all the studies above, we hypothesize that the high MUFA content (65% of total fatty acids) of CanolaDHA in our study might have blunted the increase in LDL-C that has been reported in other studies with DHA (12; 14; 15).

Numerous studies during the last few decades have demonstrated the serum total- and LDL-cholesterol lowering effects of n-6 rich oils (17; 18; 19; 20), and our findings are consistent with these reports. An advisory issued by the American Heart Association on n-6 PUFA concluded that intake of 5-10% of energy is beneficial in decreasing the risk of CHD (21), while there was a meta-analysis reported dietary n-6 to be neutral on the relative risk for CHD (1). However, another recent meta-analysis reported that diets high in n-6 fatty acids that also were very low in n-3 fatty acids increased risk of CHD and death (22). Nevertheless, conclusions of the latter meta-analysis were mainly influenced by a study with a sample size of 54 and with a higher than recommended n-6 intake (14.9% of energy). Inverse associations have been shown consistently between higher LA intakes and mortality from CVD in prospective cohort studies (23; 24) A recent large prospective study, also demonstrated an association of higher plasma phospholipid n-6 fatty acid concentrations with reduced risk of CHD incidence in men and women, while n-3 fatty acid concentrations did not show any association (25). Possible competition between n-6 and n-3 fatty acids in their shared metabolic pathways (26) might need to be considered in future studies. Indeed, the relative proportions of these fatty acids required in the diet have been a point of scientific discussion. In the present study we demonstrated that n-9 fatty acid-rich oils in combination with long chain n-3 fatty acids have the most beneficial effects on HDL-C and TG levels and in reducing estimated CHD risk compared with a combination of long chain n-6 and
n-3 rich oils. However, the beneficial changes of the two n-6 PUFA enriched diets relative to LDL-C, apo B and the TC/HDL-C ratio are also noteworthy.

Other studies evaluating the TC and LDL-C lowering effects of dietary MUFA have typically utilized Western diets or diets high in saturated fat as a control. The absence of such a control diet in this study is a limitation. However, the differential effects of saturated fat on lipid metabolism are well studied. For example, in a cross-over study, endpoint TC and LDL-C decreased by 10% and 14%, respectively, between a high MUFA diet (21% of energy from MUFA) and an average American diet (11% of energy from MUFA) over a period of 28 days (27). Gillingham et al. also showed that 28-day dietary MUFA treatment (23% of energy intake) decreased both TC and LDL-C levels compared to a typical Western diet (28). Moreover, LDL-C concentrations decreased in 3 out of 8 intervention studies reviewed by Hunter et al, where oleic acid replaced stearic acid (29). In an 8-week trial comparing diets high in either MUFA (18% of energy intake) or SFA (16% of energy intake) in young men, LDL-C levels were decreased 7.8% during the MUFA diet, while they increased 6.2 % during the SFA diet (30). In the same study, LDL-C decreased 11% on the MUFA diet with no change on the SFA diet in middle-aged men (30). Therefore, most of the available evidence supports the conclusion that higher MUFA intakes reduce LDL-C concentrations compared to SFA.

Hodson et al. reported a significant decrease in HDL-C (4%) after a diet in which more than 50% of the fatty acids were provided as MUFA, when compared to a SFA diet (31). Our study results agree with these findings, and reinforce our view that the pre-baseline diet of our participants was a typical north-American diet. Hence, decreasing SFA results in a HDL-C-lowering effect. The observation of a higher HDL-C in the CanolaDHA treatment compared to baseline agrees with the HDL-C increasing effect of DHA reported by Milte et al (14). These investigators noted
that for every 1 g/day increase in DHA intake, HDL-C levels increased by 4.4% during a 12-week free-living parallel study in which subjects were given capsules containing varying DHA concentrations versus a placebo.

Reported effects of high dietary MUFA on TG are mixed, with some studies showing decreases compared to a high carbohydrate diet (32), and some demonstrating neutral effects compared to an average American diet (33). Presently, we failed to observe any significant changes in serum TG on the Canola or CanolaOleic treatments. However, the CanolaDHA treatment reduced serum TG compared to Canola or CanolaOleic feedings. This finding is in agreement with a meta-analysis by Wei and Jacobson, who found that in 13 out of 15 studies reviewed, significant reductions from 0.03 to 0.64 mmol/L were seen for TG after DHA treatment, compared with placebo (15).

None of the diets studied served as a control in the present study because our objective was to compare the effects of different fatty acid profiles. Inclusion of a typical Western diet would have been useful to establish whether any of the treatments had adverse effects. However, the addition of a sixth period of controlled feeding would have made the protocol overly burdensome for the participants. In fact, our design could be viewed as conservative as we are comparing novel oils to other oils typically viewed as “healthy”.

The TC/HDL-C ratio is a strong predictor of CHD risk (34) and is widely used clinically. In the present study, the CanolaDHA treatment elicited the greatest reductions in the ratio compared to the other two n-9 MUFA enriched diets, Canola and CanolaOleic. In contrast, Sagara et al. made a similar comparison between olive oil (1 g/day for 5-weeks) and DHA (2 g/day for 5-weeks) and found no difference in TC/HDL-C ratio (35). The higher dose of DHA (3.5 g/day) as well as
the controlled dietary design feature of the present trial may contribute to the discrepancies observed between studies.

Reductions of both systolic and diastolic blood pressure following the CanolaDHA treatment from baseline as well as compared to other treatments is a novel finding for a canola oil blend. However, previous studies, as reviewed by Mori et al.\(^{(36)}\) describe a reduction of 0.66/0.35 mmHg/g (SBP/DBP) following feeding of n-3 fatty acids, with the blood pressure lowering effect being greatest for DHA and in hypertensive individuals. A recent study reported a dose-dependent, reversible activation of voltage activated K+ (BK) channels in vascular smooth muscle by DHA thereby demonstrating a critical vasodilatory influence which sheds light on a possible blood pressure-lowering mechanism of DHA\(^{(37)}\). Other blood pressure-lowering mechanisms potentially include increased release of nitric oxide, release of vasoactive prostanoids, and endothelium derived hyperpolarizing factor, as well as attenuation of vasoconstrictor response to noradrenaline\(^{(36)}\).

The significant reduction in FRS from baseline to endpoint following CanolaDHA treatment observed presently is mainly the result of elevated HDL, and decreased SBP, rather than decreased TC levels. A simple sensitivity analysis was conducted and resulted in the same change in FRS after the CanolaDHA treatment while holding SBP constant. However, FRS changes in other treatments became insignificant. Even though our study was not designed to evaluate blood pressure changes, since one of the objectives was to analyze endothelial function, we assumed that there might be changes related to vascular function. Therefore, we ensured that blood pressure measurements were taken in a very controlled and reproducible manner. In the statistical model using “center” as a random effect, any effects of site have been accounted for; hence, the blood pressure changes observed herein can be considered a true treatment effect. This
beneficial effect of the CanolaDHA treatment observed even over the comparatively short treatment period, underscores its potential long-term health benefits. While it is implicitly understood that it is important to avoid increasing CHD risk with DHA or fish oil consumption (4), the beneficial effects on risk status of this unique CanolaDHA oil blend have not been reported elsewhere.

Recent studies (38; 39) have yet to provide conclusive evidence of the benefits of increasing HDL-C on CHD risk. Further work is needed to determine the cardioprotective benefits of this particular oil blend, CanolaDHA, on CHD. Nevertheless, we acknowledge the beneficial effects of CanolaDHA on TG, LDL-C, blood pressure and HDL-C, and most likely other similar oil blends that provide inexpensive DHA enriched oil to the North American population for reduction of multiple major risk factors.

Based on recommendations from the American Heart Association, individuals at risk for CHD due to elevated TG should consume 2 to 4 g of fish oil supplement daily under a physician’s care (4). In addition, a recent dose-response study showed that 3.4 g/d of EPA + DHA significantly lowered average mean arterial pressure by about 2 mmHg, whereas a more moderate dose (0.85 g/d) did not (40). Therefore, the dose of DHA (3.5 g/day) used in the present study is within the recommended range for TG lowering, and allowed us to evaluate the health benefits of DHA particularly in individuals with metabolic syndrome or with CHD risk factors. However, although no adverse effects were observed in the current study, the potential for long-term adverse effects of higher doses should be considered when designing longer term trials.

Hartweg et al. demonstrated a weighted mean difference in TG of 0.35 mmol/L in trials that provided >2 g/day n-3 (EPA+DHA) and 0.57 mmol/L in trials that provided <2 g/day n-3 (EPA+DHA) (16). Therefore, intakes of DHA lower than evaluated herein would not be expected
to change the results significantly, particularly in individuals with metabolic syndrome. New plant varieties created through non-GMO double haploid genetic techniques are becoming available, which are expected to provide inexpensive crop sources of DHA such as canola which can be easily accessed in North America.

The limitations of the study are lack of a control Western diet, relatively high treatment oil supplementation, and having a per protocol type of statistical analysis where only the completer data were used for statistical analysis without data imputation for missing data. We acknowledge that this approach is biased in circumstances in which attrition is not totally at random. The strengths of the study are the large subject number, controlled feeding and crossover design as well as demonstrated adherence to the study protocol. Also, a unique feature of the study is that we compared novel oil blends that mimic new oils entering the marketplace. Thus, our study provides a proof of concept for the efficacy of these oils.

In summary, DHA enrichment significantly increased HDL-C and reduced TG, whereas n-6 and n-9 rich oils without DHA significantly reduced LDL-C levels. Nevertheless, DHA-enriched canola oil reduced baseline to endpoint FRS, to the extent that was greater than did the other treatments. The effects of these oil blends merit further investigation to confirm the relative benefits of each oil blend.
4.6 Acknowledgements

We gratefully acknowledge Dr. Rasheda Rabbani, Biostatistician at Manitoba Institute of Child Health for the statistical analysis of data. We would like thank the participants of the study. Funders were not involved in the design, conduct, management, data collection and analysis or preparation and review of the manuscript.
4.7 References


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Bridge to chapter V

Chapter IV has demonstrated health benefits of five dietary oil treatments varying in unsaturated fatty acid composition on blood lipids. Studies in nutrigenomics have revealed that diet-gene interactions can play an important role in whole body lipid and energy metabolism. Therefore, the following chapter comprises a manuscript which presents the investigation on effects of common genetic variants in *FADS* and *ELOVL* gene clusters on circulating fatty acid profiles in response to those different dietary treatment oils.
Chapter V

Genetic variants in FADS and ELOVL gene clusters modulate n-3 and n-6 fatty acid metabolism in volunteers at risk of metabolic syndrome: a randomized controlled trial

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This manuscript will be submitted to Genes and Nutrition.
5.1 Abstract

Personalized diet modification or design on the basis of interactions between nutrients and genetic variants has become a potential solution to prevent cardiovascular diseases. Genetic variants of FADS and ELVOL genes are known to associate with circulating n-6 and n-3 fatty acid profiles, but how these variants modulate levels of these fatty acid remains to be assessed. The present study was to investigate the effect of common polymorphisms of FADS and ELOVL genes on plasma fatty acid profiles in response to different vegetable oils with varying fatty acid compositions. In a multicenter, double-blind, randomized, crossover clinical trial, 129 volunteers with at risk of metabolic syndrome (69 women, 18-80 y old, BMI 22-32 kg/m²) were fed five isocaloric diets, each for 29 days with 60 g/day of: 1) conventional canola oil, Canola 2) docosahexaenoic acid (DHA)-enriched high oleic canola oil, CanolaDHA 3) corn/safflower oil blend, CornSaff 4) flax/safflower oil blend, FlaxSaff and 5) high oleic canola oil, CanolaOleic. Endpoint plasma fatty acid profiles were determined for each phase and four selected polymorphisms were genotyped including rs174561 (FADS1), rs174583 (FADS2), rs953413 (ELOVL2) and rs2397142 (ELOVL5). de novo lipogenesis were established using stable isotope tracer (deuterium water). CanolaDHA feeding increased (P<0.01) plasma DHA and eicosapentaenoic acid (EPA) levels but lowered (P<0.01) docosapentaenoic acid (DPA). The α-linolenic acid (ALA)-rich diet, FlaxSaff, increased (P<0.01) EPA and DPA levels with no difference in DHA compared to other treatments. Across all diets, minor allele homozygotes of rs174583 (TT) showed lower (P<0.01) plasma EPA and arachidonic acid (AA) levels, but no difference in DHA concentrations. Specifically, rs174583 resulted in higher DPA levels in CC and CT than those with TT after FlaxSaff (P<0.01) with no change in DHA content. There were no differences in EPA, DPA or DHA between rs174561 and rs174583 genotypes after
consuming the CanolaDHA diets. No significant effects of the two selected ELOVL polymorphisms on post-treatment fatty acid levels within each treatment were observed. DHA synthesis appears not to be influenced by DHA intake or by common genetic variants of FADS or ELOVL genes. In summary, healthy fatty acid intakes could modulate the association between common genetic variations and healthy circulating fatty acid patterns via diet-gene interactions. DHA-rich foods or supplements may be the most efficient way to increase circulating DHA concentration in humans. This trial was registered at clinicaltrials.gov as NCT01351012.
5.2 Introduction

The health benefits of consumption long chain polyunsaturated fatty acids (PUFAs) on prevention of cardiovascular diseases (CVD) have been a central theme in nutrition research, yet there remains considerable confusion about the threshold for beneficial effects of different types of PUFAs. Omega 3 (n-3) fatty acids, including plant derived α-linolenic acid (18:3n-3, ALA) and marine derived eicosapentaenoic (20:5n-3, EPA) and docosahexaenoic acid (22:6n-3, DHA) have been shown to modulate multiple cardiovascular risk factors in epidemiological studies (1), animal models (2), and human clinical investigations (3). Current positive health benefits of n-3 consumption are mainly on account of the effects of EPA and DHA on improvement of cardiovascular function (4), but not ALA. Although EPA and DHA can be converted in humans from their precursor compound ALA, this metabolic conversion is extremely inefficient and individuals are therefore largely reliant on EPA and DHA dietary intake. Such conversion is mediated by two series of enzymes, desaturases and elongases, and takes place competitively between n-3 and omega-6 (n-6) fatty acids (from dietary linoleic acid (18:2n-6, LA) to arachidonic acid (20:4n-6, AA) and beyond) which are also essential to humans. Therefore, this competition for desaturases and elongases might cause the rate-limiting changes in circulating fatty acid levels in plasma or tissues in humans.

The delta-5-desaturase (D5D) and delta-6-desaturase (D6D), encoded by the genes fatty acid desaturase 1 and 2 (FADS1 and FADS2, respectively), are responsible for the biosynthesis of n-3 and n-6 long chain PUFA from 18 carbon to 20 and 22 carbon fatty acids (5; 6). Beyond the desaturation steps, elongation steps in the pathways are regulated by several elongases of very long chain fatty acid (ELOVL). The predominant enzymes, encoded by gene clusters ELOVL2
and *ELOVL5*, are likely quantitatively most important for the elongation of 20 carbon to 22 carbon, and 18 carbon to 20 carbon fatty acids, respectively, during n-3 long chain PUFA synthesis in mammals (7; 8; 9).

Both desaturases and elongases levels can be associated with the activities of transcription factors such as peroxisome proliferator-activated receptor α (PPARα) (10) and sterol response element-binding protein 1 (SREBP-1), which, in turn, regulate the endogenous synthesis of long chain PUFA (11; 12). Recent studies suggest that common single nucleotide polymorphisms (SNPs) in *FADS1*-*FADS2* gene clusters are associated with levels of long chain PUFA in plasma, tissue and breast milk in humans (13; 14; 15). These associations indicate that the genetic variants in *FADS1*-*FADS2* gene clusters influence plasma concentrations of n-6 and n-3 fatty acids, which in turn affect essential fatty acid metabolism leading to the pathogenesis of multiple diseases (16). With regard to the elongation steps, knowledge of the regulation of *ELOVL2* and *ELOVL5* genes in mRNA/protein expression and enzymatic activities remains unclear in humans (17; 18), and additionally, the association between *ELOVL* family and concentrations of n-3 and n-6 fatty acids during the n-3 and n-6 desaturation and elongation remains unanswered (19). Genome-wide association studies (GWAS) suggest that certain polymorphisms in *ELOVL2* gene influence circulating EPA, docosapentaenoic acid (n-3, DPA), and DHA levels during n-3 long chain PUFA metabolism in humans (17; 20; 21; 22). However, how these polymorphisms modulate *de novo* lipogenesis of these fatty acids remains unclear. Thus, it is important to conduct an intervention study to verify the association between potential gene locus and fatty acid concentrations in response to the dietary fat intake (23).

The aim of the present study was, therefore, to investigate the impact of the common genetic
variants in the *FADS* and *ELOVL* genes on circulating n-3 and n-6 fatty acid composition in response to consumption of different oils with varying fatty acid profiles, as one of the major secondary outcomes of the completed Canola Oil Multicenter Intervention Trial (COMIT). We hypothesized that 1) circulating plasma fatty acid profiles will reflect the consumption of dietary oil treatments rich in long-chain n-3 (DHA), precursor n-3 (ALA) and n-6 (LA); 2) *de novo* synthesis of these target fatty acids, which can be determined using stable isotope tracer, might be influenced by common genetic variants in the *FADS1, FADS2, ELOVL2,* and *ELOVL5* genes, and 3) the diet-gene interactions play a key role in modulating the circulating fatty acid composition in humans.

5.3 Materials and methods

5.3.1 Study design

The COMIT study was a randomized, controlled, double-blinded, crossover study designed to investigate the effects of various vegetable oils in volunteers at risk of metabolic syndrome. This multicenter trial was conducted at the University of Manitoba in Winnipeg, Laval University in Québec City, and the Pennsylvania State University in University Park. This trial was registered with clinicaltrials.gov (NCT01351012). Details of the study design, recruitment, and clinical protocols have been described elsewhere (24; 25). Written consent was obtained from all participants before the start of the trial. The protocol was reviewed and approved by institutional ethics boards across the three participating clinical sites. Briefly, 130 volunteers (70 women) aged 18 years or older at risk of metabolic syndrome (abdominal obesity, high triglyceride level, low HDL level, high glucose level, or hypertension) completed the trial. Individuals were instructed to consume a consistent, weight-maintaining diet of fixed macronutrient composition
daily with an additional healthy vegetable oil treatment in a beverage (60 g/3000 Kcal) during each phase. Each of five treatment phase extended for four weeks and were separated by 4-week washout intervals. The five oil treatments included conventional canola oil (Canola; 60% oleic acid (OA), 20% LA, 10% ALA), DHA-enriched high oleic canola oil (CanolaDHA; 63% OA, 13% LA, 2% ALA, 6% DHA), a blend of corn oil/safflower oil (CornSaff; 18% OA, 69% LA, 0% ALA), a blend of flax oil/safflower oil (FlaxSaff; 18% OA, 38% LA, 32% ALA), and high oleic acid canola oil (CanolaOleic; 72% OA, 15% LA, 2% ALA).

5.3.2 Sample collection

Fasting blood samples were collected on day 29 and 30 for endpoint analyses after each dietary phase. Aliquots of plasma and sera were stored at −80°C and shipped to central laboratories for analyses, including plasma fatty acid profiles (Winnipeg), polymorphism genotyping (Winnipeg), gene expression (Québec City), and sera lipid parameters (St. Michaels Hospital, Toronto).

5.3.3 Plasma fatty acid composition

Total plasma fatty acid profiles were analyzed as previously reported \(^{(24)}\). Briefly, fatty acids were extracted from aliquots of plasma EDTA samples by the Folch method \(^{(26)}\) using chloroform-methanol containing butylated hydroxytoluene, and followed by methylation with methanolic hydrochloric acid. Fatty acid methyl esters (FAMEs) were then measured using a gas chromatography equipped with flame ionization detection (GC-FID). All fatty acid levels were calculated as percentage values of total identified fatty acid from the GC-FID measures. The activities of desaturases and elongases were estimated using product-to-precursor ratios as
follows: D5D (20:4n-6/20:3n-6) and D6D (20:3n-6/18:2n-6), *ELOVL2* (22:5n-3/20:5n-3), and *ELOVL5* (22:4n-6/20:4n-6) as previously described 

5.3.4 Fatty acid synthesis analysis using stable isotope tracers

Fatty acid synthesis rates were assessed using $^2$H labels based on the rate of deuterium incorporated from body water into n-3 and n-6 fatty acids. On day 29 of each experimental phase, a fasting blood sample (0 h) was taken as baseline prior to administration of deuterium oxide ($^2$H$_2$O) at a dose of approximately 0.7 g/kg of estimated body water, where body water is estimated to be 60% of total body weight. On day 30, another overnight fasting blood sample was taken as post-administration (24 hours) for the tracer of $^2$H. Followed by plasma fatty acid extraction and methylation, $^2$H enrichment of each fatty acid methyl ester was measured using a gas chromatography with combustion isotope-ratio mass spectrometry (GC-IRMS). Plasma water pre- and post-administration levels were analyzed using high temperature conversion elemental analyzer (TC/EA)-IRMS in order to correct for the change in the deuterium in the body water pool. Since the tracer $^2$H from $^2$H$_2$O can be taken up by ALA, EPA or any fatty acids along the pathway and be incorporated into the final product DHA, the relative isotope ratio of $^2$H/$^1$H in DHA reflects the maximum assumed conversion of ALA to DHA.

5.3.5 Calculation of de novo lipogenesis rate

Based on the model for TG-FA synthesis rate using $^2$H$_2$O, fractional synthesis rates (FSR) of target fatty acids are calculated in response to the dietary oil treatments by the following equation:

$$\text{FSR/d = } \frac{\Delta \text{FA}}{\Delta \text{plasma} \times 0.87 \times R(H)} \quad \text{Eq. 1}$$
where FSR is the fractional synthesis rate over the study period (24 hours), ΔFA is the isotopic enrichment of deuterium on fatty acids, Δplasma is deuterium enrichment in plasma water, 0.87 is the correction factor observed by Jungas (30), and R(H) represents the ratio of maximum incorporated deuterium atoms to total hydrogen atoms. Here, an assumption is made that the additional protons are all deuterated during the synthesis procedure (29). Thus, the equation estimates the maximum fractional synthesis rate of certain fatty acids depending on their potential $^2\text{H} - ^1\text{H}$ replacement.

The isotopic enrichment of deuterium on plasma water is calculated as below:

$$\Delta_{\text{plasma}} = \frac{R(\text{sample})}{R(\text{standard})} - 1 \quad \text{Eq.2}$$

where R is the ratio of deuterium to hydrogen. R(sample) is the ratio in plasma water from the blood sample, and R(standard) is the reference standard. In the current study, Standard Mean Ocean Water (SMOW) was used as the reference standard.

5.3.6 DNA extraction and genetic variants analysis

Collected buffy coat layers of the blood samples were sent to Winnipeg for the single nucleotide polymorphism genotyping analysis. A total of four common SNPs were selected and genotyped across all subjects. Two SNPs from FADS1-FADS2 gene clusters (FADS1, rs174561; FADS2, rs174583), which have been repeatedly reported in GWAS database and were chosen in our previous investigations (31), were selected; rs953413 on EVLOV2 and rs2397142 on ELOVL5 were selected as those of common tag SNPs (20, 21). Genomic DNA was extracted using commercial Qiagen DNeasy Blood and Tissue Kit according to the manufacturer’s instructions (Qiagen Sciences Inc., Toronto, ON). The quality and quantity of individual genomic DNA were
assessed by Thermo Scientific NanoDrop 2000 micro-volume spectrophotometer (Thermo Fisher Scientific Inc., Waltham, MA). Amplification and detection of DNA were conducted with the 7500 Fast Real-Time PCR System (Applied Biosystems, Life Technologies Inc., Burlington, ON) using customized Taqman SNP Genotyping Assays, (Life Technologies Inc., Burlington, ON). Data were acquired by software StepOne 2.1 (Applied Biosystems, Life Technologies Inc., Burlington, ON).

5.3.7 Gene expression analysis

Gene expression of FADS1 and FADS2 were conducted at Québec City. Blood samples on day 30 of each phase were also collected using PAXGene blood RNA tubes (Qiagen, Valencia, CA) according to manufacturer’s instructions for the measurement of the gene expression in whole blood cells as previously described (32). After RNA was isolated and purified, cDNA was synthesized via reverse transcription using the High Capacity cDNA Reverse Transcription Kit (Life Technologies Inc., Burlington, ON). Gene expression of FADS1 and FADS2 genes was conducted by real-time quantitative PCR using TaqMan® OpenArray® Real-Time PCR Plates with Inventoried Gene Expression Assays (Applied Biosystems, Life Technologies Inc., Burlington, ON) and the QuantStudio 12K Flex Software (Applied Biosystems, Life Technologies Inc., Burlington, ON). Target gene expression values were normalized to the expression of a control gene, glyceraldehyde 3-phosphate dehydrogenase (GAPDH), for all five diets in order to calculate the dCt values, and in turn, estimate the relative expression levels (33).
5.3.8 Other variables

Baseline characteristics of participants were determined at all clinical sites as previously reported \(^{(24)}\). The post-treatment sera lipid profiles were analyzed in a central laboratory at St. Michaels Hospital, University of Toronto, as previously described \(^{(25)}\).

5.3.9 Statistical analysis

Statistical analyses were performed using SAS (SAS version 9.2, SAS Institute Inc., Cary, NC). Results were expressed as least squares means with standard errors unless otherwise noted. Normality and homogeneity of the data were visually checked on the plots of residuals against predicted values. Baseline characteristics of the study subjects were tested using analysis of variance among different genotypes. Mixed-effects repeated-measures analysis of variance were used for data with treatment, genotype, age, BMI and sex as fixed effects, participants as a repeated factor, multicenter effects and sequence of treatments as random effects. Each SNP was individually analyzed by their categorized genotype groups (major/minor alleles). Treatment-by-genotype (diet-gene) interactions were examined in the mixed model with additional adjustments for potential confounders including age, sex, BMI, and treatment sequence. Tukey-adjusted P values were used to examine differences at P<0.05 for all analyses.

A sample size of 120 subjects was determined based on the primary outcome on endothelial function of the COMIT study. In this analysis of secondary outcome on diet-gene interactions, this sample size of 120 ensured that we could detect any possible differences according to our previous investigation of 36 subjects on FADS and ELOVL gene clusters with a greater power \(^{(31)}\).
5.4 Results

A total of 129 out of 130 completed subjects (60 males and 69 females, one blood sample failed to provide enough DNA yield) from the COMIT study were successfully genotyped for the four selected SNPs (Table S5.1). The baseline characteristics of all participants are summarized and presented by the genotypes of rs174583 (FADS2) (Table 5.1). Body weights were slightly higher (P=0.041) in the minor allele carriers of rs174583, and circulating serum triglycerides were higher (P=0.023) in the heterozygotes of rs174583. No other differences were observed in total cholesterol, LDL-C, or HDL-C levels between genotypes at baseline. No other differences were observed at baseline in the other three interested SNPs in the current study.

5.4.1 Plasma fatty acid profiles

Post-treatment total plasma fatty acid profiles have been reported previously (24). Here, selected n-3 and n-6 total plasma fatty acid profiles of all participants are summarized in Table 5.2. Briefly, the fatty acid profiles reflected the differences in the dietary fatty acids in the different dietary treatments. The two n-6 rich diets both resulted in higher (P<0.05) LA levels compared to the three canola oil-based treatments. FlaxSaff produced lower (P<0.05) AA levels and higher (P<0.05) ALA than other four diets. CanolaDHA resulted in higher (P<0.05) DHA levels than other treatments. Both FlaxSaff and CanolaDHA treatments had higher (P<0.05) EPA levels compared to other three treatments.
Table 5.1 Baseline characteristics of study subjects by polymorphism rs174583.  

<table>
<thead>
<tr>
<th>Anthropometric and serum components</th>
<th>All participants (n=129)</th>
<th>Genotypes of rs174583, FADS2&lt;sup&gt;2&lt;/sup&gt;</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (y)</td>
<td>46.4±2.4</td>
<td>CC (n=49) 47.5±2.0</td>
<td>0.538</td>
</tr>
<tr>
<td></td>
<td></td>
<td>CT (n=61) 45.0±1.8</td>
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<tr>
<td></td>
<td></td>
<td>TT (n=19) 48.3±3.3</td>
<td></td>
</tr>
<tr>
<td>Body weight (kg)</td>
<td>85.1±2.6</td>
<td>CC (n=49) 80.8±2.2&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.041</td>
</tr>
<tr>
<td></td>
<td></td>
<td>CT (n=61) 87.1±2.0&lt;sup&gt;a&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>TT (n=19) 89.9±3.6&lt;sup&gt;a&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>Height (m)</td>
<td>1.69±0.02</td>
<td>CC (n=49) 1.67±0.01</td>
<td>0.369</td>
</tr>
<tr>
<td></td>
<td></td>
<td>CT (n=61) 1.70±0.01</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>TT (n=19) 1.68±0.02</td>
<td></td>
</tr>
<tr>
<td>Waist circumference (cm)</td>
<td>101±2.0</td>
<td>CC (n=49) 99.5±1.7</td>
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</tr>
<tr>
<td></td>
<td></td>
<td>CT (n=61) 102±1.6</td>
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</tr>
<tr>
<td></td>
<td></td>
<td>TT (n=19) 105±2.8</td>
<td></td>
</tr>
<tr>
<td>Serum Glucose (mmol/L)</td>
<td>5.34±0.19</td>
<td>CC (n=49) 5.53±0.16</td>
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</tr>
<tr>
<td></td>
<td></td>
<td>CT (n=61) 5.27±0.15</td>
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</tr>
<tr>
<td></td>
<td></td>
<td>TT (n=19) 5.11±0.26</td>
<td></td>
</tr>
<tr>
<td>Serum Total cholesterol (mmol/L)</td>
<td>5.31±0.19</td>
<td>CC (n=49) 5.28±0.16</td>
<td>0.134</td>
</tr>
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<td></td>
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<td>CT (n=61) 5.47±0.14</td>
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</tr>
<tr>
<td></td>
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<td>TT (n=19) 4.88±0.26</td>
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<tr>
<td>Serum LDL cholesterol (mmol/L)</td>
<td>3.21±0.16</td>
<td>CC (n=49) 3.21±0.13</td>
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<td>CT (n=61) 3.28±0.12</td>
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<tr>
<td></td>
<td></td>
<td>TT (n=19) 2.96±0.22</td>
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<tr>
<td>Serum HDL cholesterol (mmol/L)</td>
<td>1.29±0.06</td>
<td>CC (n=49) 1.34±0.05</td>
<td>0.381</td>
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<tr>
<td></td>
<td></td>
<td>CT (n=61) 1.27±0.04</td>
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<td></td>
<td>TT (n=19) 1.23±0.08</td>
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</tr>
<tr>
<td>Serum Triglycerides</td>
<td>1.80±0.17</td>
<td>CC (n=49) 1.60±0.15&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.023</td>
</tr>
<tr>
<td></td>
<td></td>
<td>CT (n=61) 2.06±0.13&lt;sup&gt;a&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>TT (n=19) 1.47±0.23&lt;sup&gt;b&lt;/sup&gt;</td>
<td></td>
</tr>
</tbody>
</table>
All values are means ± SDs, n=129; Genotypes of rs174583 are CC, CT and TT. Analysis of variance was used to analyze between-genotype differences. Labeled means in a row without a common letter differ, P< 0.05. Reproduced with permission from reference 24.
Table 5.2 Selected n-3 and n-6 plasma fatty acid profiles at the end of five dietary oil treatments in participants in a crossover design (g/100g).^1

<table>
<thead>
<tr>
<th>Fatty acids</th>
<th>Treatments</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Canola</td>
<td>CanolaDHA</td>
</tr>
<tr>
<td><strong>n-3 PUFA</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C18:3n3</td>
<td>0.78±0.04^b</td>
<td>0.58±0.04^cd</td>
</tr>
<tr>
<td>C20:5n3</td>
<td>1.08±0.06^b</td>
<td>1.52±0.06^a</td>
</tr>
<tr>
<td>C22:5n3</td>
<td>0.81±0.03^b</td>
<td>0.33±0.03^e</td>
</tr>
<tr>
<td>C22:6n3</td>
<td>2.81±0.15^b</td>
<td>7.12±0.15^a</td>
</tr>
<tr>
<td><strong>n-6 PUFA</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C18:2n6</td>
<td>22.0±0.3^c</td>
<td>19.0±0.3^d</td>
</tr>
<tr>
<td>C20:3n6</td>
<td>2.48±0.17^ab</td>
<td>1.78±0.17^c</td>
</tr>
<tr>
<td>C20:4n6</td>
<td>9.32±0.39^b</td>
<td>9.72±0.39^a</td>
</tr>
<tr>
<td>C22:4n6</td>
<td>0.27±0.03^b</td>
<td>0.25±0.03^b</td>
</tr>
</tbody>
</table>

^1All values are % abundance of each fatty acid to total fatty acids given as least squares means ± SEs, n=129 for each experimental diet. Mixed-effects repeated-measures analysis of variance with treatment, age, gender and the dietary phase as fixed effect, the last
diet (sequence effect) as a random effect, and the measures for each subject by phases were used for the data analysis. Canola, conventional canola oil; CanolaOleic, high oleic canola oil; CanolaDHA, high oleic canola and DHA oil blend; CornSaff, corn and safflower oil blend; FlaxSaff, flax and safflower oil blend; PUFA, polyunsaturated fatty acids. Labeled means in a row without a common letter differ, P< 0.05.
Selected fatty acid ratios relative to the enzyme activities are presented in Figure 5.1, and their effects by corresponding SNPs are presented in Table S5.2. Calculated D5D enzyme activities were significantly different across treatments (P<0.001), and different across FADS1 genotypes (P<0.001). D6D enzyme activities were significantly different across treatments (P<0.001), but not FADS2 genotypes. The enzyme activities of ELOVL2 across treatments were significantly different (P<0.001), but not associated with ELOVL2 genotypes. ELOVL5 enzyme activities were not affected by treatments or ELOVL genotypes.

5.4.2 Fractional synthesis rates of n-3 and n-6 fatty acids

Results of deuterium enrichment of plasma AA and DHA were observed in a total of 120 subjects at 24 hours after consumption of the administered dose of deuterium water, presented in Figure 5.2. Overall, no differences were observed for the DHA synthesis rate across all five diets. The CornSaff diet, rich in LA, resulted in a higher AA synthesis rate compared to CanolaDHA, FlaxSaff, and CanolaOleic (P<0.05). It was not possible to capture the peaks with sufficient intensity for the enrichment of EPA and DPA on the GC-IRMS.
Figure 5.1 Enzyme activities using fatty acid ratios at the end of five dietary oil treatments in participants in a crossover design.

All values are least squares means ± SEs, n=129 for each experimental diet. Mixed-effects repeated-measures analysis of variance with treatment, age, gender and the dietary phase as fixed effect, the last diet (sequence effect) as a random effect, and the measures for each subject by phases were used for the data analysis. Canola, conventional canola oil; CanolaOleic, high oleic canola oil; CanolaDHA, high oleic canola and DHA oil blend; CornSaff, corn and safflower oil blend; D5D, delta-5-desaturase; D6D, delta-6-desaturase; FlaxSaff, flax and safflower oil blend. Labeled means without a common letter differ, P< 0.05.
Figure 5.2 Fractional synthesis rates of plasma AA and DHA at 24 hours after consumption of administered deuterium water at the end of five dietary oil treatments in participants in a crossover design.

All values are least squares means ± SEs, n=120 for each experimental diet. Mixed-effects repeated-measures analysis of variance with treatment, age, gender and the dietary phase as fixed effect, the last diet (sequence effect) as a random effect, and the measures for each subject by phases were used for the data analysis. Canola, conventional canola oil; CanolaOleic, high oleic canola oil; CanolaDHA, high oleic canola and DHA oil blend; CornSaff, corn and safflower oil blend; FlaxSaff, flax and safflower oil blend. Labeled means without a common letter differ, P<0.05.
5.4.3 SNP characteristics and association with endpoint circulating fatty acid levels

Several potential interactions of dietary intakes and selected SNPs on plasma n-3 fatty acid levels were evaluated in our study, and results of SNPs on FADS and ELOVL genes are reported in Table 5.3 and Table 5.4, respectively. We observed that the diet-gene interactions significantly influenced EPA levels, including rs174561 (FADS1) (P<0.001), rs174583 (FADS2) (P=0.001), and rs2397142 (ELOVL5) (P=0.009), indicating that variation in dietary oil intake interacts with common variants in these genes on EPA metabolism. Similarly, the interactions between dietary treatments and polymorphisms rs174561 and rs174583 were also observed for plasma DPA (P=0.050 and P=0.041, respectively), but not for DHA levels. In addition, plasma ALA level was modified by the ALA rich diet, FlaxSaff, with a tendency of genetic interaction of rs953413 (ELOVL2) (P=0.07), while the major allele homozygotes produced higher (P<0.05) ALA than that in minor allele carrier groups. No other interactions were observed in the selected ELOVL2 and ELOVL5 polymorphisms.

In contrast to the findings on plasma n-3 fatty acid levels between CornSaff vs. FlaxSaff (Figure 5.3), results showed that the rs174583 minor allele homozygotes (TT) manifested lower (P<0.05) circulating EPA and DPA levels than major allele carriers (CC/CT) after the FlaxSaff diet. On the other hand, no differences were observed after the CornSaff treatment while ALA and DHA both showed no influence by these genetic variants. For n-6 fatty acids, polymorphism rs174583 showed significantly lower (P<0.05) plasma LA and AA levels compared with TT and CC/CT genotypes after both CornSaff and FlaxSaff diets (Table S5.3).
Figure 5.3 Impact of polymorphism rs174583 (FADS2) on plasma n-3 fatty acid levels in CornSaff and FlaxSaff diets in participants in a crossover design.

(A), CornSaff (n-6), corn and safflower oil blend; (B), FlaxSaff (n-3), flax and safflower oil blend. All values are least squares means ± SEs, n=129 for each experimental diet. Mixed-effects repeated-measures analysis of variance with treatment, age, gender and the dietary phase as fixed effect, the last diet (sequence effect) as a random effect, and the measures for each subject by phases were used for the data analysis. Major allele homozygotes CC (n=49); heterozygotes CT (n=61); minor allele homozygotes TT (n=19). Labeled means without a common letter differ, P<0.05.
Table 5. Selected plasma n-3 PUFA composition at the end of five dietary oil treatments in participants in a crossover design
(g/100g)

<table>
<thead>
<tr>
<th>Fatty acid, genotypes</th>
<th>Treatments</th>
<th>Canola</th>
<th>CanolaDHA</th>
<th>CornSaff</th>
<th>FlaxSaff</th>
<th>CanolaOleic</th>
<th>P-treatment</th>
<th>P-genotype</th>
<th>P-interaction</th>
</tr>
</thead>
<tbody>
<tr>
<td>18:3n3, ALA</td>
<td></td>
<td></td>
<td></td>
<td></td>
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<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>FADS1 MM</td>
<td>0.77±0.06</td>
<td>0.56±0.06</td>
<td>0.46±0.06</td>
<td>1.64±0.06</td>
<td>0.60±0.06</td>
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</tr>
<tr>
<td>rs174561 Mm</td>
<td>0.77±0.06</td>
<td>0.57±0.06</td>
<td>0.49±0.06</td>
<td>1.62±0.06</td>
<td>0.65±0.06</td>
<td>&lt;0.001</td>
<td>0.634</td>
<td>0.985</td>
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</tr>
<tr>
<td>mm</td>
<td>0.70±0.12</td>
<td>0.46±0.12</td>
<td>0.42±0.12</td>
<td>1.46±0.12</td>
<td>0.54±0.12</td>
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<tr>
<td>FADS2 MM</td>
<td>0.77±0.07</td>
<td>0.56±0.07</td>
<td>0.46±0.07</td>
<td>1.58±0.07</td>
<td>0.59±0.07</td>
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<td>rs174583 Mm</td>
<td>0.75±0.06</td>
<td>0.57±0.06</td>
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<td>&lt;0.001</td>
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<td>mm</td>
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<td>Mean 2</td>
<td>Mean 3</td>
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<td>0.62±0.08</td>
<td>0.24±0.08</td>
<td>0.52±0.08</td>
<td>0.70±0.08</td>
<td>0.59±0.08</td>
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</tr>
<tr>
<td>rs174561</td>
<td>22:6N-3, DHA</td>
<td>2.83±0.12</td>
<td>7.01±0.12</td>
<td>2.63±0.12</td>
<td>2.58±0.12</td>
<td>2.74±0.12</td>
<td>&lt;0.001</td>
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</tr>
<tr>
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<td>2.87±0.25</td>
<td>7.73±0.25</td>
<td>2.83±0.25</td>
<td>2.62±0.25</td>
<td>2.77±0.25</td>
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<td></td>
</tr>
<tr>
<td>rs174583</td>
<td>FADS2</td>
<td>3.05±0.14</td>
<td>7.29±0.14</td>
<td>2.83±0.14</td>
<td>2.85±0.14</td>
<td>2.93±0.14</td>
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</tr>
<tr>
<td></td>
<td></td>
<td>2.86±0.12</td>
<td>7.21±0.12</td>
<td>2.68±0.12</td>
<td>2.63±0.12</td>
<td>2.84±0.12</td>
<td>&lt;0.001</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>2.69±0.21</td>
<td>7.02±0.21</td>
<td>2.59±0.21</td>
<td>2.44±0.21</td>
<td>2.56±0.22</td>
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<td></td>
</tr>
</tbody>
</table>
All values are % abundance of each fatty acid to total fatty acids given as least squares means ± SEs, n=129 for each experimental diet. MM: major allele homozygotes; Mm: heterozygotes; mm: minor allele homozygotes. Mixed-effects repeated-measures analysis of variance with treatment, age, gender and the dietary phase as fixed effect, the last diet (sequence effect) as a random effect, and the measures for each subject by phases were used for the data analysis. rs174561 (FADS1): MM (n=55), Mm (n=60) and mm (n=14); rs174583 (FADS2) MM (n=49), Mm (n=61) and mm (n=19). Canola, conventional canola oil; CanolaOleic, high oleic canola oil; CanolaDHA, high oleic canola and DHA oil blend; CornSaff, corn and safflower oil blend; FlaxSaff, flax and safflower oil blend. Significant differences at P< 0.05.
Table 5.4 Selected plasma n-3 PUFA composition at the end of five dietary oil treatments in participants in a crossover design (g/100g), by rs953413 (ELOVL2) and rs2397142 (ELOVL5) genotypes.

<table>
<thead>
<tr>
<th>Fatty acid, genotypes</th>
<th>Treatments</th>
<th>P-treatment</th>
<th>P-genotype</th>
<th>P-interaction</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Canola</td>
<td>CanolaDHA</td>
<td>CornSaff</td>
<td>FlaxSaff</td>
</tr>
<tr>
<td>18:3n3, ALA</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ELOVL2</td>
<td>MM</td>
<td>0.86±0.09</td>
<td>0.58±0.09</td>
<td>0.44±0.09</td>
</tr>
<tr>
<td>rs953413</td>
<td>Mm</td>
<td>0.74±0.06</td>
<td>0.57±0.06</td>
<td>0.50±0.06</td>
</tr>
<tr>
<td></td>
<td>mm</td>
<td>0.74±0.07</td>
<td>0.52±0.07</td>
<td>0.43±0.07</td>
</tr>
<tr>
<td>ELOVL5</td>
<td>MM</td>
<td>0.77±0.06</td>
<td>0.54±0.06</td>
<td>0.45±0.06</td>
</tr>
<tr>
<td>rs2397142</td>
<td>Mm</td>
<td>0.72±0.06</td>
<td>0.58±0.06</td>
<td>0.48±0.06</td>
</tr>
<tr>
<td></td>
<td>mm</td>
<td>0.81±0.10</td>
<td>0.51±0.10</td>
<td>0.48±0.11</td>
</tr>
<tr>
<td>20:5n3, EPA</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>ELOVL2</td>
<td>MM</td>
<td>1.13±0.12</td>
<td>1.54±0.12</td>
<td>0.41±0.12</td>
</tr>
<tr>
<td>rs953413</td>
<td>Mm</td>
<td>1.02±0.07</td>
<td>1.47±0.07</td>
<td>0.43±0.07</td>
</tr>
<tr>
<td></td>
<td>mm</td>
<td>1.05±0.10</td>
<td>1.49±0.10</td>
<td>0.49±0.10</td>
</tr>
<tr>
<td>ELOVL5</td>
<td>MM</td>
<td>1.13±0.08</td>
<td>1.46±0.08</td>
<td>0.49±0.08</td>
</tr>
<tr>
<td>SNP</td>
<td>Gene</td>
<td>Mm</td>
<td>mm</td>
<td>Mm</td>
</tr>
<tr>
<td>---------</td>
<td>------</td>
<td>-----</td>
<td>------</td>
<td>-----</td>
</tr>
<tr>
<td>rs2397142</td>
<td>Mm</td>
<td>0.98±0.08</td>
<td>1.61±0.08</td>
<td>0.39±0.08</td>
</tr>
<tr>
<td></td>
<td>mm</td>
<td>0.93±0.14</td>
<td>1.26±0.14</td>
<td>0.45±0.14</td>
</tr>
<tr>
<td>22:5n-3, DPA</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ELOVL2</td>
<td>MM</td>
<td>0.80±0.07</td>
<td>0.23±0.07</td>
<td>0.58±0.07</td>
</tr>
<tr>
<td>rs953413</td>
<td>Mm</td>
<td>0.76±0.04</td>
<td>0.27±0.04</td>
<td>0.58±0.04</td>
</tr>
<tr>
<td></td>
<td>mm</td>
<td>0.85±0.06</td>
<td>0.42±0.06</td>
<td>0.65±0.06</td>
</tr>
<tr>
<td>ELOVL5</td>
<td>MM</td>
<td>0.77±0.05</td>
<td>0.30±0.05</td>
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<tr>
<td>rs2397142</td>
<td>Mm</td>
<td>0.82±0.05</td>
<td>0.32±0.05</td>
<td>0.60±0.05</td>
</tr>
<tr>
<td></td>
<td>mm</td>
<td>0.76±0.08</td>
<td>0.31±0.08</td>
<td>0.60±0.09</td>
</tr>
<tr>
<td>22:6n-3, DHA</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ELOVL2</td>
<td>MM</td>
<td>2.85±0.18</td>
<td>7.17±0.18</td>
<td>2.72±0.18</td>
</tr>
<tr>
<td>rs953413</td>
<td>Mm</td>
<td>2.83±0.12</td>
<td>7.00±0.12</td>
<td>2.57±0.12</td>
</tr>
<tr>
<td></td>
<td>mm</td>
<td>3.04±0.15</td>
<td>7.56±0.15</td>
<td>2.95±0.15</td>
</tr>
<tr>
<td>ELOVL5</td>
<td>MM</td>
<td>2.85±0.12</td>
<td>7.17±0.12</td>
<td>2.66±0.12</td>
</tr>
<tr>
<td>rs2397142</td>
<td>Mm</td>
<td>2.92±0.13</td>
<td>7.25±0.13</td>
<td>2.71±0.13</td>
</tr>
<tr>
<td></td>
<td>mm</td>
<td>2.95±0.21</td>
<td>7.16±0.21</td>
<td>2.91±0.22</td>
</tr>
</tbody>
</table>
All values are % abundance of each fatty acid to total fatty acids given as least squares means ± SEs, n=129 for each experimental diet. MM: major allele homozygotes; Mm: heterozygotes; mm: minor allele homozygotes. Mixed-effects repeated-measures analysis of variance with treatment, age, gender and the dietary phase as fixed effect, the last diet (sequence effect) as a random effect, and the measures for each subject by phases were used for the data analysis. rs953413 (ELOVL2): MM (n=40), Mm (n=65) and mm (n=24); rs2397142 (ELOVL5) MM (n=61), Mm (n=51) and mm (n=17). Canola, conventional canola oil; CanolaOleic, high oleic canola oil; CanolaDHA, high oleic canola and DHA oil blend; CornSaff, corn and safflower oil blend; FlaxSaff, flax and safflower oil blend. Significant differences at P< 0.05.
5.4.4 SNP associations with plasma deuterium enrichment in fatty acids

The results of diet-gene interactions on fractional synthesis rates of plasma AA and DHA are presented on Table 5.5. Diet-gene interactions for DHA synthesis using deuterium across all treatment groups and SNPs were not observed. Interestingly, a significant interaction was observed between treatments and polymorphism rs174561 (FADS1) on the synthesis of AA in plasma (P=0.009). The major allele homozygotes (TT) in rs174561 had higher (P=0.009) AA synthesis rates after the CornSaff treatment than those after the CanolaDHA diet.

5.4.5 SNP association with gene expression levels

Results of gene expression analyses for FADS1 and FADS2 genes are presented in Figure 5.4. Overall, dietary oil treatments had no impact on expression levels of FADS1 or FADS2. The genetic variants of the related SNPs on FADS1 and FADS2 did not appear to impact the expression levels either. No diet-gene interactions were observed.
Table 5. Diet-gene interactions on fractional synthesis rates of plasma AA and DHA in response to five dietary oil treatments in participants in a crossover design.¹

<table>
<thead>
<tr>
<th>Fatty acid, genotypes²</th>
<th>Treatments</th>
<th>Canola</th>
<th>CanolaDHA</th>
<th>CornSaff</th>
<th>FlaxSaff</th>
<th>CanolaOleic</th>
<th>P-treatment</th>
<th>P-genotype</th>
<th>P-interaction</th>
</tr>
</thead>
<tbody>
<tr>
<td>20:4n6, AA</td>
<td></td>
<td></td>
<td></td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>FADS1</td>
<td>MM</td>
<td>0.22±0.04</td>
<td>0.10±0.04</td>
<td>0.26±0.04</td>
<td>0.15±0.04</td>
<td>0.13±0.04</td>
<td>0.001</td>
<td></td>
<td></td>
</tr>
<tr>
<td>rs174561</td>
<td>Mm</td>
<td>0.12±0.04</td>
<td>0.12±0.04</td>
<td>0.22±0.04</td>
<td>0.14±0.04</td>
<td>0.09±0.04</td>
<td>0.014</td>
<td>0.456</td>
<td>0.009</td>
</tr>
<tr>
<td></td>
<td>mm</td>
<td>0.29±0.08</td>
<td>0.13±0.08</td>
<td>0.13±0.08</td>
<td>0.04±0.07</td>
<td>0.29±0.07</td>
<td>0.019</td>
<td></td>
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</tr>
<tr>
<td>FADS2</td>
<td>MM</td>
<td>0.23±0.04</td>
<td>0.09±0.04</td>
<td>0.26±0.04</td>
<td>0.15±0.04</td>
<td>0.12±0.04</td>
<td>0.001</td>
<td></td>
<td></td>
</tr>
<tr>
<td>rs174583</td>
<td>Mm</td>
<td>0.17±0.04</td>
<td>0.14±0.04</td>
<td>0.24±0.04</td>
<td>0.13±0.04</td>
<td>0.14±0.04</td>
<td>0.032</td>
<td>0.057</td>
<td>0.352</td>
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<tr>
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<td>mm</td>
<td>0.02±0.07</td>
<td>0.07±0.06</td>
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<td>0.11±0.06</td>
<td>0.08±0.06</td>
<td>0.837</td>
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</tr>
<tr>
<td>ELOVL2</td>
<td>MM</td>
<td>0.20±0.06</td>
<td>0.12±0.06</td>
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<td>0.15±0.06</td>
<td>0.14±0.06</td>
<td>0.043</td>
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<tr>
<td>rs953413</td>
<td>Mm</td>
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<td>0.13±0.04</td>
<td>0.22±0.04</td>
<td>0.14±0.04</td>
<td>0.14±0.04</td>
<td>0.024</td>
<td>0.129</td>
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<tr>
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<td>0.09±0.04</td>
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<tr>
<td>ELOVL5</td>
<td>MM</td>
<td>0.11±0.04</td>
<td>0.11±0.04</td>
<td>0.21±0.04</td>
<td>0.13±0.04</td>
<td>0.09±0.04</td>
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<tr>
<td>rs2397142</td>
<td>Mm</td>
<td>0.22±0.04</td>
<td>0.11±0.04</td>
<td>0.23±0.04</td>
<td>0.13±0.04</td>
<td>0.16±0.04</td>
<td>0.022</td>
<td>0.142</td>
<td>0.366</td>
</tr>
<tr>
<td>Gene</td>
<td>Major Allele</td>
<td>Heterozygote</td>
<td>Minor Allele</td>
<td>p-value</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>--------</td>
<td>--------------</td>
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<td>---------</td>
<td></td>
<td></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>22:6n3 DHA</td>
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<td></td>
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<td>0.047</td>
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<td></td>
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<td></td>
</tr>
<tr>
<td>FADS1</td>
<td>MM 0.04±0.03</td>
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<td>0.09±0.02</td>
<td>0.048</td>
<td>0.476</td>
<td>0.753</td>
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</tr>
<tr>
<td></td>
<td>Mm 0.06±0.02</td>
<td>0.05±0.02</td>
<td>0.05±0.02</td>
<td>0.244</td>
<td>0.661</td>
<td>0.568</td>
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</tr>
<tr>
<td></td>
<td>mm 0.09±0.06</td>
<td>0.01±0.06</td>
<td>0.09±0.05</td>
<td>0.884</td>
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<td></td>
</tr>
<tr>
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<td>MM 0.04±0.03</td>
<td>0.01±0.03</td>
<td>0.04±0.03</td>
<td>0.244</td>
<td>0.661</td>
<td>0.568</td>
<td></td>
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<td></td>
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<tr>
<td></td>
<td>Mm 0.06±0.02</td>
<td>0.06±0.02</td>
<td>0.05±0.02</td>
<td>0.884</td>
<td></td>
<td></td>
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</tr>
<tr>
<td></td>
<td>mm 0.02±0.05</td>
<td>0.01±0.05</td>
<td>0.04±0.05</td>
<td>0.884</td>
<td></td>
<td></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>ELOVL2</td>
<td>MM 0.01±0.04</td>
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<td>-0.01±0.04</td>
<td>0.212</td>
<td>0.198</td>
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<tr>
<td></td>
<td>Mm 0.08±0.02</td>
<td>0.08±0.02</td>
<td>0.05±0.02</td>
<td>0.866</td>
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<td></td>
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<td></td>
</tr>
<tr>
<td></td>
<td>mm 0.03±0.03</td>
<td>0.00±0.03</td>
<td>0.05±0.03</td>
<td>0.866</td>
<td></td>
<td></td>
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</tr>
<tr>
<td>ELOVL5</td>
<td>MM 0.03±0.02</td>
<td>0.01±0.02</td>
<td>0.03±0.02</td>
<td>0.230</td>
<td>0.266</td>
<td>0.648</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Mm 0.08±0.03</td>
<td>0.06±0.03</td>
<td>0.06±0.03</td>
<td>0.230</td>
<td>0.266</td>
<td>0.648</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>mm 0.03±0.05</td>
<td>0.04±0.05</td>
<td>0.07±0.05</td>
<td>0.230</td>
<td>0.266</td>
<td>0.648</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

1All values are least squares means ± SEs, n=129 for each experimental diet. 2MM: major allele homozygotes; Mm: heterozygotes; mm: minor allele homozygotes. Mixed-effects repeated-measures analysis of variance with treatment, age, gender and the dietary
phase as fixed effect, the last diet (sequence effect) as a random effect, and the measures for each subject by phases were used for the data analysis. rs174561 (FADS1): MM (n=55), Mm (n=60) and mm (n=14); rs174583 (FADS2) MM (n=49), Mm (n=61) and mm (n=19); rs953413 (ELOVL2): MM (n=40), Mm (n=65) and mm (n=24); rs2397142 (ELOVL5) MM (n=61), Mm (n=51) and mm (n=17). Canola, conventional canola oil; CanolaOleic, high oleic canola oil; CanolaDHA, high oleic canola and DHA oil blend; CornSaff, corn and safflower oil blend; FlaxSaff, flax and safflower oil blend. Significant differences at P< 0.05.
Figure 5.4 Gene expression of FADS1 and FADS2 at the end of five dietary oil treatments in participants in a crossover design.

(A), FADS1; (B), FADS2. All values are least squares means ± SEs, n=66 for each experimental diet. Mixed-effects repeated-measures analysis of variance with treatment, age, gender and the dietary phase as fixed effect, the last diet (sequence effect) as a random effect, and the measures for each subject by phases were used for the data analysis. Canola, conventional canola oil; CanolaOleic, high oleic canola oil; CanolaDHA, high oleic canola and DHA oil blend; CornSaff, corn and safflower oil blend; FlaxSaff, flax and safflower oil blend. No significant differences were observed, P< 0.05.
Table S5.1 SNP characteristics of the selected SNPs (n=129)

<table>
<thead>
<tr>
<th>Gene</th>
<th>SNP</th>
<th>Alleles (Major/minor)</th>
<th>Genotypes(^{1,2}) MM</th>
<th>MAF(%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>FADS1</td>
<td>rs174561</td>
<td>T/C</td>
<td>55</td>
<td>60</td>
</tr>
<tr>
<td>FADS2</td>
<td>rs174583</td>
<td>C/T</td>
<td>49</td>
<td>61</td>
</tr>
<tr>
<td>ELOVL2</td>
<td>rs953413</td>
<td>G/A</td>
<td>40</td>
<td>65</td>
</tr>
<tr>
<td>ELOVL5</td>
<td>rs2397142</td>
<td>C/G</td>
<td>61</td>
<td>51</td>
</tr>
</tbody>
</table>

\(^1\)Number of subjects for each genotype. \(^2\)MM: major allele homozygotes; Mm: heterozygotes; mm: minor allele homozygotes. MAF: minor allele frequency; SNP: single nucleotide polymorphism.
Table S5.2. Effects of SNPs on their corresponding enzyme activities (n=129)

<table>
<thead>
<tr>
<th>Enzyme activities, SNPs</th>
<th>Genotypes</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>MM</td>
<td>Mm</td>
</tr>
<tr>
<td><strong>D5D</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>rs174561</td>
<td>6.05±0.36</td>
<td>4.73±0.30</td>
</tr>
<tr>
<td><strong>D6D</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>rs174583</td>
<td>0.09±0.00</td>
<td>0.10±0.00</td>
</tr>
<tr>
<td><strong>ELOVL2</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>rs953413</td>
<td>0.05±0.13</td>
<td>0.08±0.08</td>
</tr>
<tr>
<td><strong>ELOVL5</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>rs2397142</td>
<td>0.09±0.08</td>
<td>0.21±0.09</td>
</tr>
</tbody>
</table>

1MM: major allele homozygotes; Mm: heterozygotes; mm: minor allele homozygotes. All values are least squares means ± SEs, n=129 for each experimental diet. SNP: single nucleotide polymorphism. Significant differences at P<0.05.
Table S5.3 Impact of polymorphism rs174583 (*FADS2*) on plasma n-6 fatty acid levels (g/100g) in CornSaff and FlaxSaff diets (n=129).

<table>
<thead>
<tr>
<th>n-6 fatty acids</th>
<th>Genotypes(^1)</th>
<th>Treatment(^2)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>CornSaff (n-6)</td>
<td>FlaxSaff (n-3)</td>
</tr>
<tr>
<td>18:2n6 LA</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CC</td>
<td>24.5±0.4(^b)</td>
<td>23.8±0.4(^b)</td>
</tr>
<tr>
<td>CT</td>
<td>25.8±0.3(^b)</td>
<td>25.0±0.3(^b)</td>
</tr>
<tr>
<td>TT</td>
<td>28.9±0.6(^a)</td>
<td>28.0±0.6(^a)</td>
</tr>
<tr>
<td>20:4n6 AA</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CC</td>
<td>11.2±0.3(^a)</td>
<td>9.4±0.3(^a)</td>
</tr>
<tr>
<td>CT</td>
<td>9.4±0.2(^b)</td>
<td>8.3±0.2(^b)</td>
</tr>
<tr>
<td>TT</td>
<td>7.2±0.4(^c)</td>
<td>6.5±0.4(^c)</td>
</tr>
</tbody>
</table>

\(^1\)Major allele homozygotes CC (n=49); hetrozygotes CT (n=61); minor allele homozygotes TT (n=19);  
\(^2\)Two selected treatments: CornSaff (n-6), corn and safflower oil blend; FlaxSaff (n-3), flax and safflower oil blend. All values are least squares means ± SEs, n=129 for each experimental diet. Labeled means with a column without a common letter differ, P< 0.05.
5.5 Discussion

The present study shows that genetic variants of *FADS1, FADS2, ELOVL2* and *ELOVL5* genes associate with differences in n-3 and n-6 PUFA concentrations. In addition, diet-gene interactions were found to play an important role in modulating plasma fatty acid composition in humans. Although dietary ALA can significantly increase EPA and DPA but not DHA levels in plasma, *de novo* synthesis of DHA remained unchanged in response to all treatments regardless of common genetic variants. Thus, consuming n-3 rich diets which can interact with the genetic variants in *FADS* and *ELOVL* genes appears to be an effective way to improve circulating fatty acid profile in the general population.

Dietary n-3 and n-6 intake could interact with common genetic variants to modulate circulating fatty acid levels. In a Swedish study investigating interactions between different PUFA intakes and genetic variants in *FADS* gene on fasting blood lipid profile, results from 4,635 subjects suggested that different PUFA intakes could modulate the association of genetic variation in *FADS* gene on lipid parameters \(^{(34)}\). Recently, a Chinese study by Liu et al. \(^{(35)}\) confirmed such finding in a Chinese population of 1,278 subjects that dietary n-3 intake (EPA + DHA) could modulate the association between the polymorphism rs174547 in *FADS1* gene and coronary artery disease. Here, our data also showed these genetic variations exists in requirements for n-3 and n-6 fatty acid intakes on the fatty acid profile. Previously, the impact of SNPs in *FADS* and *ELOVL* genes has been investigated intensively. In the CHARGE study \(^{(17)}\), a meta-analysis of GWAS in populations of European ancestry, results from 8,866 subjects showed that genetic variants in *FADS1, FADS2* and *ELOVL2* genes were associated with plasma phospholipid levels of the major n-3 PUFAs including ALA, EPA, DPA and DHA. It has been reported that minor
alleles of SNPs in *FADS* gene clusters were associated with higher levels of ALA, and lower levels of EPA and DPA, while minor alleles of rs953413 in *ELOVL2* were associated with higher levels of EPA and DPA, but lower levels of DHA. In the GOLDN (USA) and the InCHIANTI (Italy) studies\(^{(20)}\), researchers predicted that the variant of rs953413 in *ELOVL2*, located in intron 1 region, might be functional, and associated with lower expression of the *ELOVL2* or result in a lower enzyme activity in the elongation of EPA to DHA. However, in the present study, when we showed that EPA, DPA and DHA were significantly shifted by the n-3 rich dietary treatments, we did not observe the interaction between rs953413 genotypes and n-3 fatty acid profiles. Our data support that the polymorphism rs953413 does not interact with dietary fatty acids and does not influence circulating EPA, DPA or DHA levels.

The present study clearly shows that increased intake of ALA elevated circulating ALA, EPA and DPA, but not DHA. The FSR values of DHA across five treatments did not differ, indicating that dietary ALA intake did not influence circulating DHA levels. Human studies exploring the conversion of dietary ALA consistently show that ALA can effectively increase circulating ALA, EPA, and DPA but does not affect DHA\(^{(36; 37; 38)}\). In addition, Burdge and Calder\(^{(39)}\) concluded that there are some exceptions about *de novo* synthesis of DHA between women who show some conversion whereas men do not, which may be due to the effect of hormonal regulation. In the present study, the FSR values of DHA across all diets were not different between women and men. Furthermore, our data on diet-gene interactions between *FADS/ELOVL* gene clusters and treatment oils only showed significant effects on EPA, DPA levels but not DHA content. In fact, one of the most important implications of the present study is that only consumption of DHA enriched diet (CanolaDHA) effectively elevated circulating DHA levels. One study by Molto-Puigmart et al.\(^{(15)}\) reported that DHA in plasma phospholipids was elevated with increasing fish
and fish-oil intake regardless of their genotypes in FADS1-FADS2 gene cluster. Overall, our finding showing increased plasma DHA after consumption of CanolaDHA, irrespective of the genotype, was consistent with this previous study, and demonstrated that DHA supplementation is most effective way to boost circulating DHA levels in humans.

The surrogate markers, product-to-precursor ratios, AA/LA clearly revealed lower levels in minor allele homozygotes of rs174583 compared with major allele carriers, suggesting reduced FADS2 activity in such minor allele homozygotes group. This observation agrees with the findings of Martinelli et al. (40). Their study investigated 13 SNPs (including rs174561 and rs174583) and showed that individuals with FADS haplotypes tended to have higher desaturase activity with higher AA/LA and EPA/ALA ratios. Another study by Bokor et al. (41) investigating genetic variants in FADS region on D6D and D5D activities estimated by serum fatty acid ratios in 1,144 European adolescents, suggested that minor SNPs in FADS gene clusters were responsible to higher D6D and lower D5D activities. Recently, Abdelmagid et al. (42) investigated the interaction of ethnicity, sex and multiple FADS polymorphisms in Caucasian and East Asian males and females using aggregate desaturase indices (DHA/EPA and DHA/ALA) and DHA concentrations. Results identified the ethnic- and sex-specific effects of FADS polymorphisms on desaturase activities DHA concentrations. The lack of agreement on enzyme activities between using calculated product-to-precursor ratios and gene expression in whole blood has been observed previously (43). The gene expression data for FADS1 and FADS2 from 66 subjects showed no dietary treatment effects at endpoints, while the calculated D5D and D6D enzyme activity data from 129 subjects clearly indicated treatments effects. In addition, we also observed a strong diet-gene interaction in terms of the enzyme activities observed, but not on gene expression data across all diets. Recently, Chisaguano et al. reported that gene expression of
FA
dS1 and FA

dS2 in peripheral blood were positively associated with PUFA levels in children
(44), although the relative coefficients were not strong. Several studies exploring the disparity
between gene expression levels and enzyme activities have suggested that different underlying
regulatory mechanisms may contribute to the expression of the genes (45; 46), and, therefore, it is
possible that the levels of gene expression are not congruent with enzyme activities. Due to the
different sub-group analyses, we were unable to observe direct correlations between FA
DS gene

e xpression levels and their calculated enzyme activities. The lack of impact of either genotypes
or dietary treatments on FA
DS1 and F
AD
S2 expression is suggestive that whole blood may not
in fact serve as a good biomarker of hepatic expression. Furthermore, we cannot rule out the fact
that the numbers of subjects analyzed for gene expression were lower compared with those for
fatty acid ratio calculations, which might indicate insufficient statistical power in the current
analysis. Overall, our data indicate that fatty acid ratios can be used to estimate D5D and D6D
activities and might better reflect the actual enzyme functions instead of measuring gene
expression.

One of our previous clinical studies (31) showed the impact of rs174561 (FA
DS1) on plasma EPA
levels in 36 subjects, but only included 4 homozygous minor allele carriers (CC). Importantly,
the present work was sufficiently powered to detect diet-gene interactions with a larger sample
size (n=129), and confirmed the previous findings in two SNPs, rs174561 and rs174583. Unlike
the previous study which used 13C labeled ALA as a tracer, one limitation of our study was that
the estimated FSR of DHA using deuterium enrichment was not an optimal surrogate marker of
DHA synthesis, which could not directly reflect the hepatic conversion of ALA to DHA. The
relative isotope ratio of 2H/1H in final product, DHA, represents the maximum assumed
conversion of ALA to DHA. In the COMIT study, all participants self-identified as having
European ancestry. Therefore, we were unable to assess the differences among diverse populations; however, our results clearly indicate that the impact of genetic variants can also be recognized within the same ethnicity, in this case, Caucasian.

In summary, our results demonstrate that the impact of genetic variants in the specific \textit{FADS1}-\textit{FADS2} gene clusters on enzyme activities and circulating fatty acid composition is very promising and consistent to GWAS studies. The diet-gene interactions appeared to largely influence AA, EPA and DPA levels, but not DHA content, because \textit{de novo} synthesis of DHA seems not to be affected by different genotypes in our cohort. Therefore, we suggest that dietary n-3 fatty acid intakes could module the association between genetic variations in \textit{FADS} and \textit{ELOVL} on circulating fatty acid profile via diet-gene interaction; increasing dietary DHA intake may be the most effective strategy for increasing circulating n-3 PUFA levels if this fatty acid is required to lower CVD risks for the general population irrespective of their genetic background.
5.6 Acknowledgements

We are indebted to Haifeng Yang for optimizing the performance of the GC-IRMS, TC/EA-IRMS, and GC-FID. We also acknowledge Dr. Dylan MacKay for his interpretation on the isotope enrichment data.
5.7 References


34. Hellstrand S, Sonestedt E, Ericson U et al. (2012) Intake levels of dietary long-chain PUFAs modify the association between genetic variation in FADS and LDL-C. *Journal of lipid research* 53, 1183-1189.


Chapter V has demonstrated that common genetic variants in *FADS* and *ELOVL* gene clusters can largely influence n-3 and n-6 fatty acid metabolism and dietary recommendations based on people’s genetic make-ups may become an effective strategy for management of CVD risk. A group of endogenous lipid molecules, fatty acid ethanolamides (FAEs) are known to play a role in molecular signaling associated with physiological actions and multiple diseases. The following chapter comprises a manuscript which presents the investigation on the response in circulating FAE levels after those different dietary oil treatments. In addition, genetic variants on the FAE-related genes are also examined in chapter VI, which provides information that identifying diet-gene interactions in sub-populations is able to help people to maximize the health benefits from dietary intakes.
Chapter VI

Interactions between dietary oil treatments and genetic variants modulate fatty acid ethanolamides in plasma and body weight composition

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This manuscript has been published in British Journal of Nutrition. (2016) Jan 25:1-12.
6.1 Abstract

Fatty acid ethanolamides (FAEs), a group of lipid mediators derived from long-chain fatty acids (FAs), mediate biological activities including activation of cannabinoid receptors, stimulation of fat oxidation, and regulation of satiety. However, how circulating FAE levels are influenced by FA intake in humans remains unclear. The study objective was to investigate the response of six major circulating FAEs to various dietary oil treatments in a 5-period, cross-over, randomized, double-blind clinical study in volunteers with abdominal obesity. Treatment oils (60 g/3000 Kcal per day) provided for 30 days were: conventional canola oil, high oleic canola oil, high oleic canola oil enriched with docosahexaenoic acid (DHA), flax/safflower oil blend, and corn/safflower oil blend. Two single nucleotide polymorphisms (SNPs) associated with FAE degradation and synthesis were studied. Post-treatment results showed overall that plasma FAE levels were modulated by dietary FAs and were positively correlated with corresponding plasma FA levels; minor allele (A) carriers of SNP rs324420 in gene FAAH produced higher circulating oleoylethanolamide (P=0.0209) and docosahexaenoylethanolamide levels (P=0.0002). In addition, elevated plasma docosahexaenoylethanolamide levels in response to DHA intake tended to associate with lower plasma oleoylethanolamide and an increased gynoid fat mass. In summary, data suggest that the metabolic and physiological responses to dietary FAs may be influenced via circulating FAEs. Genetic analysis of rs324420 might help identify a sub-population who appears to benefit from increased consumption of DHA and oleic acid.
6.2 Introduction

Fatty acid ethanolamides (FAEs), also referred to as N-acylethanolamines, are a group of endogenous ethanolamides of different fatty acids that were first identified in the late 1970s\(^1\). N-arachidonoylethanolamide (AEA, also called anadamide), the derivative of arachidonic acid (AA), is the first isolated and identified FAE compound\(^2\) and has been characterized in placenta, fetal membranes as well as human plasma, amniotic fluid\(^3\) and the nervous system\(^4\). AEA serves as an endogenous ligand of cannabinoid receptors (CB)\(^5\). Oleoylethanolamide (OEA), a derivative of oleic acid (OA), is thought to regulate satiety and body weight\(^6\) by activating peroxisome proliferator-activated receptor α (PPAR-α) which is responsible for energy expenditure and energy intake through lipolysis in adipocytes\(^7\). Consequently, administration of OEA may become part of the treatment of eating disorders and body weight maintenance\(^8\).

Other FAEs, including palmitoylethanolamide (PEA) and linoleoylethanolamide (LEA) over a range of concentrations are believed to have anti-inflammatory properties\(^9\;10\;11\). Little is known, however, about docosahexaenoylethanolamide (DHEA; derivative of docosahexaenoic acid (DHA)) or alpha-linolenylethanolamide (ALEA; derivative of alpha-linolenic acid (ALA)), although it was observed that a high dietary intake of DHA increased brain DHEA levels in piglets, suggesting a similar metabolism of AEA in the nervous system\(^12\). Recently, it has been reported that DHEA-dependent pathways may be associated with hippocampal neurodevelopment and synaptic activity\(^13\). However, the biological importance of omega-3 (n-3) derived FAEs remains to be resolved.

The endogenous FAEs are involved in numerous biological activities and primarily modulated by their biosynthesis and degradation\(^14\). Generally, the enzymes N-
acylphosphatidylethanolamine-hydrolyzing phospholipase D (NAPE-PLD) and fatty acid amide hydrolase (FAAH) appear to be responsible for the synthesis and hydrolysis of FAE, respectively, in turn influencing circulating FAE levels \textit{in vivo}\textsuperscript{(15; 16)}. With the growing knowledge of nutrigenomics, the common genetic variants of NAPE-PLD and FAAH genes may modulate circulating FAE levels in humans. Investigations of common single nucleotide polymorphisms (SNPs) in humans have demonstrated that major allele carriers of SNP rs324420 of FAAH are more likely associated with overweight/obesity and metabolic disorders\textsuperscript{(17; 18)}, although the functional significance of these differences remain unclear over time\textsuperscript{(19; 20)}. It was proposed that this polymorphism might impact specific neural mechanisms through CB signaling which, in turn, increase the risk of reward deficiency syndrome that causes obesity\textsuperscript{(21)}. Meanwhile, although little is known about NAPE-PLD, a study has shown that a common haplotype of NAPE-PLD gene may be protective against obesity\textsuperscript{(22)}. Most recently, Geurts et al.\textsuperscript{(23)} proposed an important mechanism of adipose tissue NAPE-PLD on whole-body metabolism using NAPE-PLD knock-out mice, indicating that NAPE-PLD may play an essential role in regulating energy homeostasis and be responsive to cold-induced browning. In other words, alterations of the enzyme activity of NAPE-PLD may be beneficial as an antiobesity treatment.

In addition, evidence has demonstrated that intake of high monounsaturated fatty acid (MUFA) diets elicits weight loss and/or body fat mass reduction compared to consuming high saturated fatty acid (SFA) diets in humans\textsuperscript{(24; 25; 26; 27)}. Also, oral administration of OEA appeared to beneficially affect health, resulting in weight loss in humans\textsuperscript{(16; 28)}. Therefore, a knowledge gap exists concerning whether endogenous OEA, which converted from dietary OA, plays a role on body fat distribution. In addition, it remains to be determined whether the consumption of fatty acids other than OA can contribute to changes in body fat composition via their corresponding
Therefore, the purpose of the present study was to answer the following questions; first, how major FAEs shift in response to dietary oil treatments in humans; second, whether common genetic variants in FAAH and NAPE-PLD genes affect plasma FAE levels; third, whether possible diet-gene interactions exist in population with risk of metabolic syndrome; and last, whether associations exist between changes in circulating FAE concentrations, especially OEA, and body fat composition after the dietary interventions. Therefore, we hypothesize that consumption of various dietary oil treatments with differing fatty acid composition can lead to corresponding shifts in plasma FAE levels, and the diet-gene interaction may play an important role on the levels of FAEs in response to dietary treatments, resulting in potential changes in body fat composition.
6.3 Materials and methods

6.3.1 Clinical design

This study was conducted as part of the Canola Oil Multicenter Intervention Trial (COMIT) study, a dietary intervention in adults with abdominal obesity, and at least one criterion for metabolic syndrome\(^{(29)}\). The COMIT study was conducted at the Richardson Centre for Functional Foods and Nutraceuticals (RCFFN) at the University of Manitoba, the Institute of Nutraceuticals and Functional Foods (INAF) at the Laval University, and the Department of Nutritional Sciences at the Pennsylvania State University (PSU) between September 2010 and March 2012, as previously described\(^{(30)}\). The study was registered at clinicaltrials.gov (NCT01351012). All participants provided written consent before the study started. The study was reviewed and approved by Institutional Review Board or Committee at all participating cites.

The COMIT study investigated effects of a daily intake of 60 g of dietary oils low in SFA and high in MUFA or polyunsaturated fatty acids (PUFA). A randomized cross-over study design was implemented in which participants were randomly assigned to five novel vegetable oil treatments. Each treatment phase was 30 days in duration and separated by a 4-week washout period. The diets were designed to maintain body weight, and energy needs for each participant were calculated using the Mifflin equation\(^{(31)}\). With the exception of the treatment oils, a 7-day rotating menu for the foods in the full-feeding diets was served identically across all treatments, with a fixed macronutrient composition (35% fat, 50% carbohydrate, 15% protein). The five oil treatments were: canola oil (Canola; 60% OA, 20% linoleic acid (LA), 10% ALA), high oleic acid canola oil (CanolaOleic; 72% OA, 15% LA, 2% ALA), DHA enriched canola-oil (CanolaDHA; 63% OA, 13% LA, 6% DHA (from an algal oil)), a blend of corn oil/safflower oil
(CornSaff; 18% OA, 69% LA), and a blend of flax oil/safflower oil (FlaxSaff; 18% OA, 38% LA, 32% ALA) (Table 6.1). Three canola-based diets were rich in MUFA while the two safflower oil blends were high in n-3 PUFA or n-6 PUFA. Compliance of the feeding was assessed by clinical coordinators as participants were required to drink at least one of their two treatment beverages under supervision daily and return meal bags with non-consumed food for off-site consumption in the following day. Compliance was further confirmed by measuring post-treatment plasma fatty acid profiles.
Table 6. Fatty acid composition of five treatment oils (60 gram based on 3000 kcal diet per day).

<table>
<thead>
<tr>
<th>Fat type (gram)</th>
<th>Canola</th>
<th>CanolaOleic</th>
<th>CanolaDHA</th>
<th>FlaxSaff</th>
<th>CornSaff</th>
</tr>
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<tbody>
<tr>
<td>SFA</td>
<td>4.33</td>
<td>3.91</td>
<td>5.19</td>
<td>4.87</td>
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<tr>
<td>c16:0</td>
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<td>2.20</td>
<td>3.15</td>
<td>2.94</td>
<td>3.52</td>
</tr>
<tr>
<td>c18:0</td>
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<td>1.10</td>
<td>1.02</td>
<td>1.90</td>
<td>1.14</td>
</tr>
<tr>
<td>MUFA</td>
<td>37.69</td>
<td>43.19</td>
<td>38.25</td>
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<tr>
<td>c18:1n-9</td>
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<td>42.88</td>
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<td>13.97</td>
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<td>11.72</td>
<td>8.84</td>
<td>7.56</td>
<td>22.48</td>
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<td>c18:3n-3</td>
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<td>1.18</td>
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<td>c22:6n-3</td>
<td>0</td>
<td>0</td>
<td>3.48</td>
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</tbody>
</table>

SFA: saturated fatty acids; MUFA: monounsaturated fatty acids; PUFA: polyunsaturated fatty acids; Canola: conventional canola oil; CanolaOleic: high oleic canola oil; CanolaDHA: DHA enriched canola oil; FlaxSaff: a blend of flax oil and safflower oil; CornSaff: a blend of corn oil and safflower oil.
6.3.2 Subjects

One hundred and seventy volunteers were recruited using media advertisements. The inclusion criteria required an increased waist circumference (>94 cm for men and >80 cm for women) or at least one of the following criteria: triglycerides (TG) >1.7 mmol/L; HDL cholesterol (HDL-C) <1 mmol/L (men) or <1.3 mmol/L (women); blood pressure ≥130 mmHg (systolic, SBP) and/or ≥85 mmHg (diastolic, DBP); and glucose ≥5.5 mmol/L. Exclusion criteria included history of thyroid disease, diabetes mellitus, kidney disease, and liver disease; current smokers, consuming more than two alcoholic drinks per week, and taking lipid-lowering medications or supplements of any kind in the past two weeks.

6.3.3 Blood sample collection

The intervention was designed to conduct the endpoint-to-endpoint comparisons in all blood sample measures as it presented the systematic comparison of the relative effects of all treatment oils after the 4-wk treatment periods. Therefore, on days 29 and 30 of each phase, twelve-hour fasting blood samples were collected in EDTA coated tubes and centrifuged at 3000 rpm for 20 min at 4°C. Separated plasma and leukocyte samples were transferred to labeled aliquot tubes and stored at -80°C until further analyzed. After the completion of the trial, samples for the present study were shipped to the RCFFN for analysis.
6.3.4 Measurement of plasma fatty acid profiles

Plasma fatty acid concentrations at endpoint of each phase were examined. Individual plasma fatty acid classes were extracted from plasma-EDTA aliquots using the Folch method\(^{32}\), followed by methylation using methanolic hydrogen chloride as previously described\(^{30}\). Fatty acid methyl esters were separated on a DB-225 column (30m x 250 um with 0.25 um film thickness; Agilent Technologies, Mississauga, ON, Canada) using an Agilent 6890N gas chromatograph equipped with a flame ionization detector (Agilent Technologies, Mississauga, ON, Canada). The oven was programmed from 70 to 240°C in five temperature steps (70°C for 1 min, rise of 30°C/min to 180°C, rise of 10°C/min to 200°C, rise of 2°C/min to 220°C and hold for 9.5 min, rise of 40°C/min to 240°C). Samples were run with a 20:1 split ratio, and helium was used as the carrier gas with a column flow rate of 1 ml/min. Temperatures for the injector and detector were set at 280 and 300°C, respectively. Individual fatty acids were identified by comparison with GLC 461 standard (Nu-Check Prep, Elysian, MN). The internal fatty acid standard heptadecanoic acid (C17:0) (Sigma-Aldrich, St. Louis, MO) was introduced into original plasma samples to quantify the amount of each fatty acid in plasma. The proportion of substrate fatty acids converted to fatty acid products was calculated based on the peak area relative to the total area and expressed as the percentage of total fatty acids.

6.3.5 Total plasma fatty acid ethanolamide analysis

Analysis of FAEs was conducted using a previously described method\(^{33}\). The six targeted FAE in plasma samples (PEA, OEA, LEA, AEA, ALEA, and DHEA) were quantified according to ratio of the known deuterated FAEs in the internal standard mixture, with an expected relative standard deviation value of less than 5%. Standards including PEA, OEA, LEA, AEA, ALEA,
DHEA, PEA-d4, OEA-d4, LEA-d4, AEA-d8, and DHEA-d4 were purchased from Cayman Chemicals Company (Ann Arbor, MI). ALEA-d4 was synthesized in our laboratory using alpha-linolenic acid chloride (Nuchek Prep, Elysian, MN) dissolved in methylene chloride with ethanolamide-d4 (Cambridge Isotope Laboratories, Tewksbury, MA)\(^2\). Relative ratios of FAEs and their deuterated isotopes in the internal standard mixture were generated by serial dilution when the internal standard mixture was adjusted to the optimal concentration.

Aliquots of plasma samples at endpoint of each phase were used for FAE extraction using a solid-phase extraction method\(^34\) with some modifications. The stability of FAEs in frozen plasma samples were previously validated in our laboratory\(^35; 36\). Briefly, plasma samples that were mixed with internal standards on ice were filtered under gentle vacuum at a low flow rate through an activated Oasis HLB cartridge (Waters Corporation, Milford, MA). Extracts were then washed and eluted with acetonitrile, followed by analysis on an ultra-performance liquid chromatography tandem Quattro micro API mass spectrometry (UPLC-MS/MS, Waters Corporation, Milford, MA), as published by Lin et al.\(^33\). A Kinetex XB-C18 column (100 x 2.1 mm, 1.7 µm, Phenomenex, Torrance, CA) was used for separation at a flow rate of 0.2 ml/min. Data were acquired and processed using MassLynx 4.1 (Waters Corporation, Milford, MA).

6.3.6 Measures of body fat mass

Regional changes in body composition of study participants were assessed at both the initiation and termination of each dietary phase by dual-energy X-ray absorptiometry (DEXA) scanning according to the manufacturer’s recommendations (GE Healthcare, Madison, WI; QDR-4500W; Hologic Corp, Waltham, MA). The equipment, room supplies, and room set-up were checked on a regular basis. All maintenance work including calibration was performed according to the
manufacturer’s recommendations. Licensed DEXA technicians and coordinators performed the scan examinations on participant at each phase. Participants were required to lie down with the same posture each time in the center of the measurement box from head to toe, face up and keep arms at their sides during the scans. The regions of interest, including android fat mass, gynoid fat mass, and the ratio of android to gynoid fat, were manually adjusted on the scan images. All data were then automatically calculated using the software Lunar Prodigy Advance enCORE and APEX System.

6.3.7 Single nucleotide polymorphism analysis

To investigate the relationship between genetic variants of FAAH and NAPE-PLD genes and plasma FA and FAE levels, all subjects were genotyped for 2 selected SNPs (rs324420 for FAAH and rs12540583 for NAPE-PLD). According to the GWAS and NCBI dbSNP database, the selected SNPs both belong to missense mutations with a higher minor allele frequency and might lead to significant functional changes in the corresponding enzymes. Genomic DNA was extracted from leukocyte samples using a commercial Qiagen DNeasy Blood and Tissue Kit according to the manufacturer’s instructions (Qiagen Sciences Inc, Toronto, ON). The quality and quantity of individual genomic DNA were assessed by Thermo Scientific NanoDrop 2000 micro-volume spectrophotometer (Thermo Fisher Scientific, Waltham, MA). Amplification and detection of DNA were conducted with the 7500 Fast Real-Time PCR System (Applied Biosystems, Life Technologies, Burlington, ON) using optical-grade 96-well plates. Each well contained 5-ul PCR reaction, containing customized Taqman SNP Genotyping Assays, Taqman SNP genotyping Master Mix (Life Technologies Inc, Burlington, Ontario), and pre-diluted DNA
samples. Data were acquired by software StepOne 2.1 (Applied Biosystems, Life Technologies, Burlington, ON).

6.3.8 Statistical analyses

Only participants who completed all five dietary phases were included in the statistical analyses. A per-protocol approach was used to avoid the need for multiple imputations for missing data during the analyses. The power calculation was performed based on previous results on post-treatment plasma OEA levels in the clinical intervention by Jones et al. 2014 (36), indicating a power of 100% to detect significant differences on OEA levels between three MUFA rich diets and two PUFA rich diets. Statistical analyses were performed using SAS 9.2 (SAS, Cary, NC). Statistical significance was determined using the adjusted Tukey test for multiple comparisons with P < 0.05. The results are expressed as least square means ± SEMs unless noted elsewhere.

The effects of dietary treatment on plasma fatty acid profiles, plasma FAE levels and body composition were analyzed by using a mixed model with repeated measures analysis of time. Treatment, age and sex were considered as fixed factors. Sequence of treatments and center were used as random effects in the model. Tukey-adjusted P values were used to examine difference between treatments. Pearson correlation analyses were conducted to test associations between plasma FA and FAE, and between plasma FAE and fat mass change.

Effects of different genotypes on FAE levels were analyzed in mixed models. Treatment, sex, age, genotype, genotype-by-sex interaction, and treatment-by- genotype interaction were entered as fixed effects, sequence of treatments were random effects, and clinical sites were selected as random effects. Repeated measures by five dietary phases on subjects were used to investigate
the effects of treatments, genotypes, and their interactions. Two individual SNPs were analyzed separately.
6.4 Results

6.4.1 Subject characteristics

One hundred and thirty participants (60 male; 70 female; 76.5% of the total randomized subjects) completed the 5-phase intervention study. The men and women were 46.5 ± 14.2 and 47.6 ± 14.5 years and had a body mass index (BMI) of 29.8 ± 4.4 and 29.0 ± 4.2 kg/m² (mean ± SD), respectively. Baseline characteristics of the study population have been previously published and are shown in Table 6.2.

6.4.2 Plasma fatty acid profiles

The effects of the diet interventions on plasma fatty acid profiles are summarized in Table 6.3. Overall, all changes in fatty acid composition agreed with our expectation based on the fatty acid profile of each intervention diet. The Canola and CanolaOleic diets produced the highest level of total MUFA (P<0.05) while CornSaff and FlaxSaff rich in n-6 and/or n-3 PUFA contents resulted in higher (P<0.05) levels of PUFA than in the other three diets. The CornSaff group showed the lowest (P<0.05) ALA, eicosapentaenoic acid (EPA) and docosapentaenoic acid (DPA) levels compared to all other four treatments. Post-treatment DHA concentrations were higher (P<0.0001) after the CanolaDHA treatment than after the other four treatments, while no differences were observed across the other four treatments, indicating good dietary compliance across all centers.
Table 6.2 Baseline characteristics of participants at baseline of the dietary intervention.

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Male (n=60)</th>
<th>Female (n=70)</th>
<th>Total (n=130)</th>
<th>P-value&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>BMI (kg/m&lt;sup&gt;2&lt;/sup&gt;)</td>
<td>30.7±0.6</td>
<td>29.0±0.5</td>
<td>29.8±4.3</td>
<td>0.0254</td>
</tr>
<tr>
<td>Age (years)</td>
<td>45.2±1.8</td>
<td>47.6±1.7</td>
<td>46.5±14.2</td>
<td>0.3308</td>
</tr>
<tr>
<td>Total cholesterol (mmol/L)</td>
<td>5.2±0.1</td>
<td>5.4±0.2</td>
<td>5.3±1.1</td>
<td>0.3560</td>
</tr>
<tr>
<td>HDL-cholesterol (mmol/L)</td>
<td>1.2±0.0</td>
<td>1.4±0.0</td>
<td>1.3±0.3</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>LDL-cholesterol (mmol/L)</td>
<td>3.1±0.1</td>
<td>3.3±0.1</td>
<td>3.2±0.9</td>
<td>0.4276</td>
</tr>
<tr>
<td>Triglycerides (mmol/L)</td>
<td>2.0±0.2</td>
<td>1.6±0.1</td>
<td>1.8±1.0</td>
<td>0.0119</td>
</tr>
<tr>
<td>Glucose (mmol/L)</td>
<td>5.3±0.1</td>
<td>5.4±0.2</td>
<td>5.4±1.1</td>
<td>0.8448</td>
</tr>
<tr>
<td>Body weight (kg)</td>
<td>95.1±1.8</td>
<td>76.5±1.4</td>
<td>85.1±12.8</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Waist circumference (cm)</td>
<td>106.6±1.3</td>
<td>96.6±1.4</td>
<td>101.2±10.9</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Systolic BP (mmHg)</td>
<td>128.9±2.2</td>
<td>120.2±2.0</td>
<td>124.3±16.7</td>
<td>0.0041</td>
</tr>
<tr>
<td>Diastolic BP (mmHg)</td>
<td>81.0±1.7</td>
<td>78.6±1.3</td>
<td>79.7±11.8</td>
<td>0.2659</td>
</tr>
</tbody>
</table>

<sup>a</sup>All values are means ± SEM, n=130. ANOVA was used to analyze between-sex differences in continuous variables. P<0.05 was considered significant.
Table 6.3 Selected plasma fatty acid profiles of participants at endpoints of each dietary phase (g/100g).

<table>
<thead>
<tr>
<th>Fatty acids</th>
<th>Treatments</th>
<th>Canola</th>
<th>CanolaOleic</th>
<th>CanolaDHA</th>
<th>FlaxSaff</th>
<th>CornSaff</th>
</tr>
</thead>
<tbody>
<tr>
<td>c16:0</td>
<td></td>
<td>27.04±0.20&lt;sup&gt;b&lt;/sup&gt;</td>
<td>27.40±0.20&lt;sup&gt;b&lt;/sup&gt;</td>
<td>28.10±0.20&lt;sup&gt;a&lt;/sup&gt;</td>
<td>27.41±0.20&lt;sup&gt;b&lt;/sup&gt;</td>
<td>27.35±0.20&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>c18:0</td>
<td></td>
<td>11.83±0.10&lt;sup&gt;c&lt;/sup&gt;</td>
<td>11.79±0.10&lt;sup&gt;c&lt;/sup&gt;</td>
<td>12.28±0.10&lt;sup&gt;b&lt;/sup&gt;</td>
<td>12.51±0.10&lt;sup&gt;a&lt;/sup&gt;</td>
<td>12.34±0.10&lt;sup&gt;ab&lt;/sup&gt;</td>
</tr>
<tr>
<td>c18:1n9</td>
<td></td>
<td>14.90±0.19&lt;sup&gt;b&lt;/sup&gt;</td>
<td>15.52±0.19&lt;sup&gt;a&lt;/sup&gt;</td>
<td>13.36±0.19&lt;sup&gt;c&lt;/sup&gt;</td>
<td>12.10±0.18&lt;sup&gt;d&lt;/sup&gt;</td>
<td>11.62±0.18&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
<tr>
<td>c18:2n6</td>
<td></td>
<td>22.00±0.23&lt;sup&gt;c&lt;/sup&gt;</td>
<td>21.52±0.23&lt;sup&gt;c&lt;/sup&gt;</td>
<td>18.68±0.23&lt;sup&gt;d&lt;/sup&gt;</td>
<td>25.13±0.23&lt;sup&gt;b&lt;/sup&gt;</td>
<td>25.93±0.23&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>c18:3n3</td>
<td></td>
<td>0.79±0.03&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.63±0.03&lt;sup&gt;c&lt;/sup&gt;</td>
<td>0.57±0.03&lt;sup&gt;cd&lt;/sup&gt;</td>
<td>1.61±0.03&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.49±0.03&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
<tr>
<td>c20:4n6</td>
<td></td>
<td>9.28±0.15&lt;sup&gt;b&lt;/sup&gt;</td>
<td>9.67±0.15&lt;sup&gt;a&lt;/sup&gt;</td>
<td>9.70±0.15&lt;sup&gt;a&lt;/sup&gt;</td>
<td>8.27±0.15&lt;sup&gt;c&lt;/sup&gt;</td>
<td>9.59±0.15&lt;sup&gt;ab&lt;/sup&gt;</td>
</tr>
<tr>
<td>c20:5n3</td>
<td></td>
<td>1.09±0.04&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.86±0.04&lt;sup&gt;c&lt;/sup&gt;</td>
<td>1.53±0.04&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1.45±0.04&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.49±0.04&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
<tr>
<td>c22:5n3</td>
<td></td>
<td>0.81±0.03&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.72±0.03&lt;sup&gt;c&lt;/sup&gt;</td>
<td>0.34±0.03&lt;sup&gt;c&lt;/sup&gt;</td>
<td>0.97±0.03&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.62±0.03&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
<tr>
<td>c22:6n3</td>
<td></td>
<td>2.84±0.09&lt;sup&gt;b&lt;/sup&gt;</td>
<td>2.79±0.09&lt;sup&gt;b&lt;/sup&gt;</td>
<td>7.21±0.10&lt;sup&gt;a&lt;/sup&gt;</td>
<td>2.59±0.09&lt;sup&gt;b&lt;/sup&gt;</td>
<td>2.66±0.09&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Total SFA</td>
<td></td>
<td>41.74±0.19&lt;sup&gt;c&lt;/sup&gt;</td>
<td>41.92±0.19&lt;sup&gt;c&lt;/sup&gt;</td>
<td>43.28±0.19&lt;sup&gt;a&lt;/sup&gt;</td>
<td>42.71±0.19&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>42.51±0.19&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Total MUFA</td>
<td></td>
<td>18.05±0.22&lt;sup&gt;a&lt;/sup&gt;</td>
<td>18.50±0.22&lt;sup&gt;a&lt;/sup&gt;</td>
<td>16.20±0.22&lt;sup&gt;b&lt;/sup&gt;</td>
<td>14.78±0.22&lt;sup&gt;c&lt;/sup&gt;</td>
<td>14.49±0.22&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>Total PUFA</td>
<td></td>
<td>40.21±0.22&lt;sup&gt;b&lt;/sup&gt;</td>
<td>39.54±0.22&lt;sup&gt;c&lt;/sup&gt;</td>
<td>40.47±0.22&lt;sup&gt;b&lt;/sup&gt;</td>
<td>42.57±0.22&lt;sup&gt;a&lt;/sup&gt;</td>
<td>43.04±0.22&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Total n-6 PUFA</td>
<td></td>
<td>34.67±0.23&lt;sup&gt;c&lt;/sup&gt;</td>
<td>34.46±0.23&lt;sup&gt;c&lt;/sup&gt;</td>
<td>30.92±0.23&lt;sup&gt;d&lt;/sup&gt;</td>
<td>35.92±0.23&lt;sup&gt;b&lt;/sup&gt;</td>
<td>38.79±0.23&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Total n-3 PUFA</td>
<td></td>
<td>5.53±0.10&lt;sup&gt;c&lt;/sup&gt;</td>
<td>5.03±0.10&lt;sup&gt;d&lt;/sup&gt;</td>
<td>9.62±0.11&lt;sup&gt;a&lt;/sup&gt;</td>
<td>6.64±0.10&lt;sup&gt;b&lt;/sup&gt;</td>
<td>4.24±0.10&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

All values are least squares means ± SEM, n=130. Mix model with repeated measures was used to analyze between-treatment differences in continuous variables. P<0.05 was considered significant. SFA: saturated fatty acids; MUFA: monounsaturated fatty acids; PUFA: polyunsaturated fatty acids; Canola: conventional canola oil; CanolaOleic: high oleic canola oil; CanolaDHA: DHA enriched canola oil;
6.4.3 Total plasma fatty acid ethanolamides

The endpoint concentrations of OEA, AEA, PEA, LEA, ALEA, and DHEA (n=121) are successfully measured and shown in Figure 6.1. Samples from 9 participants were excluded from this analysis due to failures in the extraction of samples being run through the cartridges or in the FAE measurement using UPLC-MS/MS. Overall, plasma PEA and OEA were the two major FAE. No differences were observed in plasma PEA levels across the five dietary treatments. Three MUFA-rich treatments, Canola, CanolaDHA and CanolaOleic resulted in higher (P<0.05) OEA levels compared to the two PUFA-rich diets, CornSaff and FlaxSaff. However, despite the similar OA content of the three canola based diets, the CanolaDHA treatment appeared to lower the OEA levels compared to the CanolaOleic diet (P<0.05). The plasma AEA level after the CanolaOleic diet was higher (P<0.05) compared to the CanolaDHA, CornSaff, and FlaxSaff diets, but no difference was observed between the CanolaOleic and Canola treatments. The two PUFA-rich diets had higher (P<0.05) LEA levels compared to the CanolaDHA and CanolaOleic treatments. The FlaxSaff diet had the highest (P<0.0001) ALEA levels and the DHEA level was substantially higher (P<0.0001) in response to the CanolaDHA diet compared to the other four diets.
Figure 6.1 Total plasma fatty acid ethanolamide levels at the endpoint of five dietary treatments (n=121).

Values are presented in ng/ml as least squares means ± SEM. Different lower letters within each graph indicate significantly differences between treatments, P<0.05. (A) PEA:
palmitoylethanolamide; (B) OEA: oleoylethanolamide; (C) LEA: linoleoylethanolamide; (D) AEA: arachidonoyl etanolamide; (E) ALEA: alpha-linolenoyl ethanolamide; (F) DHEA: docosahexaenoyl ethanolamide. Canola: conventional canola oil; Canola Oleic: high oleic canola oil; Canola DHA: DHA enriched canola oil; Flax Saff: a blend of flax oil and safflower oil; Corn Saff: a blend of corn oil and safflower oil.
6.4.4 Correlation between plasma fatty acid ethanolamides and plasma fatty acids

Pearson correlation coefficients between plasma FAE and their corresponding FA are presented in Table 6.4. Overall, positive correlations existed between the five major FAEs and their individual precursor FAs (P<0.0001), while DHEA-DHA (r=0.52, P<0.0001) and ALEA-ALA (r=0.51, P<0.0001) showed the strongest correlations. Only AEA failed to produce a significant association with AA (r=0.04, P=0.3547).

Table 6.4 Pearson correlation between fatty acid ethanolamides and their corresponding precursor fatty acids in plasma (n=121).

<table>
<thead>
<tr>
<th>Fatty acids</th>
<th>Fatty acid ethanolamides</th>
<th>Correlation coefficients</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>PA</td>
<td>PEA</td>
<td>0.34504</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>OA</td>
<td>OEA</td>
<td>0.36434</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>LA</td>
<td>LEA</td>
<td>0.18462</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>AA</td>
<td>AEA</td>
<td>0.03785</td>
<td>0.3547</td>
</tr>
<tr>
<td>ALA</td>
<td>ALEA</td>
<td>0.51068</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>DHA</td>
<td>DHEA</td>
<td>0.51879</td>
<td>&lt;0.0001</td>
</tr>
</tbody>
</table>

PA: palmitic acid; PEA: palmitoylethanolamide; OA: oleic acid; OEA: oleoylethanolamide; LA: linoleic acid; LEA: linoleoylethanolamide; AA: arachidonic acid; AEA: arachidonoylethanolamide; ALA: alpha-linolenic acid; ALEA: alpha-linolenoylethanolamide; DHA: docosahexaenoic acid; DHEA: docosahexaenoylethanolamide.
6.4.5 Body composition and correlation with plasma fatty acid ethanolamides

A subgroup of 27 (male n=7; female n=20) volunteers at RCFFN completed all the baseline and endpoint DEXA measures at each phase, and, therefore, their data were used for the exploratory analysis on the correlations between body composition and plasma FAE levels. The baseline characteristics of the subjects are presented in Table S6.1. Total fat mass, android fat mass, gynoid fat mass and the ratio of android to gynoid fat mass at baseline, endpoint and changes in each dietary phase are reported in Table 6.5. No significant differences were observed except for the endpoint gynoid fat mass (P=0.0503). It was mostly attributed to higher (P<0.05) gynoid fat mass for CanolaDHA compared to CanolaOleic. Changes of gynoid fat mass from baseline were tended to differ (P=0.0803), due to an increase after CanolaDHA and a decrease after CanolaOleic.

Correlations between endpoint FAE levels and android fat mass change (endpoint vs. baseline) were tested in the subset (n=27) using Pearson correlations (Table 6.6). Although we noticed that this subgroup had an unbalanced sex ratio, no differences on android fat mass change were observed. Overall, negative correlations between plasma OEA (r=-0.24, P=0.0049) (Figure 6.2), AEA (r=-0.24, P=0.0059) and LEA (r=-0.20, P=0.0225) levels and android fat mass changes across five diets were observed. Also, similar negative correlations were observed between the OEA/DHEA ratio and android fat mass change (r=-0.20, P=0.0187). No correlations were shown between ALEA, DHEA or PEA and android fat mass change.
Table S6.1 Subgroup baseline characteristics of participants at baseline of the dietary intervention for android fat mass analyses.

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Male (n=7)</th>
<th>Female (n=20)</th>
<th>Total (n=27)</th>
<th>P-value$^a$</th>
</tr>
</thead>
<tbody>
<tr>
<td>BMI (kg/m$^2$)</td>
<td>32.0±5.8</td>
<td>29.0±4.0</td>
<td>29.8±4.6</td>
<td>0.1346</td>
</tr>
<tr>
<td>Age (years)</td>
<td>43.9±14.8</td>
<td>57.0±9.3</td>
<td>53.6±12.2</td>
<td>0.0112</td>
</tr>
<tr>
<td>Total cholesterol (mmol/L)</td>
<td>5.8±0.7</td>
<td>6.1±1.1</td>
<td>6.0±1.0</td>
<td>0.6562</td>
</tr>
<tr>
<td>HDL-cholesterol (mmol/L)</td>
<td>1.1±0.2</td>
<td>1.4±0.3</td>
<td>1.3±0.3</td>
<td>0.0230</td>
</tr>
<tr>
<td>LDL-cholesterol (mmol/L)</td>
<td>3.4±0.5</td>
<td>3.8±1.1</td>
<td>3.7±0.9</td>
<td>0.3279</td>
</tr>
<tr>
<td>Triglycerides (mmol/L)</td>
<td>3.0±0.9</td>
<td>2.0±0.8</td>
<td>2.2±0.9</td>
<td>0.0056</td>
</tr>
<tr>
<td>Glucose (mmol/l)</td>
<td>4.9±1.2</td>
<td>5.4±2.3</td>
<td>5.3±2.1</td>
<td>0.5926</td>
</tr>
<tr>
<td>Body weight (kg)</td>
<td>98.6±17.5</td>
<td>78.1±11.9</td>
<td>83.4±16.1</td>
<td>0.0019</td>
</tr>
<tr>
<td>Waist (cm) Circumference</td>
<td>109.4±11.9</td>
<td>93.7±11.6</td>
<td>97.8±13.4</td>
<td>0.0052</td>
</tr>
<tr>
<td>SBP (mmHg)</td>
<td>127.7±21.2</td>
<td>124.5±17.1</td>
<td>125.3±17.9</td>
<td>0.6909</td>
</tr>
<tr>
<td>DBP (mmHg)</td>
<td>79.6±9.7</td>
<td>79.4±11.8</td>
<td>79.4±11.1</td>
<td>0.9680</td>
</tr>
</tbody>
</table>

$^a$All values are means ± SEM, n=27. ANOVA was used to analyze between-sex differences in continuous variables. P<0.05 was considered significant.
Table 6.5 Body fat mass composition at the beginning, the end, and changes of each dietary phase.

<table>
<thead>
<tr>
<th>Body fat profile</th>
<th>Treatments</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Canola</td>
<td>CanolaOleic</td>
</tr>
<tr>
<td>Baseline</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total fat mass (kg)</td>
<td>33.72  ±2.10</td>
<td>33.35  ±2.10</td>
</tr>
<tr>
<td>Android fat mass (kg)</td>
<td>3.42   ±0.22</td>
<td>3.37   ±0.22</td>
</tr>
<tr>
<td>Gynoid fat mass (kg)</td>
<td>5.61   ±0.43</td>
<td>5.58   ±0.43</td>
</tr>
<tr>
<td>Android/gynoid ratio</td>
<td>0.63   ±0.03</td>
<td>0.62   ±0.03</td>
</tr>
<tr>
<td>Endpoint</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total fat mass (kg)</td>
<td>33.11  ±2.08</td>
<td>32.81  ±2.08</td>
</tr>
<tr>
<td>Android fat mass (kg)</td>
<td>3.39   ±0.22</td>
<td>3.33   ±0.22</td>
</tr>
<tr>
<td>Gynoid fat mass (kg)</td>
<td>5.47   ±0.41</td>
<td>5.43   ±0.41</td>
</tr>
<tr>
<td>Android/gynoid ratio</td>
<td>0.63   ±0.03</td>
<td>0.63   ±0.03</td>
</tr>
<tr>
<td>Changes</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total fat mass (kg)</td>
<td>-0.42  ±0.23</td>
<td>-0.15  ±0.23</td>
</tr>
<tr>
<td>Android fat mass (kg)</td>
<td>-0.02  ±0.03</td>
<td>-0.04  ±0.03</td>
</tr>
</tbody>
</table>
Gynoid fat mass (kg)  & -0.11 ± 0.06 & -0.11 ± 0.06 & 0.06 ± 0.06 & 0.01 ± 0.06 & -0.06 ± 0.06 & 0.0803 \\
Android/gynoid ratio & 0.00 ± 0.01 & 0.01 ± 0.01 & -0.01 ± 0.01 & -0.01 ± 0.01 & 0.00 ± 0.01 & 0.1464 \\

*All values are least squares means ± SEM, n=27 (Male=7; Female=20) from RCFFN. Mixed-model ANOVA corrected by sex and post hoc Tucky's test were used to analyze treatment effects on different fat mass profiles. P<0.05 was considered significant. Canola: conventional canola oil; CanolaOleic: high oleic canola oil; CanolaDHA: DHA enriched canola oil; FlaxSaff: a blend of flax oil and safflower oil; CornSaff: a blend of corn oil and safflower oil.*
Table 6.6 Pearson correlation between endpoint fatty acid ethanolamide levels and android fat mass changes of each dietary treatment (n=27).

<table>
<thead>
<tr>
<th>Fatty acid ethanolamides</th>
<th>Correlation coefficients</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>PEA</td>
<td>-0.14</td>
<td>0.1037</td>
</tr>
<tr>
<td>OEA</td>
<td>-0.24</td>
<td>0.0049</td>
</tr>
<tr>
<td>LEA</td>
<td>-0.20</td>
<td>0.0225</td>
</tr>
<tr>
<td>AEA</td>
<td>-0.24</td>
<td>0.0059</td>
</tr>
<tr>
<td>ALEA</td>
<td>-0.08</td>
<td>0.3564</td>
</tr>
<tr>
<td>DHEA</td>
<td>0.08</td>
<td>0.3564</td>
</tr>
<tr>
<td>OEA/DHEA ratio</td>
<td>-0.20</td>
<td>0.0187</td>
</tr>
</tbody>
</table>

PEA: palmitoylethanolamide; OEA: oleoylethanolamide; LEA: linoleoylethanolamide; AEA: arachidonoylethanolamide; ALEA: alpha-linolenoylthanolamide; DHEA: docosahexaenoylethanolamide.
6.4.6 Single nucleotide polymorphism characteristics and association with fatty acid ethanolamides

Genomic DNA was genotyped for 129 of 130 subjects (one blood sample failed to provide enough DNA yield). Analyses of the two SNPs rs12540583 in NAPE-PLD and rs324420 in FAAH are summarized in Table 6.7.

Since numbers of rare homozygotes in both polymorphisms were relatively low, the genotypes were grouped into two categories, common homozygous and minor allele carriers. Results of the NAPE-PLD polymorphism rs12540583 (Table 6.8a) showed no differences in FAE levels across all treatments, except the C-allele carriers had a higher (P=0.0010) DHEA level after the CanolaDHEA diet, while a strong treatment-by-genotype interaction for DHEA was also observed (P=0.0004). In general, analyses of polymorphism rs324420 in FAAH (Table 6.8b) indicated that the A-allele carriers had higher (P=0.0002) DHEA levels than the CC genotype carrier. In particular, after the Canola and CanolaDHA treatments, DHEA levels of A-allele carriers were higher (P<0.05) than the homozygous C genotype group. In addition, a strong treatment x genotype interaction for DHEA was also observed (P<0.0001). DHEA levels after the CanolaDHA diet were observed to be higher in both genotypes compared to other treatments, and were significantly different between two genotypes. No differences were observed in the corresponding plasma DHA levels between the two genotypes.
Table 6.7 Characteristics of the selected SNPs associated with metabolism of fatty acid ethanolamides (n=129).

<table>
<thead>
<tr>
<th>SNP</th>
<th>Gene</th>
<th>Annotation</th>
<th>Alleles (major/minor)</th>
<th>Genotype</th>
<th>MAF (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>rs12540583</td>
<td>NAPE-PLD</td>
<td>missense</td>
<td>A/C</td>
<td>MM 103</td>
<td>Mm 25</td>
</tr>
<tr>
<td>rs324420</td>
<td>FAAH</td>
<td>missense</td>
<td>C/A</td>
<td>MM 85</td>
<td>Mm 39</td>
</tr>
</tbody>
</table>

SNP: single nucleotide polymorphism; NAPE-PLD: N-acyl phosphatidylethanolamine phospholipase D; FAAH: fatty acid amide hydrolase; MM: homozygous for the major allele; Mm: heterozygous; mm: homozygous for the minor allele; MAF: minor allele frequency.
Figure 6.2 The correlation between OEA and change of android fat mass (endpoint vs. baseline) across all five diets (n=27).

The weak negative but significant correlation was observed ($r=-0.24$, $P=0.0049$). OEA: oleoylethanolamide.
Table 6.8a Selected plasma fatty acid ethanolamides at endpoint of five dietary treatments, by rs12540583 in NAPE-PLD (n=120).

<table>
<thead>
<tr>
<th>FAE</th>
<th>Genotype</th>
<th>Treatments</th>
<th>Overall</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Canola</td>
<td>CanolaOleic</td>
</tr>
<tr>
<td>PEA (ng/ml)</td>
<td>AA</td>
<td>3.2538</td>
<td>3.3977</td>
</tr>
<tr>
<td></td>
<td>AC/CC</td>
<td>3.0356</td>
<td>2.9576</td>
</tr>
<tr>
<td></td>
<td>P value</td>
<td>0.9867</td>
<td>0.4697</td>
</tr>
<tr>
<td></td>
<td>P-interaction</td>
<td></td>
<td></td>
</tr>
<tr>
<td>OEA (ng/ml)</td>
<td>AA</td>
<td>2.0240</td>
<td>2.2076</td>
</tr>
<tr>
<td></td>
<td>AC/CC</td>
<td>2.1030</td>
<td>2.0432</td>
</tr>
<tr>
<td></td>
<td>P value</td>
<td>0.9999</td>
<td>0.9764</td>
</tr>
<tr>
<td></td>
<td>P-interaction</td>
<td></td>
<td></td>
</tr>
<tr>
<td>LEA (ng/ml)</td>
<td>AA</td>
<td>0.7924</td>
<td>0.8027</td>
</tr>
<tr>
<td></td>
<td>AC/CC</td>
<td>0.9368</td>
<td>0.7444</td>
</tr>
<tr>
<td></td>
<td>P value</td>
<td>0.6667</td>
<td>0.9990</td>
</tr>
<tr>
<td>----------------</td>
<td>--------------</td>
<td>--------</td>
<td>--------</td>
</tr>
<tr>
<td>P-interaction</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AEA (ng/ml)</td>
<td>AA</td>
<td>0.4790</td>
<td>0.5288</td>
</tr>
<tr>
<td></td>
<td>AC/CC</td>
<td>0.4450</td>
<td>0.4379</td>
</tr>
<tr>
<td></td>
<td>P value</td>
<td>0.9960</td>
<td>0.2991</td>
</tr>
<tr>
<td></td>
<td>P-interaction</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ALEA (ng/ml)</td>
<td>AA</td>
<td>0.05240</td>
<td>0.04416</td>
</tr>
<tr>
<td></td>
<td>AC/CC</td>
<td>0.05189</td>
<td>0.03951</td>
</tr>
<tr>
<td></td>
<td>P value</td>
<td>1.0000</td>
<td>0.9998</td>
</tr>
<tr>
<td></td>
<td>P-interaction</td>
<td></td>
<td></td>
</tr>
<tr>
<td>DHEA (ng/ml)</td>
<td>AA</td>
<td>1.0434</td>
<td>1.0953</td>
</tr>
<tr>
<td></td>
<td>AC/CC</td>
<td>1.1485</td>
<td>1.0234</td>
</tr>
<tr>
<td></td>
<td>P value</td>
<td>0.9903</td>
<td>0.9995</td>
</tr>
</tbody>
</table>
Values are presented as least squares means. Different letters in the same row are significantly different between treatment groups, P<0.05. P value shows the differences between two genotypes within the same treatment. P-interaction shows the gene-treatment interaction in the entire population. NAPE-PLD: N-acyl phosphatidylethanolamine phospholipase D; Canola: conventional canola oil; CanolaOleic: high oleic canola oil; CanolaDHA: DHA enriched canola oil; FlaxSaff: a blend of flax oil and safflower oil; CornSaff: a blend of corn oil and safflower oil.
Table 6.8b Selected plasma fatty acid ethanolamides at endpoint of five dietary treatments, by rs324420 in FAAH (n=120).

<table>
<thead>
<tr>
<th>FAE</th>
<th>Genotype</th>
<th>Treatments (ng/ml)</th>
<th>Overall</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Canola</td>
<td>CanolaOleic</td>
</tr>
<tr>
<td>PEA (ng/ml)</td>
<td>CC</td>
<td>3.0678</td>
<td>3.1518</td>
</tr>
<tr>
<td></td>
<td>P value</td>
<td>0.2108</td>
<td>0.1865</td>
</tr>
<tr>
<td></td>
<td>P-interaction</td>
<td>0.8483</td>
<td></td>
</tr>
<tr>
<td>OEA (ng/ml)</td>
<td>CC</td>
<td>1.9085</td>
<td>2.0534</td>
</tr>
<tr>
<td></td>
<td>CA/AA</td>
<td>2.2849</td>
<td>2.3984</td>
</tr>
<tr>
<td></td>
<td>P value</td>
<td>0.0826</td>
<td>0.1527</td>
</tr>
<tr>
<td></td>
<td>P-interaction</td>
<td>0.0607</td>
<td></td>
</tr>
<tr>
<td>LEA (ng/ml)</td>
<td>CC</td>
<td>0.7448</td>
<td>0.7416</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>--------</td>
<td>--------</td>
<td>--------</td>
<td>--------</td>
</tr>
<tr>
<td></td>
<td>CC</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CA/AA</td>
<td>0.9668</td>
<td>0.8751</td>
<td>0.7295</td>
</tr>
<tr>
<td>P value</td>
<td>0.0346</td>
<td>0.6084</td>
<td>0.9998</td>
</tr>
<tr>
<td>P-interaction</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AEA (ng/ml)</td>
<td>CC</td>
<td>0.4447</td>
<td>0.4947</td>
</tr>
<tr>
<td>CA/AA</td>
<td>0.5156</td>
<td>0.5273</td>
<td>0.4579</td>
</tr>
<tr>
<td>P value</td>
<td>0.5242</td>
<td>0.9937</td>
<td>0.9978</td>
</tr>
<tr>
<td>P-interaction</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ALEA (ng/ml)</td>
<td>CC</td>
<td>0.04573</td>
<td>0.04013</td>
</tr>
<tr>
<td>CA/AA</td>
<td>0.06474</td>
<td>0.04863</td>
<td>0.04256</td>
</tr>
<tr>
<td>P value</td>
<td>0.0832</td>
<td>0.9430</td>
<td>1.0000</td>
</tr>
<tr>
<td>P-interaction</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>DHEA (ng/ml)</td>
<td>CC</td>
<td>0.9742</td>
<td>1.0427</td>
</tr>
<tr>
<td>CA/AA</td>
<td>1.2361</td>
<td>1.1554</td>
<td>2.2018</td>
</tr>
<tr>
<td>P value</td>
<td>0.0848</td>
<td>0.9561</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>-----------</td>
<td>--------</td>
<td>--------</td>
<td>---------</td>
</tr>
<tr>
<td>P-interaction</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Values are presented as least squares means. Different letters in the same row are significantly different between treatment groups, P<0.05. P value shows the differences between two genotypes within the same treatment. P-interaction shows the gene-treatment interaction in the entire population. FAAH: fatty acid amide hydrolase; Canola: conventional canola oil; CanolaOleic: high oleic canola oil; CanolaDHA: DHA enriched canola oil; FlaxSaff: a blend of flax oil and safflower oil; CornSaff: a blend of corn oil and safflower oil.
6.5 Discussion

The present study demonstrated that circulating FAE levels reflected their precursor fatty acids from the dietary intake in this clinical feeding study. Polymorphisms of rs324420 (FAAH) may play a role in modulating the circulating levels of OEA and DHEA, which might further influence the body fat mass distribution. Given the growing evidence for the importance of FAEs in governing energy metabolism, the implications of these trial results could be substantial.

Accumulating evidence suggests that circulating FAE levels are influenced by genetic variants, which account for the FAE synthesis and degradation \textit{in vivo} \cite{37}. Here we have extended the genetic investigation by examining the differences among endpoint plasma FAEs in response to various dietary fatty acids. Our results for the polymorphism rs12540583 with a missense mutation in humans also indicated a very limited effect of NAPE-PLD mutations on circulating FAEs levels, except for the CanolaDHA treatment. To the best of our knowledge, this is the first study that has reported an analysis of the association between this polymorphism and circulating FAE levels. One clinical study reported that a common haplotype in NAPE-PLD was protective against obesity \cite{22}, but no other human studies have assessed whether NAPE-PLD deficiency influences circulating FAEs. In animal models, it has been reported that FAE levels were unaltered in NAPE-PLD-deficient mice \cite{38}, suggesting that FAEs might be synthesized by both NAPE-PLD-dependent and –independent pathways based on the NAPE. Recently, Geurts et al. \cite{23} reported \textasciitilde 60\% reduction of PEA, OEA, and stearoylethanolamide levels in the adipose tissues, but not in the brain, of NAPE-PLD-deleted mice compared to wild type mice when AEA remained unchanged in both genotypes. The important evidence on research of the NAPE-PLD gene suggests the existence of an alternative pathway of FAE synthesis \cite{39}. Interestingly, the
lack of positive correlation between circulating AA and AEA in the present study suggests that the similar alternative synthesis pathway of AEA may exist in humans. It may also suggest that plasma AA may not reflect the amount of AA intake, but associate to the level of its precursor, LA, and the levels of its derivatives, eicosanoids.

Apart from synthesis of FAEs, the regulation of FAE levels also involves degradation. Enzymatic hydrolysis can degrade FAE to fatty acid and ethanolamide by FAAH in virtually all mammalian tissues \(^{(40)}\). FAAH is an integral membrane enzyme that can catalyze the hydrolysis of all FAEs. As such, genetic variants of the FAAH gene could alter the functionality of enzyme activity. The genetic missense polymorphism rs324420 in the FAAH gene has been extensively studied as an endocannabinoid risk factor in overweight/obesity and potential cannabinoid antagonist treatment in obese population \(^{(17)}\). However, the direct association between this SNP and obesity has not been evaluated. One study in overweight/obese subjects with binge eating disorder (BED) or non-BED demonstrated a positive correlation between A allele carriers of rs324420 with overweight/obesity people, but no differences between BED and non-BED subjects \(^{(18)}\). In addition, it was reported that the polymorphism rs324420 variants may be associated with early onset, but not adult obesity \(^{(41)}\). Our study did not show any direct association between rs324420 and BMI types (data not shown). In the current study, the A allele carriers of rs324420 in FAAH had a higher circulating DHEA level. It suggests that the A allele carriers may have a lower activity rate for hydrolyzing DHEA, resulting in a relatively higher level of DHEA, especially in response to consumption of DHA-enriched diets. Interestingly, such diet-gene interactions were observed only in DHEA and ALEA, indicating this SNP rs324420 may only affect DHEA and ALEA levels. Although little is known about the potential bioactive compound DHEA and ALEA, studies reported that the beneficial functions of DHA or
DHEA could be attributed to major DHEA derivatives which both have potent anti-inflammatory and organ-protective properties\(^{(42; 43)}\). As such, this finding concerning the SNP rs324420 might be useful for further research on the beneficial effects of DHA consumption.

Based on animal studies, OEA is of pharmaceutical interest because of the effects it has on the endocannabinoid system, which could be a therapy for treating severe obesity by inhibiting appetite. Recently, a study compared the efficacy for anti-obesity treatment using orally administered OEA and rimonabant, an anorectic antiobesity drug approved for use in Europe\(^{(6)}\). Results indicated that OEA might represent a novel alternative to cannabinoid antagonists for the control of appetite; and rimonabant might also affect food intake and share a similar mechanism. Our results demonstrate a negative correlation between circulating OEA levels and the change in android fat mass from baseline across all five diets. This finding indicates that endogenous OEA from dietary OA might play a role in reducing fat mass. It is possible that the reduction of body weight, especially gynoid fat mass, by consuming diets high in OA is due to OEA: (a) suppression of appetite or (b) increased fat oxidation, resulting in decreased food intake. However, in our study, food intake was controlled and subjects on all diets consume the same number of calories across phases\(^{(30)}\). Thus, in the present study, our observations may be due to a different mechanism(s). Nevertheless, a different study conducted by our group showed a trend for increasing food intake and body weight in response to consumption of a DHA-enriched high oleic canola oil compared to corn oil or high oleic canola oil in golden hamsters\(^{(35)}\). In the present study, the CanolaDHA diet did not reduce android fat mass, but increased gynoid fat mass compared to the other two oleic acid-rich diets, resulting in the lowest total fat mass change. Consistent findings about the OEA/DHEA ratio change have been reported in CD1 mice\(^{(44)}\), while our results reported lower plasma OEA levels in response to the DHA-rich diet. Recent
evidence showed that DHEA might be a potent mediator for neurogenic differentiation (45), and plays a role in reduction of headache pain and psychological distress in humans (46), although we are not aware how such bioactivity of DHEA on neurological system could influence the OEA metabolism. Therefore, our observation provides indirect evidence to support the discovery of the effects of FAEs on appetite and satiety that has been reported by Piomelli et al. (48). Also, we speculate that the elevated DHEA in response to the dietary DHA interferes with the synthesis and function of OEA to potentially suppress appetite and food intake which would prevent weight loss.

In the present study, the plasma fatty acid profile reflected the dietary fatty acids in the treatments oils. The good compliance of participants across the three clinical sites has been previously reported (30). As expected, corresponding elevations in endpoint FAE levels were also observed after the dietary interventions. Our current results indicate that individuals with elevated plasma FA levels tend to have higher corresponding FAE levels. Results of a clinical study by Joosten et al. (49) conformed to our findings and further suggested that the nonesterified fatty acids (NEFA) level may be positively correlated with both circulating fasting or non-fasting FAEs. It is possible that the influences of NEFA on circulating FAEs are based on their mobilization from adipose and clearance in the fasting state. Nevertheless, no further evidence would support the physiological stimuli involved in the changes of NEFA levels after meal consumption. In addition, since FAEs are mostly converted in the tissues and then released into the circulating pool, the enzyme activities of FAE synthesis and degradation are more crucial for circulating FAE levels (15; 23). As such, our important findings demonstrate that the fat types in the diet can positively influence the corresponding FAE levels in humans. These results also provide evidence supporting the conclusion that dietary fatty acids lead to increased
circulating FAEs in both piglets (12) and humans (36, 49), although it remains to be established whether such changes in plasma FAE levels further alter the FAE levels in target tissues such as brain, liver, and intestines.

Polyunsaturated long-chain FAEs are a relatively minor group compared to saturated and monounsaturated FAEs, such as PEA and OEA which are the major FAEs in plasma and tissues (50). Evidence shows that feeding a high saturated fat diet to mice (51) and rats (47) resulted in lower levels of PEA and OEA. Joosten et al. (49) reported that the two most abundant FAEs, PEA and OEA levels, were approximately 7-fold higher than AEA levels in a clinical study. Our observation concurs with the findings with similar absolute values for PEA and OEA ranging from 0.1 to 4.0 ng/ml. Due to the intervention design in our clinical trial, we were unable to demonstrate the effect of dietary saturated fat on PEA and OEA. However, we noticed that although post-treatment plasma LA, LEA’s precursor, levels were higher than OA, OEA’s precursor, levels in our population, the overall plasma LEA levels were lower than OEA. It was clear that the correlation coefficient between LA and LEA was lower than that between OA and OEA. It indicated that the endogenous conversion from OA to OEA might be faster compared with LA to LEA. One possible explanation is because of the variations on the positional distribution of fatty acids on triglyceride molecules. The negative correlations between android fat mass changes and FAEs (OEA, LEA and AEA) indicated that these endogenous FAE levels may be involved in regulation of food intake and energy balance as suggested by Diep et al. (47).

This study has strengths and limitations. Firstly, in contrast to a few previous human studies which relied on small subject number on the post-treatment FAE response, this study was powered to examine a larger group of people in North America. Furthermore, to the best of our
knowledge, this is the first study ever to show that DHEA may have a suppression effect on OEA level, potentially resulting in a failure in body fat loss. Unfortunately, we were unable to assess the baseline FAE contents in plasma which can establish the initial FAE profiles of the participants for further comparison. Although the crossover design of our trial clearly showed the treatment effects on different circulating FAE levels, lacking of baseline values restricted our prediction on the association between change of FAE and change of body fat mass in response to different oil treatments. Nonetheless, the correlation analysis was only performed in a small subgroup of 27 subjects with an unbalanced sex ratio due to the limited design of satellite sites in such multicenter trial, and thus, our attempt was not able to assess the gender effects and the question of how the shifts of FAE influence the body fat response requires further investigation.

In summary, our study reports several novel findings that clarify our understanding of the metabolic and physiological responses associated with different dietary fatty acid classes. Our finding on the genetic variants indicates that the polymorphism rs324420 in FAAH may influence the beneficial function of FAEs in humans by altering the circulating FAE levels. Furthermore, the elevated circulating DHEA in response to dietary DHA may suppress OEA levels and, in turn, interfere with the function of OEA which may disrupt regulation of body weight.
6.6 Acknowledgements

We are indebted to Haifeng Yang for optimizing the performance of the UPLC-MS/MS and GC-FID. We also acknowledge Dennis Labossiere for his interpretation on the plasma fatty acid chromatograph. We especially thank the personnel at all participating centers including investigators, clinical coordinators, kitchen staff, analytical teams and study volunteers.

This study was conducted under the clinical project of COMIT which was supported by the Canola Council of Canada, Flax Council of Canada, Agriculture and Agri-Food Canada and Dow AgroSciences, Canada Research Chairs, and Western Grains Research Foundation through the Growing Forward program of Agriculture and Agri-Food Canada. The current study was also supported by the Manitoba’s Agri-Food Research and Development Initiative (ARDI) (ID: GRWFWD ARDI#12-1186).
6.7 References


49. Joosten MM, Balvers MG, Verhoeckx KC et al. (2010) Plasma anandamide and other N-acylethanolamines are correlated with their corresponding free fatty acid levels under both fasting and non-fasting conditions in women. *Nutr Metab (Lond)* **7**, 49.

Bridge to chapter VII

Chapter VI has demonstrated that dietary fatty acid intakes may influence the metabolic and physiological response via the group of endogenous lipid molecules, fatty acid ethanolamides. Dietary intake is known to have impact on gut microbiota profile over time. However, effects of different unsaturated fatty acid intakes on these gut microorganisms are not clear. The following chapter comprises a manuscript which presents the associations between dietary fatty acid intakes and gut microbiota composition.
Chapter VII

Effects of dietary monounsaturated and polyunsaturated oils on human gut microbiome profiles in the Canola Oil Multicenter Intervention Trial (COMIT)

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This manuscript has been submitted to PLoS ONE and currently under review.
7.1 Abstract

Long-term dietary fatty acid intake is believed to induce changes in the human gut microbiome which might be associated with human health or obesity status; however, considerable debate remains regarding the most favorable ratios of fatty acids to optimize these processes. The objective of this sub-study of a double-blinded randomized crossover clinical study, the canola oil multicenter intervention trial (COMIT), was to investigate effects of five different novel oil blends fed for 30 days each on the intestinal microbiota in 25 volunteers with risk of metabolic syndrome. The 60 g treatments included three MUFA-rich diets: 1) conventional canola oil (Canola); 2) DHA-enriched high oleic canola oil (CanolaDHA); 3) high oleic canola oil (CanolaOleic); and two PUFA-rich diets: 4) a blend of corn/safflower oil (25:75) (CornSaff); and 5) a blend of flax/safflower oil (60:40) (FlaxSaff). Stool samples were collected at the end of each period. DNA was extracted and amplified for pyrosequencing. A total of 17 phyla and 187 genera were identified. While five novel oil treatments failed to alter bacterial phyla composition, obese participants produced a higher proportion of Firmicutes to Bacteroidetes than overweight or normal weight groups ($P = 0.01$). Similarly at the genus level, overall bacterial distribution was highly associated with subjects’ body mass index (BMI). Treatment effects were observed between MUFA- and PUFA-rich diets, with the three MUFA diets elevating Parabacteroides, Prevotella, Turicibacter, and Enterobacteriaceae (F)’s populations, while the two PUFA-rich diets favored the abundance of Isobaculum. High MUFA content feedings also resulted in an increase of Parabacteroides and a decrease of Isobaculum in obese, but not overweight subjects. Data suggest that BMI is a predominant factor in characterization of human gut microbiota profiles, and that MUFA-rich and PUFA-rich diets impact the populations of specific genera. High dietary MUFA content may increase Parabacteroides levels in obese populations.
7.2 Introduction

The prevalence of metabolic syndrome (MetS) is dramatically increasing worldwide (1), with major attention directed on examining effects of dietary fat types, and particularly the optimal ratio of saturated fatty acid (SFA), monounsaturated fatty acid (MUFA), and polyunsaturated fatty acid (PUFA). High amounts of n-6 PUFA, mainly linoleic acid (LA) replacing the SFA contents found in present Western diets, have been shown to prevent cardiovascular diseases (2). Other types of PUFA including n-3 PUFA-rich diets also resulted in weight loss in humans (3). The Mediterranean diet has long been associated with decreased prevalence of obesity and diabetes, and increased longevity (4), partly because of the favorable effects of its MUFA content from olive oil, mainly oleic acid (OA). Therefore, although current dietary recommendations suggest reductions in intakes of SFA, they fall short of providing the optimal ratios of unsaturated fatty acids needed to prevent MetS development. Novel modified oils, designed as sources of blended MUFA and PUFA contents, are expected to significantly improve risk factors of MetS, including central obesity, body mass index (BMI), blood pressure, impaired glucose tolerance, lipid profile, age and lifestyle (5). There is recent evidence that the composition of gut microbiota may play a role in metabolism and adiposity, implicated in MetS risk (6).

Each individual houses a specific and diverse composition of gut microbiome, which potentially impacts on their health. Differences in the gut microbiota patterns have been observed between normal weight and obese animals (7; 8; 9). In humans, the gut microbiota has also been reported to impact the physiological state of obesity (10). Although the links between the composition of gut microbiota and specific conditions associated with obesity are not clearly understood, an increased ratio of two dominant bacterial phyla, Firmicutes to Bacteroidetes appears to associate
with obese-type humans and further correlate to obesity and MetS development \(^{11}\). Therefore, the relationship between the gut microbiome and obesity status may be anticipated possessing potential diagnostic and therapeutic implications for maintaining human health.

The composition of the gut microbiota can change dramatically in response to long-term dietary intake as nutrients obtained through foods are essential to these bacteria. A recent study comparing the gut microbiota of children from Burkina Faso and Italy revealed that the ratio of two dominant phyla, Bacteroidetes and Firmicutes, were entirely different between the two groups \(^{12}\). Here, differences in diets presumably contributed to the shifts in microbiota, which in turn influenced overall metabolism. Studies on traditional Western diets rich in SFA but low in fiber content demonstrated that the diets rich in SFA possessed stronger effects than unsaturated fatty acids in shifting gut microbiota profiles towards those in obese individuals \(^{13; 14}\). On the other hand, very few studies to date have investigated the impacts of novel vegetable oils rich in MUFA or PUFA on gut microbiota in a longer-term human clinical trial.

Therefore, the cause-and-effect relationships between any shift of gut microbiota, the obese state itself, and the diet in obesity remain unknown \(^{15}\). Here, we hypothesized that healthy dietary oil treatments with diverse fatty acid compositions, particularly those rich in MUFA or PUFA, may influence the bacterial composition in human gut. Objectives of present study were to investigate 1) whether significant differences in the gut microbiota composition could be produced in response to MUFA or PUFA oil blends; 2) how the obese status influences the gut bacterial communities during the interventions; and 3) whether the change in gut microbiota composition is correlated with the change in MetS biomarkers.
7.3 Materials and Methods

7.3.1 Clinical design

The Canola Oil Multicenter Intervention Trial (COMIT) was conducted at Richardson Centre for Functional Foods and Nutraceuticals (RCFFN) at University of Manitoba, the Institute of Nutraceuticals and Functional Foods (INAF) at Laval University, and the Department of Nutritional Sciences at Pennsylvania State University (PSU) between September 2010 and March 2012. The intervention studies were reviewed and approved by institutional ethics boards in respective participating universities. Written consents were obtained from all subjects as prescribed by Research Ethics Boards at all clinical centers. The protocol of the present sub-study of COMIT was approved by Biomedical Research Ethics Board (BREB) at University of Manitoba. The clinical trial was registered with clinicaltrials.gov (NCT01351012).

The COMIT study was designed as a random, controlled, double-blind, crossover clinical trial on volunteers at risk of MetS, as previously described (16). Adult men and women with at least one of the following cardiovascular risk factors were recruited for the study: waist circumference ≥ 94 cm for men and ≥ 80 cm for women, triglyceride (TG): ≥ 1.7 mmol/L, HDL cholesterol (HDL-C): < 1 mmol/L (men) or < 1.3 mmol/L (women), blood pressure: ≥ 130 mmHg (systolic) and/or ≥ 85 mmHg (diastolic) and glucose: ≥ 5.5 mmol/L. Participants with thyroid disease, diabetes mellitus, kidney disease, liver disease, current smokers, or people drinking more than 2 alcoholic beverages per week were excluded from the study. During the trial, volunteers who took any medication known to affect endothelial function during the trial were released from protocol, but those who were taking blood pressure medication with a constant dose were included. All participants provided written consent before clinical trial started. The endpoint data
for serum lipid variables [Total Cholesterol (TC), HDL-C, LDL Cholesterol (LDL-C), TG] for the 25 subjects included in the microbiome study were extracted from previously reported COMIT data (17), reanalyzed in the context of their correlations with microbial changes evaluated in this research.

The five-phase randomized full-feeding study design provided subjects with a consistent, individual weight-maintaining diet with a fixed 7-day rotation isocaloric menu of three meals and two snacks a day, including 50% carbohydrate 15% protein and 35% fat of total energy of 3000 Kcal per day. A daily intake of 60 g dietary oils was equally distributed to two identical sizes of beverage shakes at breakfast and supper. Five oil treatments included: 1) canola oil (Canola; 63% MUFA, 20% LA, 10% α-linolenic acid (ALA)); 2) DHA enriched canola-oil (CanolaDHA; 64% MUFA, 13% LA, 6% docosahexaenoic acid (DHA)); 3) high OA canola oil (CanolaOleic; 72% MUFA, 15% LA, 2% ALA); 4) a blend of corn oil/safflower oil (CornSaff; 18% MUFA, 69% LA); and 5) a blend of flax oil/safflower oil (FlaxSaff; 18% MUFA, 38% LA, 32% ALA) (Table 7.1). All five diets were low in SFA, but replaced with different combinations of unsaturated fatty acids. Three canola-based diets were rich in MUFA while two safflower oil blends were high in n-3 PUFA or n-6 PUFA. To compare the impacts of MUFA-rich diets (Canola, CanolaDHA, and CanolaOleic) with those of PUFA-rich diets (CornSaff and FlaxSaff) on the gut microbiota a contrast analysis was performed. Comparisons of subgroups between CanolaDHA and CanolaOleic, and CornSaff and FlaxSaff were also conducted. All the meals and shakes were prepared fresh in the metabolic kitchen. Participants were instructed to visit the research site and consume at least one meal containing one dose of treatment beverage (usually breakfast) under the supervision of the clinical staff in the cafeteria on weekdays, while the remaining meals and weekend proportions were packed for off-site consumption. No outside
food was allowed to consume during the intervention period. Compliance was assessed by clinical staff as rate of completion of meals provided under supervision as well as by the presence of food not consumed in their returned meal bags packed for off-site consumption. Both participants and study coordinators were blinded to the treatments. Each treatment phase lasted 30 days in duration and separated with 4 week washout periods.

Table 7.1 Fatty acid composition of five dietary oil treatments (g) consumed at 60 g/d (35% of energy intake based on 3000 kcal/d)

<table>
<thead>
<tr>
<th>Fatty acids</th>
<th>Treatments¹</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Canola</td>
</tr>
<tr>
<td></td>
<td>g (% of energy)</td>
</tr>
<tr>
<td>SFA²</td>
<td></td>
</tr>
<tr>
<td>C12:0</td>
<td>0.05</td>
</tr>
<tr>
<td>C14:0</td>
<td>0.04</td>
</tr>
<tr>
<td>C16:0</td>
<td>2.44</td>
</tr>
<tr>
<td>C17:0</td>
<td>0.04</td>
</tr>
<tr>
<td>C18:0</td>
<td>1.10</td>
</tr>
<tr>
<td>C20:0</td>
<td>0.39</td>
</tr>
<tr>
<td>C22:0</td>
<td>0.19</td>
</tr>
<tr>
<td>C24:0</td>
<td>0.11</td>
</tr>
<tr>
<td>Total SFA</td>
<td>4.36 (6.6)</td>
</tr>
<tr>
<td>MUFA³</td>
<td></td>
</tr>
<tr>
<td>C16:1</td>
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</tr>
</tbody>
</table>

²SFA: Saturated Fatty Acids
³MUFA: Monounsaturated Fatty Acids
<table>
<thead>
<tr>
<th></th>
<th>C17:1</th>
<th>C18:1</th>
<th>C20:1</th>
<th>C22:1</th>
<th>Total MUFA</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0.07</td>
<td>0.10</td>
<td>0.12</td>
<td>0.00</td>
<td>36.15 (17.6)</td>
</tr>
<tr>
<td></td>
<td>35.17</td>
<td>37.95</td>
<td>42.88</td>
<td>10.56</td>
<td>38.84 (17.8)</td>
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<tr>
<td></td>
<td>0.73</td>
<td>0.62</td>
<td>0.72</td>
<td>0.02</td>
<td>43.89 (19.3)</td>
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<tr>
<td></td>
<td>0.03</td>
<td>0.04</td>
<td>0.04</td>
<td>0.00</td>
<td>10.60 (9.5)</td>
</tr>
<tr>
<td></td>
<td>0.03</td>
<td>0.04</td>
<td>0.04</td>
<td>0.00</td>
<td>10.72 (9.6)</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th>Total PUFA</th>
</tr>
</thead>
<tbody>
<tr>
<td>PUFA&lt;sup&gt;4&lt;/sup&gt;</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>11.72</td>
<td>7.65</td>
<td>8.84</td>
<td>41.61</td>
<td>22.48</td>
</tr>
<tr>
<td></td>
<td>5.86</td>
<td>1.18</td>
<td>1.38</td>
<td>0.17</td>
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</tr>
<tr>
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<td>0.04</td>
<td>0.00</td>
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<td>17.58</td>
<td>13.86</td>
<td>10.22</td>
<td>41.78</td>
<td>41.67</td>
</tr>
</tbody>
</table>

<sup>1</sup>Dietary oil treatments are Canola: conventional canola oil; CanolaDHA: high oleic canola oil with DHA (85:15); CanolaOleic: high oleic canola oil; CornSaff: corn oil and safflower oil blend (25:75); FlaxSaff: flax oil and safflower oil blend (60:40).

<sup>2, 3, 4</sup>SFA: saturated fatty acid; MUFA: monounsaturated fatty acid; PUFA: polyunsaturated fatty acid.
7.3.2 Stool collection

Twenty-five participants from RCFFN (Winnipeg, Manitoba) arm of the COMIT trial agreed to provide 5 g stool samples at the end of each intervention phase for the present study (Table S7.1). Subjects were instructed to collect their own samples from only one bowel movement in the privacy of their home the day before they visited RCFFN on either day 29 or day 30 of their endpoint visits. Subjects were directed to store fecal specimens immediately after collection at -20 °C freezer until collector tubes were handed over to clinical coordinators at RCFFN. Fecal specimens subsequently labelled and stored at -80 °C until further analysis.

7.3.3 DNA extraction

The frozen fecal specimens in collector tubes were wrapped in individual zipper bags and placed in a cooler with ice packs at RCFFN. After transport to the Gut Microbiome and Large Animal Biosecurity Laboratories at Department of Animal Science, University of Manitoba, fecal samples were thoroughly thawed and homogenized at room temperature. Genomic DNA was then extracted from approximately 250 mg fecal mass using ZR Fecal DNA Kit (D6010, Zymo Research Corp., Orange, CA), which included a bead-beating step to lyse microbial cells, according to the manufacturer’s protocol. To match the concentration requirement for pyrosequencing, DNA quantity was determined under a Beckman DU/800 spectrophotometer (Beckman Coulter, Inc., Fullerton, CA). Genomic DNA was normalized to achieve a concentration of 20 ng/μL and quality checked by PCR amplification of the 16S ribosomal RNA (rRNA) gene, 27f (5'-GAAGAGTTTGATCATGGCTCAG-3') and 342r (5'-CTGCTGCCTCCGTCAG-3') (18; 19). Amplicons were verified by agarose gel electrophoresis.
7.3.4 Pyrosequencing

Approximately 200 ng aliquots of high quality, inhibitor-free genomic DNA were sent to the Research and Testing Laboratories (Lubbock, TX) for bacterial rRNA gene tag-encoded GS FLX-Titanium amplicon pyrosequencing \(^{(20)}\). Briefly, a mixture of Hot Start, HotStar high-fidelity Taq polymerases, and Titanium reagents were used to perform a one-step PCR (35 cycles). Pyrosequencing was performed using a 454 GS FLX-Titanium Sequencing System (454 Life Sciences, Roche Company, Branford, CT). The primers 28f (GAGTTTGATCMTGGCTCAG) and 519r (GTNTTACNGCGGCKGCTG) were utilized to target the variable regions V1-V3 of the bacterial 16S rRNA genes. The sequencing data are uploaded into the Sequence Read Archive (SRA) of NCBI (http://www.ncbi.nlm.nih.gov/sra).

7.3.5 Sequence classification and diversity analysis

After sequencing, the data were edited, categorically transformed and classified as described previously \(^{(21)}\). In general, all failed sequence reads, low-quality sequence ends, tags, and non-bacterial ribosome sequences and chimeras were removed from the dataset. Using software “mothur (version 1.30.2)” \(^{(22)}\), the second round of sequence quality control and assignments of operational taxonomic unit (OTU) were performed. All sequences less than 200 bp or sequences having one or more ambiguous base or containing a homopolymer length more than 8 bp were excluded from the data set. High quality 16S rRNA bacterial sequences were identified and aligned using the comprehensive rRNA database Silva \(^{(23)}\) to reduce the noise from pyrosequencing data. The remaining sequence position columns and sequences were used to build a distance matrix with a distance threshold of 0.15. Using the furthest neighbor algorithm with a cutoff of 95% similarity, these sequences were clustered to OTU.
Within community diversity (α-diversity) was conducted based on OTU counts to evaluate the biodiversity of the bacterial population in the fecal samples at the genus level. Richness indices, Chao1 and abundance based coverage estimation (ACE), Shannon index and Simpson diversity were calculated to estimate the number of OTU that were present in each individual sample. Appropriate statistical considerations were used to exclude samples with extremely high or low diversity as outliers. Rarefaction curve which allows the calculation of sequence richness were also performed for five treatment groups and BMI groups using mothur (22) based on randomly re-sampling the pool of sequence numbers.

To evaluate the differences in community composition among different treatments and BMI populations, β-diversity was measured by calculating the Bray-Curtis distance (24). Principal coordinate analysis (PCoA) was applied on resulting distance matrices to generate two-dimensional plots using PRIMER v6 software (25). Permutational multivariate analysis of variance (PERMANOVA) was performed to assess significant differences of β-diversity among the treatments and BMI groups on the basis of distance measures using permutation methods (26).

7.3.6 Statistical analysis

Based on the outcomes from mothur, the phyla and genera in relatively low abundance (under 0.1%) were removed from further analysis. SAS version 9.2 (SAS Institute Inc., Cary, NC) was used for data analysis. Normality of residuals for α-diversity indices was tested by Kolmogorov-Smirnov tests and histograms were used to test the Gaussian nature of the dataset. The significance of differences between treatments was analyzed using mixed model with treatment followed by Tukey adjustment.
Normal distribution was tested by Kolmogorov-Smirnov tests and histograms, and Box-Cox power transformation macro was used if necessary when analyzing statistical differences among treatments or contrasts at phylum and genus levels. Depending on the distribution of the residuals, MIXED or GLIMMIX models in SAS with mixed-effect analysis of variance with treatment as fixed effect, and subject as a random effect followed by Tukey adjustment, were conducted to estimate the significant differences. Differences between treatments were considered significant at $P < 0.05$ while trends were observed at $P < 0.1$.

The non-parametric Spearman correlation matrix was generated and Spearman’s $r$ and $P$-values were calculated to investigate the correlations between fecal microbiome and serum lipid profiles using the PAST software (PAleontological Statistics; (27)). The results were visualized over heatmap using core plot package of R (28). Taxa with relative abundance greater than 0.1% of population were included in the analysis. Strong and weak correlations were presented by dark and light colors, respectively. The spearman $r$ denotes whether the correlation between the taxa of interest and the selected parameter was positive (closer to 1, blue squares) or negative (closer to -1, red squares).

Partial least square discriminant analysis (PLS-DA) was performed on bacterial genera to identify the effects of dietary oil treatments on the bacterial community, according to our previously described analytical method with modifications (21). For this analysis, data were scaled using Unit Variance in SIMCA-P+ 13.0 (Umetrics, Umea, Sweden). Cross-validation and permutation testing were conducted to determine significant PLS components in the optimal model. To avoid over parameterization of the model, a cut-off level was set for variable influence on projection (VIP) value in each model to improve $R^2$ and $Q^2$ with better prediction of
the bacterial distribution. Significant differences across treatments or BMI groups were expressed in scatter and score plots, according to the PLS regression coefficients. Each individual genus 95% confidence interval, therefore, determined the positive or negative correlations with dietary treatments or BMI groups.

7.4 Results

7.4.1 Sample assessment by pyrosequencing

After the 5-phase clinical trial, a total of 99 samples were collected from 25 subjects. Missing samples resulted from participants either forgetting to collect samples or failing to provide samples at expected sampling dates. During the DNA extraction process, another 33 out of 99 DNA samples were discarded due to poor quality or purity, resulting in 66 useable samples for pyrosequencing assessment and sequence classification (Table S7.1). In total, 209,490 sequences were first generated. The minimum, median and maximum lengths of sequences were 250, 393 and 698 bp, respectively. Screening, filtering, and pre-clustering processes resulted in 142,887 sequences.

7.4.2 Alpha and β-diversity analyses

Bacterial richness and diversity in individual samples under different oil treatments and BMI were calculated (Table 7.2). Oil treatments and BMI had no significant impact on Chao1, ACE, Shannon and Simpson indices of α-diversity (Figure 7.1a and Table 7.2). However, the rarefaction curves showed higher richness and diversity in overweight and obese subjects compared to the normal weight participants (Figure 7.1b). Similarity or differences in the gut microbiota among different oil treatments and BMI (β-diversity) were compared using PCoA and
PERMANOVA analyses of Bray-Curtis distances (Figures S7.1-S7.6). Comparisons included MUFA vs. PUFA ($P = 0.84$), normal weight vs. overweight ($P = 0.11$), normal weight vs. obese ($P < 0.01$), overweight vs. obese ($P = 0.03$), MUFA vs. PUFA within overweight group ($P = 0.57$), and MUFA vs. PUFA within obese group ($P = 0.99$). The $\beta$-diversity did not change among individual oil treatments.
Table 7. 2 Summary statistics for sequences using 16S rRNA gene pyrosequencing.

<table>
<thead>
<tr>
<th>Variables²</th>
<th>Sequence Number</th>
<th>OTU Number</th>
<th>Coverage (%)</th>
<th>Richness⁴</th>
<th>Diversity⁵</th>
</tr>
</thead>
<tbody>
<tr>
<td>Treatments</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Canola</td>
<td>2521</td>
<td>121</td>
<td>98.38</td>
<td>173.86</td>
<td>218.10</td>
</tr>
<tr>
<td>CanolaDHA</td>
<td>2497</td>
<td>112</td>
<td>98.98</td>
<td>126.19</td>
<td>129.81</td>
</tr>
<tr>
<td>CanolaOleic</td>
<td>2486</td>
<td>116</td>
<td>98.46</td>
<td>162.87</td>
<td>201.23</td>
</tr>
<tr>
<td>CornSaff</td>
<td>2174</td>
<td>139</td>
<td>97.63</td>
<td>232.30</td>
<td>296.89</td>
</tr>
<tr>
<td>FlaxSaff</td>
<td>2414</td>
<td>111</td>
<td>98.37</td>
<td>149.42</td>
<td>181.24</td>
</tr>
<tr>
<td>SEM</td>
<td>14.78</td>
<td>0.26</td>
<td>13.67</td>
<td>46.67</td>
<td>0.15</td>
</tr>
<tr>
<td>P-value</td>
<td>0.69</td>
<td>0.01</td>
<td>0.15</td>
<td>0.15</td>
<td>0.53</td>
</tr>
<tr>
<td>BMI groups</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Normal</td>
<td>2401</td>
<td>121</td>
<td>98.50</td>
<td>150.15</td>
<td>162.20</td>
</tr>
<tr>
<td>Overweight</td>
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<td>134</td>
<td>98.30</td>
<td>186.84</td>
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</tr>
<tr>
<td>Obese</td>
<td>2277</td>
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<td>98.39</td>
<td>164.07</td>
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<td>26.72</td>
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<tr>
<td>-------</td>
<td>-------</td>
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</tr>
<tr>
<td>P-value</td>
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<td>0.88</td>
<td>0.65</td>
<td>0.66</td>
<td>0.49</td>
</tr>
</tbody>
</table>

¹Means are from statistical models based on 66 fecal samples across five treatments. ²Dietary oil treatments are Canola: conventional canola oil; CanolaDHA: high oleic canola oil with DHA (85:15); CanolaOleic: high oleic canola oil; CornSaff: corn oil and safflower oil blend (25:75); FlaxSaff: flax oil and safflower oil blend (60:40). BMI groups are normal, overweight, and obese subjects. ³OTU: operational taxonomic units. ⁴Based on Chao1 and abundance based coverage estimation (ACE) richness indices. ⁵Based on Shannon and Simpson diversity estimators.
Figure 7.1a and 7.1b Rarefaction analysis for the observed species.
The rarefaction curve is generated using Chao1 richness estimator according to oil treatments (1a) or subjects’ body mass index (BMI; 1b). Samples have been rarified at an even depth of 21,000 sequences per oil treatment or BMI group.
7.4.3 Overall gut microbiota composition

A total of 15 phyla were identified in all the samples from 99.8% of all sequences (Table S7.2), of which four phyla were considered as abundant within the population (larger than 1%), including Firmicutes, Bacteroidetes, Actinobacteria, and Proteobacteria (Table 7.3). The abundance of Aquificae, Fusobacteria, and Verrucomicrobia were between 0.1 and 1% of population. The remaining eight phyla, which were in low abundance (under 0.1%) included Acidobacteria, Chrysiogenetes, Cyanobacteria, Deinococcus-Thermus, Nitrospira, Synergistetes, TM7, and Tenericutes. Overall, the phylum distribution did not fluctuate across the five oil treatments or among MUFA vs. PUFA groups. However, BMI status had an impact on the microbiota composition at the phylum level with obese group having greater population of Firmicutes ($P = 0.02$) compared to the combined group of normal and overweight subjects (data not shown). The average ratio of Bacteroidetes to Firmicutes was 0.15 across five diets with no difference among dietary interventions (data not shown).
Table 7.3 Relative abundances of bacterial phyla from pyrosequenced 16S rRNA sequences.

<table>
<thead>
<tr>
<th>Phyla</th>
<th>Percentages of Sequences in Treatments</th>
<th>Contrasts (P-value)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Canola</td>
<td>CanolaDHA</td>
</tr>
<tr>
<td>-----------------</td>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>Actinobacteria</td>
<td>2.85</td>
<td>3.23</td>
</tr>
<tr>
<td>Bacteroidetes</td>
<td>11.60</td>
<td>11.77</td>
</tr>
<tr>
<td>Firmicutes</td>
<td>82.37</td>
<td>82.23</td>
</tr>
<tr>
<td>Proteobacteria</td>
<td>1.96</td>
<td>1.42</td>
</tr>
<tr>
<td>Aquificae</td>
<td>0.58</td>
<td>0.63</td>
</tr>
<tr>
<td>Fusobacteria</td>
<td>0.26</td>
<td>0.25</td>
</tr>
<tr>
<td>Verrucomicrobia</td>
<td>0.19</td>
<td>0.16</td>
</tr>
</tbody>
</table>

1 Dietary oil treatments are Canola: conventional canola oil; CanolaDHA: high oleic canola oil with DHA (85:15); CanolaOleic: high oleic canola oil; CornSaff: corn oil and safflower oil blend (25:75); FlaxSaff: flax oil and safflower oil blend (60:40). 2 Means for each

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phylum under five treatments are individually reported. The Mean and SEM are also reported across five treatments. \(^3\)\(P\)-values adjusted by Tukey are significantly different at \(P < 0.05\). \(^4\) The contrast of different treatment groups or comparison between selected treatment groups. MUFA/PUFA is the contrast of the combination of Canola, CanolaDHA, and CanolaOleic compared to the combination of FlaxSaff and CornSaff. CanolaDHA/CanolaOleic is the comparison between CanolaDHA and CanolaOleic. CornSaff/FlaxSaff is the comparison between CornSaff and FlaxSaff. \(P\) values adjusted by Tukey are significantly different at \(P < 0.05\). *Percentage of sequences below 0.1.
At the genus level, 187 genera were determined using 16S rRNA gene pyrosequencing. Majority of sequences were identified at the Genus (g.) level while some sequences could only be classified up to phylum (p.), class (c.), order (o.), or family (f.) levels. Tables S7.3 and S7.4 present the distribution of taxa with a relative abundance > 0.1% and < 0.1% of population, respectively, among diets. The abundance of g. *Faecalibacterium* tended to differ across all the treatments ($P = 0.06$) where CanolaOleic feeding was associated with the highest level of *Faecalibacterium* and CanolaDHA feeding resulted in lowest population. Data also showed a tendency for higher population in g. *Parabacteroides* ($P = 0.09$) but lower population in g. *Isobaculum* ($P = 0.08$) after the three MUFA-rich diets compared to two PUFA-rich diets. Genus *Blautia* was observed to favor the CanolaOleic feeding compared to the CanolaDHA diet ($P = 0.09$). No differences were observed between n-6 PUFA-rich treatment CornSaff and n-3 PUFA-rich diet FlaxSaff.

The PLS-DA analysis (given for the cut-off value of 1.0) showed further differences at the lower taxonomical levels. Genera *Parabacteroides*, *Prevotella*, *Turicibacter*, and family *Enterobacteriaceae* were correlated to MUFA-rich diets, while g. *Isobaculum* was correlated to PUFA-rich diets ($R^2 = 0.43$, $Q^2 = 0.07$; Figure 7.2). Comparison between CanolaDHA and CanolaOleic indicated that CanolaDHA was correlated to f. Lachnospiraceae and p. Firmicutes, whereas CanolaOleic was associated with g. *Faecalibacterium* and *Coprobacillus* ($R^2 = 0.78$, $Q^2 = 0.45$; Figure 7.3). The comparison between two PUFA-rich diets showed CornSaff treatment had an impact on the population of g. *Eggerthella*, *Slackia*, *Soehngenia*, *Anaerostipes*, *Robinsoniella*, *Phascolarctobacterium*, while FlaxSaff failed to have such significant impact ($R^2 = 0.67$, $Q^2 = 0.22$; data not shown).
Figure 7.2 Partial least square discriminant analysis (PLS-DA) loading plot based on the relative abundances of bacterial genera in the gut microbiota and their association with MUFA-rich or PUFA-rich diets.

The presenting genera are chosen at VIP cutoff of 1.0 and colored according to their corresponding phyla. The size of circles is indicative of taxa abundance. Taxa closer to MUFA or PUFA indicate positive association with either treatment group. The $R^2 (= 0.43)$ and $Q^2 (= 0.07)$ estimates were calculated based on three components. Some sequences could only be affiliated to phylum (P) class (C), order (O), or family (F) levels.
Figure 7.3 Partial least square discriminant analysis (PLS-DA) loading plot based on the relative abundances of bacterial genera in the gut microbiota and their association with CanolaDHA or CanolaOleic diets.

The presenting genera are chosen at VIP cutoff of 1.0 and colored according to their corresponding phyla. The size of circles is indicative of taxa abundance. Taxa closer to CanolaDHA or CanolaOleic diets indicate positive association with either treatment group. The $R^2 (= 0.78)$ and $Q^2 (= 0.45)$ estimates were calculated based on three components. Some sequences could only be affiliated to phylum (P) class (C), order (O), or family (F) levels.
7.4.4 Gut microbiota profiles within individual obese status

At the phylum level, a significantly higher population of Firmicutes was observed in obese group (BMI > 30) compared to the combined group of normal weight (BMI < 25) and overweight subjects (BMI between 25 and 30; $P = 0.02$; data not shown). At the genus level, PLS-DA analysis confirmed a significant difference in the composition of bacteria among three BMI phenotype groups ($R^2 = 0.60$, $Q^2 = 0.32$; Figure 7.4).

Since the number of subjects in the normal weight category was insufficient, the analysis on contrasts and subgroups only focused on two representative populations, overweight and obese groups. In overweight people, *g. Streptococcus, Tepidimicrobium, Robinsoniella*, and *Turicibacter* were correlated to MUFA-rich diets, while *g. Coriobacterium* and *Mogibacterium* were associated with PUFA-rich diets ($R^2 = 0.69$, $Q^2 = 0.26$; Figure 7.5). In the comparison between CanolaDHA and CanolaOleic, *g. Adlercreutizia, Coriobacterium, Alistipes*, and *Robinsoniella* showed strong correlations with CanolaDHA while *g. Lactobacillus* was associated with CanolaOleic ($R^2 = 0.90$, $Q^2 = 0.60$; data not shown). In the comparison between the two PUFA-rich diets, CornSaff was highly associated with *g. Adlercreutizia* while FlaxSaff was correlated with *g. Collinsella, Barnesiella, Streptococcus, Roseburia, Coprobacillus*, and family Peptostreptococcaceae ($R^2 = 0.98$, $Q^2 = 0.74$; data not shown).

In obese subjects, *g. Parabacteroides, Prevotella, Flexithrix, Fusibacter*, f. Enterobacteriaceae, and p. Firmicutes were correlated to MUFA-rich diets, but no specific taxa was associated with PUFA-rich diets ($R^2 = 0.66$, $Q^2 = -0.20$; Figure 7.6). In comparison of CanolaDHA and CanolaOleic, only *g. Parasutterlla* correlated with CanolaDHA ($R^2 = 0.91$, $Q^2 = 0.29$; data not shown). Between the two PUFA-rich diets, *g. Collinsella, Hydrogenobaculum*, and
*Parabacteroides* were impacted by the CornSaff, while *g. Clostridium* was correlated to the FlaxSaff diet ($R^2 = 0.98$, $Q^2 = 0.63$; data not shown).

7.4.5 Correlations between serum lipid variables and gut microbiota profiles

The endpoint data for serum lipid variables of the 25 subjects included in this sub-study were extracted from previously reported COMIT data (17) and reanalyzed. All changes in lipid profiles followed the same trends of the entire study population (Figure S7.7). The correlations between lipid profiles and bacterial phyla are presented in Table S7.5. Across treatments, serum TG was negatively correlated with *p. Aquificae* ($r = -0.27$, $P = 0.02$) but positively correlated with *p. Cyanobacteria* ($r = 0.24$, $P = 0.05$). In contrast, LDL-C was positively correlated with *p. Proteobacteria* ($r = 0.28$, $P = 0.01$) and similar tendency was observed for TC ($r = 0.21$, $P = 0.08$). HDL-C, however, was positively correlated with *p. Verrucomicrobia* ($r = 0.21$, $P = 0.08$). In CanolaDHA treatment, TC levels trended to negatively correlate with *p. Bacteroidetes* ($r = -0.51$, $P = 0.06$) and positively correlated with *p. Firmicutes* ($r = 0.55$, $P = 0.04$), resulting in negative correlation trend with the ratio of Bacteroidetes to Firmicutes ($r = -0.51$, $P = 0.06$). In CornSaff treatment, TC levels were positively correlated with *p. Bacteroidetes* ($r = 0.64$, $P = 0.02$) and *Bacteroidetes* to *Firmicutes* ratio ($r = -0.65$, $P = 0.02$). The correlations between serum lipid variables and bacterial composition at the lower taxonomical levels are presented in Figure 7.7.
Figure 7.4 Partial least square discriminant analysis (PLS-DA) score scatter plot of the gut microbiota comparing subjects with different body mass index.

The distribution of fecal samples (n = 66) indicated a significant difference in the composition of bacterial genera among obese (BMI > 30), overweight (BMI between 25 and 30), and normal weight (BMI < 25) groups. The $R^2$ (= 0.60) and $Q^2$ (= 0.32) estimates were calculated based on three components.
Figure 7.5 Partial least square discriminant analysis (PLS-DA) loading plot based on the relative abundances of bacterial genera in the gut microbiota and their association with MUFA-rich or PUFA-rich diets in overweight population (BMI between 25 and 30).

The presenting genera are chosen at VIP cutoff of 1.0 and colored according to their corresponding phyla. The size of circles is indicative of taxa abundance. Taxa closer to MUFA or PUFA indicate positive association with either treatment group. The $R^2$ ($= 0.69$) and $Q^2$ ($= 0.26$) estimates were calculated based on three components. Some sequences could only be affiliated to phylum (P) class (C), order (O), or family (F) levels.
Figure 7.6 Partial least square discriminant analysis (PLS-DA) loading plot based on the relative abundances of bacterial genera in the gut microbiota and their association with MUFA-rich or PUFA-rich diets in obese population (BMI > 30).

The presenting genera are chosen at VIP cutoff of 1.0 and colored according to their corresponding phyla. The size of circles is indicative of taxa abundance. Taxa closer to MUFA or PUFA indicate positive association with either treatment group. The $R^2$ ($= 0.66$) and $Q^2$ ($= -0.20$) estimates were calculated based on three components. Some sequences could only be affiliated to phylum (P) class (C), order (O), or family (F) levels.
Figure 7.7 Correlations between fecal microbiome compositions at the phylum level and serum lipid profiles.

The non-parametric Spearman correlation matrix is presented as Spearman’s $r$ and $P$-values in heatmap. Strong and weak correlations are presented by dark and light colors, respectively. The intensity bar shows positive and negative correlation from blue to red. F.B: ratio of Firmicutes to Bacteroidetes.
7.5 Discussion

We investigated the impact of five different healthy unsaturated fatty acid oil treatments with a dietary fat content of less than 35% energy intake on gut microbiota profiles of volunteers with risks of MetS. Our experimental diets included three MUFA-rich (Canola, CanolaDHA, and CanolaOleic) and two PUFA-rich (CornSaff and FlaxSaff) diets and were designed to maintain the body weight during the treatment periods. Major findings on post-treatment outcomes indicate that diet, metabolic profiles, obesity state, and individual responses are all contributing factors to the shifts in the gut microbiota compositions but at different degrees. Overall, the impact of our healthy unsaturated oil treatments on the microbiota composition was relatively minor at the phylum level and mainly associated with microbiota shifts at the lower taxonomical levels, while BMI contributed to a significant shift at the phylum level with greater Firmicutes and less Bacteroidetes in obese group compared to the combined group of normal and overweight subjects.

Several studies have investigated the effects of high fat diets mainly from SFA on the gut microbiota composition. Turnbaugh et al. (8) showed that diet-induced obesity can quickly develop in mice fed prototypic Western diets with high SFA content. Ley et al. (11) described that the relative proportion of p. Bacteroidetes decreased in obese people compared with lean people, and this proportion also increased when the obese individual lost weight due to either carbohydrate-restricted or fat-restricted diets. In a study comparing different types of high fat diets on the profile of gut bacteria in a mouse model, Liu et al. (14) observed that consumption of a SFA-rich diet resulted in a significant decrease in the abundance of p. Bacteroidetes compared to either n-3 PUFA-rich or n-6 PUFA-rich diets. Supplementation of fish oil for one month in
human infants influenced the intestinal microbiota composition suggesting that dietary n-3 fatty acids may influence the bacterial adhesion to the intestinal mucosa, which in turn associates to the immune response and fat accumulation\(^{(29)}\). Furthermore, Andersen et al. \(^{(30)}\) reported a significant increase in p. Bacteroidetes following the n-6 PUFA treatment. In the present study, we did not have a control low fat or a control high fat treatment, while the proportion of dietary fat content was less than 35% of total energy intake across all 5 treatments, which is the recommended level of fat intake to maintain body weight. As a result, we did not observe a major difference at the phylum level in the bacterial composition among n-3 PUFA (FlaxSaff), n-6 PUFA (CornSaff) and DHA-rich (CanolaDHA) treatments, or the combination of MUFA-rich vs. PUFA-rich diets. The differences observed were at the lower taxonomical levels, for example MUFA-rich diets increased the population of g. *Prevotella*, and *Parabacteroides* within p. Bacteroidetes, and their impacts were more pronounced in the obese people compared to combined group of normal and overweight subjects.

The individuals’ BMI has a major impact on gut microbiota. A study by Ley et al. \(^{(7)}\) used obese mice with ob/ob, lean ob/+, and wild-type siblings, and their ob/+ mothers to investigate the impact of obese genotypes and phenotypes on the gut microbiota. Results showed that ob/ob mice had a decreased abundance of Bacteroidetes and an increased population of Firmicutes. Authors also reported that the ratio of p. Bacteroidetes to Firmicutes dynamically reflects the overall weight condition in mice model. A follow-up study to extend these observations to humans showed that the relative proportion of Bacteroidetes is reduced in obese participants compared to the lean controls \(^{(11)}\). A human study by Turnbaugh et al. \(^{(10)}\) on 154 volunteers confirmed that obesity is in relation to phylum-level changes in the microbiota, and the total diversity of the gut microbiota is decreased in obese individuals with increased food intake. In
the present study, subjects’ recruitment was based on the presence of at least one of the MetS risk factors including waist circumference, blood pressure, TG, HDL-C, and glucose levels. As a result we didn't have a balanced number of subjects at different BMI groups. As such, we only observed a tendency of higher ratio of Bacteroidetes to Firmicutes in normal and overweight people compared to that of obese subjects. Nevertheless, when the obese states were classified as subgroups, the correlation coefficients were significantly improved indicating higher predictability of our models for detection of microbiota pattern in response to MUFA- or PUFA-rich diets.

Comparing the MUFA- and PUFA-rich diets, one interesting finding was the shift in g. *Parabacteroides* and *Isobaculum* populations suggesting that additional dietary OA can result in an increase in the population of *Parabacteroides* and a decrease in *Isobaculum*. When we chose the overweight and obese groups for subgroup analysis, contrasting MUFA vs. PUFA, a positive correlation between *Parabacteroides* and MUFA-rich diets was found to exist only in obese but not in overweight subjects. Further analysis comparing across CanolaOleic and CornSaff or FlaxSaff oil phases also showed consistent outcomes that this potential correlation with *Parabacteroides* was significant but was only observed in obese but not overweight volunteers. Thus, our study indicated that the MUFA-rich diets (high OA contents) can increase the population of *Parabacteroides* in human gut, and people with obese status may express a stronger response to such shift. Unfortunately, there is not much known about the function of *Parabacteroides* and *Isobaculum* and further investigation on the potential roles of these genera in development of obesity is needed.

Our results show that g. *Faecalibacterium* population was higher in CanolaOleic treatment
compared to CanolaDHA. Among different species of *g. Faecalibacterium, F. prausnitzii* is considered as an indicator of intestinal health due to its anti-inflammatory effects (31). Based on our findings, we can, therefore, hypothesize that additional intake of OA may increase the anti-inflammatory properties in human intestines by increasing the population of *F. prausnitzii* although further investigation is required.

The gut microbiome profile in humans is host-specific. Fecal communities in the human gut can change dramatically in response to new diets. Recent studies have shown that gut microbiome can rapidly and reproducibly respond to short-term dietary changes (32). These studies suggest that the human gut microbiome can rapidly switch between herbivorous and carnivorous functional profiles, which might be reflective of past selective pressures during human evolution (32). Turnbaugh et al. (33) using a humanized mice model showed that switching from a low-fat, plant polysaccharide-rich diet to a high-fat/high-sugar “Western” diet shifted the structure of the microbiota within a single day, changed the representation of metabolic pathways in the microbiome, and altered microbiome gene expression. The authors also indicated that the gut microbiota composition was stabilized 7 days following the dietary switch. As such, the four-week dietary interventions used in this study should have been sufficient to stabilize diet-induced changes in the microbiota. It is clear now that each individual is relatively unique in terms of bacterial species of microbiota in their gut, no matter what obese state the individual belongs to, or what dietary oil treatments are provided. This may partly explain the variations in the microbiota profiles among the participants of our study and the variations in their responses to the dietary treatments.

Evidence showed dietary DHA oil intends to increase LDL-C levels in humans (34; 35), although
the reason remains unclear. We observed similar elevated LDL-C levels after CanolaDHA treatment in the present study, which was significantly correlated with the increase in Firmicutes and the decrease in Bacteroidetes phyla within this treatment. These observations are supported by a mouse study (36), which reported that a diet supplemented with n-3 fatty acids (Eicosapentaenoic acid + DHA) significantly increased the abundance of Firmicutes and reduced the percentage of Bacteroidetes compared to a diet supplemented with OA. It can be speculated that the changes in metabolic parameters after DHA oil intake could be the result of interactions between gut microbiota and DHA metabolites potentially through the enterohepatic circulation of bile salts (37; 38). However, our study was not designed to test this hypothesis and further investigation on the bile excretion analysis is required to proof or disproof this hypothesis.

That being said, our study also had several limitations. First, given an intervention trial period of more than 9 months of duration during which multiple samples were collected at different time points, some participants forgot to provide a fecal sample on the specific sampling day. Second, the small proportion of fecal samples might not be able to represent the total fecal materials. Even after the homogenization, the specimens could still produce large variations in the bacterial composition and, in addition, were limited to reflect the dynamic condition in the intestine. Third, the targeted sequencing region may not reflect the authentic outcome of species in the fecal samples because the sequence divergences are not evenly distributed along the 16S rRNA regions. Fourth, the inclusion criteria of our study were based on the risks of MetS, but not the obese state. Given the lower numbers of participants with normal weight and overweight, the statistical analyses were not optimal to compare the direct variations among all participants.

In summary, the current study investigated the impact of five different healthy unsaturated fatty
acid oil treatments on volunteers with risks of MetS. Results indicate that the human gut microbiota profiles strongly relate to the body obesity states, but also exhibit host-specificity. MUFA-rich and PUFA-rich diets failed to shift the composition of gut microbiota at the phylum level in a 30-day treatment period, but the populations of specific genera can slightly alter in response to the feedings due to unclear mechanisms. Further studies may clarify whether there is a cause-and-effect relationship between dietary fatty type and shifts in human gut microbiota.
7.6 Acknowledgments

Authors are indebted to the late Denis Krause for the special contribution to the design of the study. Unfortunately, Denis passed away on Oct 16, 2011 when the clinical intervention was ongoing. We appreciated his ambition, intelligence and experience in this successful study. We also would like to thank Wenhua Tang for technical assistance on human fecal sample analysis. Authors also acknowledge Shucong Li, Navjot Nandha and Hooman Derakhshani for their helpful discussions and interpretation on statistics and bioinformatics. Special thanks to the study participants, clinical coordinators, and kitchen staff at RCFFN for their contributions in the clinical study.
7.7 References


Chapter VIII

Overall conclusions

8.1 Summary and implications

Results of the present study have implications for the optimal intake levels of n-9, n-6 and short-and long-chain n-3 fatty acids containing vegetable oils for management of cardiovascular disease (CVD) risk. Current recommendations for CVD risk reduction focus primarily on lipid lowering. All five low saturated fatty acid diets showed promising improvements on blood lipid profiles compared to the baseline levels. Importantly, the novel DHA-enriched high oleic canola oil (CanolaDHA) was observed with outstanding performance on blood lipids, and blood pressure thereby reducing Framingham risk scores. Moreover, although the endpoint LDL-C level was highest after CanolaDHA compared with other treatments, the percentage change from baseline was still significantly improved. Our results indicated that while dietary oils rich in n-6 and n-9 fatty acids significantly improve LDL-C levels, the contribution of DHA in the diet enhances HDL-C levels, reduces total triglycerides and decreases both diastolic and systolic blood pressure profiles over as short as a 4-wk period without increasing LDL-C levels.

Present data also provide the promising evidence that the diet-gene interaction plays an important role in modulating circulating fatty acid composition in humans, which further favorably influences whole body lipid metabolism. The elevated plasma EPA and DPA levels can be largely influenced by both the ALA rich diet and genetic variants in FADS and ELOVL gene clusters, but plasma DHA levels can be only boosted by dietary DHA intake regardless of
the genetic make-up. This is because de novo synthesis of DHA seems not to be affected by different genotypes in our cohort. Therefore, it is suggested that people with minor alleles in FADS and ELOVL gene clusters may not be able to obtain expected elevating EPA or DPA levels after consuming ALA rich diets. Instead, increasing dietary long-chain n-3 PUFA, particularly DHA intakes may be the most effective strategy for boosting circulating n-3 PUFA levels which is recommended to lower CVD risk for the general population irrespective of their genetic background.

The present study also demonstrates that circulating levels of FAEs, a group of lipid mediators derived from fatty acids, are influenced by the concentrations of their precursor fatty acids arising from dietary intakes during the 4-wk feeding period. The rs324420 A-allele in FAAH was associated with higher FAE levels and lower responsiveness of ALEA and DHEA after consuming ALA and DHA rich diets, respectively. In addition, elevated plasma DHEA levels in response to DHA intake tended to associate with lower plasma OEA levels and an increased gynoid fat mass, indicating that diet-gene interactions may further influence the body fat mass distribution. Given the growing evidence for the importance of FAEs in governing energy metabolism, genetic analysis of rs324420 might help identify sub-populations who appear to benefit from increased consumption of novel vegetable oils varying in unsaturated fatty acids, in particular ALA and DHA, for management of CVD risk.

Moreover, our investigation on gut microbiota composition in response to the five different unsaturated fatty acid oil feedings suggests that diet, metabolic profiles, obesity state, and individual response are all contributing factors to the shifts in characterization of human gastrointestinal tract but at different levels. Obesity state was a predominant factor at the phylum
level with greater Firmicutes and less Bacteroidetes in obese group compared to the combined group of normal and overweight subjects, while the impact of our healthy unsaturated oil treatments on the microbiota composition was relatively minor at the phylum level and mainly associated with microbiota shifts at the lower taxonomical levels. The human gut microbiota is a complex ecosystem and has a collective metabolic activity including helping digestion of nutrients, stimulating cell growth, creating immune response. Our data showed MUFA-rich or PUFA-rich diets can slightly alter the populations of specific genera according to unclear mechanisms. Therefore, the composition of these microorganisms reflects natural selection at both the microbial and host levels which are relevant to dietary intakes, human health status and to the extent of host-specificity.

Based on the observations of our study pertaining to the relationship between genetic make-ups and dietary responsiveness, a clear conclusion can be drawn that one-size-fits-all dietary recommendations are no longer sufficient. Personalized diet or nutrition will become the future “healthy eating” recommendation, where lifestyle, environmental factors, psychosocial factors, and genetic information are all necessary to optimize their own diets and thereby maximize health benefits.

8.2 Strengths and limitations

The completed trial represented a significant success which can be attributed to the dedication and commitment of all study participants, investigators, clinical coordinators, and supporting staff. The strengths of the study were the large subject number, the controlled full-feeding, and the crossover design. The multicenter nature of the trial ensured the robustness of results across different clinical sites. The full-feeding and crossover design eliminated the effects of variable
habitual, background diets on study outcomes and minimized the effect of individual differences on response to treatments which significantly reduced the required sample size. Also, a unique feature of the research was that we were able to compare novel oil blends that represent newest dietary oils entering the marketplace across North America. The completion of the trial provided a proof of concept for the efficacy of these oils.

One limitation of this trial was that none of the diets were designated as a control due to the fact that the objective was to compare across a series of healthy dietary oils, based on their fatty acid profiles. Inclusion of a typical Western diet which was high in saturated fat low in unsaturated fat would have been useful to establish the contrast between whether any of the treatments increased risk of adverse health consequences. However, our study design could be considered conservative as treatment oils were compared with other “healthy” oils, attempting to answer the question of which combination of fatty acids produced the optimal outcomes for CHD risk factors. In addition, requiring a sixth controlled feeding period would have made the protocol overly onerous for volunteers.

Furthermore, the study design required a strong assumption of no carry-over effects. In other words, the 4-wk duration and 4-wk washout period might not have been long enough to determine the long-term benefits of individual oil treatments. Another consideration was that the plasma fatty acid may reflect the dietary fatty acids within 4 weeks, but the fatty acid profile in tissues may have not been easily altered in response to those treatments swiftly enough.

Fortunately, such a design was made based on the many trials employing experimental diets with fatty acid profiles similar to those we evaluated in the current study. No harmful effects were expected from our treatments and no adverse effects with the diets selected presently were
observed at the end of the trial. As such, any carry-over effects from the previous treatment were minimized by the following treatment in the design.

Another point relates to the limitations of conducting subgroup analyses on body fat mass change and gut microbiota composition. Both of these measures were considered as secondary outcomes while the primary outcomes of the COMIT study were to measure the changes in endothelial function and major blood lipid responses for prevention of CVD risk. Therefore, the body weight maintenance design limited the potential changes in the fat mass distribution in different body regions. In addition, the DEXA measures required a virtual adjustment using the software by the technician who operated the scan. Even though only one licensed personnel was performing the measure at each clinical sites, the multicenter model which employed three different technicians overall increased the chance of introducing systematic errors when analyzing the body composition data across all centers. Therefore, only a subgroup analysis was carried out to test the body fat mass change in the current study. Regarding the gut microbiota profile analysis, the entire trial duration of more than 9 months was a challenge to retain subjects to collect fecal samples on all sampling days. As a result, only a small group subjects were consented to participate in the sub-study. In addition, the inclusion criteria of our study were based on the risk of metabolic syndrome, but not the obese state. Therefore, given the lower numbers of people with normal weight and overweight, the smaller sample size might not be able to fully represent the precise changes in gut microbiota community among all subjects.

8.3 Final conclusions and future directions

The successful completion of 130 participants through the study protocol of this full-feeding study represents a significant achievement. Results showed consumption of a novel DHA-
enriched high oleic canola oil improved blood lipid profile and reduced Framingham risk scores compared with other oils varying unsaturated fatty acid composition. Examinations on diet-gene interactions may help identify sub-populations who tend to benefit from increased consumption of DHA and oleic acid. Future studies investigating the genetic heterogeneity of responsiveness to dietary oil treatments might be conducted which possess a pre-screening procedure for particular genotypes with target SNPs during the recruitment so that the prospective response associated with genetic factors can be determined precisely. In addition, our data showed that the metabolic and physiological responses to dietary fatty acids may be influenced via circulating FAEs, especially the interaction between OEA and DHEA on body fat mass distribution. Further clinical studies of longer duration can be specifically designed to investigate such interaction among individual FAE compounds in response to dietary feedings varying in fatty acid composition. Also, using longer term durations will help to determine the action of altered gut microbiota profiles in terms of specific physiological effect of the dietary MUFA and/or PUFA intakes.
Appendices

Appendix I: Ethics approval for study

Principal Investigator: Dr. P. Jones
Sponsor: Canola Council of Canada/
Agriculture and Agri-Food Canada

Protocol Title: Canola and flax oils in modulation of vascular function and biomarkers of cardiovascular disease risk (Linked to B2007:071)

The following is/are approved for use:

- Protocol, Version 1 dated April 12, 2010
- Research Subject Information and Consent Form, Version 2 dated May 5, 2010
- Additional Research Subject Information and Consent Form for Genetic Analysis, Version 2 dated May 5, 2010
- Poster 1, Version 2 dated 5/6/2010
- Poster 2, Version 1 dated April 12, 2010
- Blood screening and general information sheet, Version 1 dated April 12, 2010

The above was approved by Dr. Nicholas Anthonisen, Chair, Biomedical Research Board, Bannatyne Campus, University of Manitoba on behalf of the committee per your letter dated May 5, 2010. The Research Ethics Board is organized and operates according to Heath Canada/CH Good Clinical Practices, Tri-Council Policy Statement, and the applicable laws and regulations of Manitoba. The membership of this Research Ethics Board complies with the membership requirements for Research Ethics Boards defined in Division 5 of the Food and Drug Regulations of Canada.

This approval is valid for one year from the date of the meeting at which it was reviewed. A study status report must be submitted annually and must accompany your request for re-approval. Any significant changes of the protocol and informed consent form should be reported to the Chair for consideration in advance of implementation of such changes. The REB must be notified regarding discontinuation or study closure.

This approval is for the ethics of human use only. For the logistics of performing the study, approval should be sought from the relevant institution, if required.

Sincerely yours,

[Signature]

Nicholas Anthonisen, MD, Ph.D
Chair,
Biomedical Research Ethics Board
Bannatyne Campus

Please quote the above Ethics Reference Number on all correspondence.
Inquiries should be directed to the REB Secretary Telephone: (204) 789-3254 Fax: (204) 789-3414
Appendix II: Study forms

Study advertisements

Information Package for

Nutrition Studies

Canola/Flax: Dr. Todd Rideout
Herb/Spice: Dr. Vanu Ramprasath
Plant Sterol: Mr. Dylan MacKay
Barley: Dr. Scott Harding/Ms. Yanan Wang

204-298-5483
rcffn@cc.umanitoba.ca
www.rcffn.ca
Study recruitment presentation

1/20/2010

Information & Screening Session

AGENDA
- What is the RCFRN?
- Types of Nutrition Studies
- Current Nutrition Studies
- Benefits & Expectations

Food

Health

Foods and Nutraceuticals We Are Studying

Research Question
Can you improve your cholesterol levels, arterial health and lower body fat with dietary factors?

Information Package for Clinical Studies

Schematic of Generalized Trial Design

Benefits and Expectations
- Benefits to you
  - Learn about diet and health
  - Social networking
  - Nutritional health information
  - Free groceries
  - Financial compensation
- Expectations from us
  - Dietary compliance
  - Blood draw
  - Input on improving studies

Information to Clinical Studies
- Contact: Dr. Todd Hendy
- Office: 294-390-5869
Subject consent form

RESEARCH SUBJECT INFORMATION AND CONSENT FORM

Title of Study:

Canola and flax oils in modulation of vascular function and biomarkers of cardiovascular disease risk

Investigator: Peter Jones, PhD
Richardson Centre for Functional Foods and Nutraceuticals
University of Manitoba
196 Innovation Drive, Smartpark
Winnipeg, Manitoba R3T 6C5
Phone: 204 474 9787

Sponsor: Canola Council of Canada, 400-167 Lombard Avenue, Winnipeg, Manitoba, R3B OT6

You are being asked to participate in a research study. Please take your time to review this Information and Consent Form and discuss any questions you may have with the study staff. You may take your time to make your decision about participating in this clinical trial and you may discuss it with your regular doctor, friends and family. This consent form may contain words that you do not understand. Please ask the study doctor or study staff to explain any words or information that you do not clearly understand.

The study doctor and institution are receiving professional fees and financial support to conduct this study.

Purpose of study

Although much is known about how total dietary fat consumption affects health, much less is understood about the role of different types of dietary fat in human health and disease prevention. The purpose of the study is 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 arterial function, blood fat

December 3, 2010
Version 5
Subject Consent Form: Canola and flax oils in modulation of vascular function and biomarkers of cardiovascular disease risk

content, 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.

**Study procedures**

**Pre-screening procedures**

If you agree to take part in this study, you will be asked to give a fasting (nothing to eat or drink 12 hours before the test) blood sample (approximately two teaspoons) to measure your blood fat levels and additionally biochemistry parameters. In addition, we will measure your blood pressure and waist to hip circumference. All baseline values must be normal as verified by the study physician prior to enrollment in the study and any abnormality in tests performed at screening will result in exclusion. An electrocardiogram (EKG) may be performed at the discretion of the physician in charge. Prior to beginning the study, you will undergo a physical examination by a physician to ensure that you are in good health. During the physical examination, the physician will measure your vital signs, examine the normality of body systems and ask you some questions regarding your medical history. The study physician and or study staff will review medical history and ask questions to determine whether you are eligible to participate. Any change in your health status at any point during the study needs to be reported to the study investigators.

**Study procedures**

The study will consist of 5 phases of 30 days each during which you will consume a fixed composition of a precisely controlled weight-maintaining diet. Each study phase will be followed by a washout period of 4 weeks where you can consume your habitual diets. During each study phase, you will be provided with a balanced, precisely controlled weight-maintaining diet (35% energy from fat, 50% carbohydrate, and 15% protein). Each phase will differ only in the dietary oils provided as part of the controlled diet as follows:

1) Control phase: Dietary fat consumed will provide 35% of total energy and will be comprised of a mix of *corn oil* (25%) and *safflower oil* (15%).

2) Canola oil phase: Dietary fat consumed will provide 35% of total energy and will be comprised of up to 60% *canola oil*.

3) 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.

4) DHA canola oil 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.

5) Flax oil phase: Dietary fat consumed will provide 35% of total energy and will be comprised of a mix of *flax oil* (60%) and *safflower oil* (40%).

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Subject Consent Form: Canola and flax oils in modulation of vascular function and biomarkers of cardiovascular disease risk

This study is a double-blind design, which means that neither you nor the study staff will know which oil variety you are receiving in each phase. In the unlikely event of an emergency, this information will be made available.

Study diets will be prepared in the metabolic kitchen of the RCFFN. You will consume at least 1 of 3 daily meals at the RCFFN under supervision. The other meals will be prepared and packed to be taken out. The treatment oils will be provided as a part of the meals given as appropriate for each phase. You will be asked to consume only the food provided by our metabolic kitchen during each study phase. Additionally, we ask that you do not consume alcohol or caffeinated beverages during the study periods.

We will measure the amount of fat in your body using a procedure called dual energy x-ray absorptiometry (DEXA). These analyses will be performed 10 times in total during the study, once at the beginning of each phase and once at the end of each phase. For this procedure, you will need to lie in a horizontal position for about 5-10 minutes while the scan arm passes from your head to your feet. The radiation from this test is very low dosage (equivalent to approximately 1 day of natural background radiation). The dosage is 1000 times less than the limit for trivial exposure. You will be asked not to wear anything metal (metal may affect bone density values which will affect body composition calculations). In addition, you will need to ensure that you will not undergo barium tests/exams, or a nuclear medicine scan or injection with an x-ray dye within two weeks prior to your DEXA scan. If you are female and are not post-menopausal you will be asked to take a pregnancy test prior to beginning the study and subsequently before each DEXA scan.

To measure abdominal and visceral fat, you may be asked to undergo a magnetic resonance imaging (MRI) scan prior to the initiation of the study. The MRI will be performed at National Research Council Institute of Biodiagnostics or at St. Boniface General Hospital Research Centre under the direction of trained technical staff. MRI is a medical imaging technique that uses a powerful magnetic field to visualize detailed internal structure of the body. MRI is not painful, does not use radiation, and does not pose any known biological hazards to humans.

During the first and fourth week of each dietary phase, you will undergo a fingertip test to measure the health of your endothelial cells (the cells that line your blood vessels). The test, known as EndoPat, is a standard system used by health practitioners worldwide to measure blood flow through your fingertip as an index of endothelial function and early heart disease risk. The test takes approximately 15 minutes to complete, is easy to perform and is non-invasive.

During days 1, 2, 28, 29 and 30 of each four-week test diet phase, fasting blood samples (approximately 8 teaspoons) will be obtained for assessment of blood fat, fatty acid profile and other CVD biomarkers including insulin glucose concentrations and inflammatory markers, oxidative stress markers and markers of adiposity. On day 28, you will be required to consume three tablespoons of tagged water (known as deuterium). The movement of these tagged materials will permit assessment the quantity of long chain

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Subject initials_____
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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.

At the end of each phase, a fecal sample will be collected using a take home stool collection kit to assess changes in intestinal bacterial populations in response to the dietary oils.

Each blood draw will take approximately 5 minutes. The total amount of blood drawn during each phase of the study will be approximately 10 tablespoons. The total blood volume required for this trial will be approximately 4 cups.

Risks and discomforts
As with any clinical trial, there may be as yet unknown or unforeseen risks of taking part.

The dietary oils contained within the meals at the proposed level has been shown to have no known direct negative side effects on health in several dozen existing animal and human experiments. Some known risks, although rare, are associated with placing a needle into a vein. These include the possibility of infection, perforation or penetration of the needle through the vein, and bleeding, pain, or bruising at the site. In case you feel any discomfort during the experimental trial a physician, Dr. Kesselman, will be available to contact at any time. Dr. Kesselman can be reached at 204 954 4486.

Benefits
You may not benefit from participation in this research; however, the study should contribute to a better understanding of the effects of dietary oils on blood fat levels as well as cardiovascular diseases (CVD) biomarkers. You will also receive access to your test results when they become available.

Costs
All clinic and professional fees, diagnostic and laboratory tests that will be performed as part of this study are provided at no cost to you. There will be no cost for the study treatment that you will receive.

Participation for participation
You will receive up to a maximum of $1,250.00 for your time and inconvenience of the study schedule. This amount will be provided as $150.00 remunerated at the end of each phase and an additional $650.00 provided upon completion of the 5th and final phase. If you withdraw early from the study, you will receive an appropriate pro-rated fraction of this amount.

Alternatives
You are not obligated to participate in this study. The study coordinators, physician and principal investigator will answer any questions you have about the experimental group of this study.

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Subject initials_____
Confidentiality
Medical records that contain your identity will be treated as confidential in accordance with the Personal Health Information Act of Manitoba. The RCFFN staff involved with your care may review/copy medical information that may reveal your identity. With your permission, the study doctor will also write to your Family Doctor to tell him/her that you are taking part in a study or to obtain further medical information. The Biomedical Research Ethics Board at the University of Manitoba and National Research Council Research Ethics Board may also review your research-related records for quality assurance purposes. If you are a research subject from National Research Council Institute of Biodiagnostics or St. Boniface General Hospital, your research related records may be reviewed by National Research Council Institute of Biodiagnostics or St. Boniface General Hospital for quality assurance purposes. Other agencies that may review your research related records for quality assurance and data analysis include; University of Toronto, Ontario, University of Laval, Quebec, Pennsylvania State University, Pennsylvania, United States, Canola Council of Canada and Agriculture and Agri-foods Canada. But these agencies will not be able to link your research related data with your personal information. If the results of the trial are published, your identity will remain confidential. Personal information such as your name, address, telephone number and/or any other identifying information will not leave the Richardson Centre for Functional Foods and Nutraceuticals.

Study samples will be stored in a locked freezer at the RCFFN. Only the study coordinators and the principal investigator will have access to the samples. Your samples will not be used for any additional analyses, nor stored for any longer than 5 years, nor shared with any other group, other than is indicated in the protocol, without your specific consent.

Voluntary participation/withdrawal from the study
Your decision to take part in this study is voluntary. You may refuse to participate or you may withdraw from the study at any time. Your decision to not participate or to withdraw from the study will not affect your other medical care.

Your participation in this study may be terminated without your consent by the study coordinators, physician or principal investigator. The study staff will withdraw you if he/she feels that participation is no longer in your best interest, or if you fail to follow the directions of the study staff.

If you decide to participate, you will agree to cooperate fully with the study visit schedule, and will follow the study staff’s instructions.

We will tell you about new information that may affect your health, welfare, or willingness to stay in this study.

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Subject initials______
Subject Consent Form: Canola and flax oils in modulation of vascular function and biomarkers of cardiovascular disease risk

Should you wish to withdraw your participation from the study, you must inform the study coordinators so that your file can be officially closed.

**Medical care for injury related to study**
In the event of an injury that occurs to you as a direct result of participating in this study, or undergoing study procedures you should immediately notify the study physician, Dr. Kesselman at 204 954 4486 or go to your nearest emergency room to receive necessary medical treatment. You are not waiving any of your legal rights by signing this consent form nor releasing the investigator or the sponsor from their legal and professional responsibilities. If any health abnormalities are identified in the clinical tests conducted during this experiment, Dr. Kesselman will be contacted, who will inform you of the results.

**Questions**
You are free to ask any questions that you may have about your treatment and your rights as a research subject. If any questions come up during or after the study or if you have a research-related injury, contact the study doctor and the study staff.

<table>
<thead>
<tr>
<th>Investigator:</th>
<th>Dr. Peter Jones</th>
<th>Tel No.</th>
<th>204 474 9787</th>
</tr>
</thead>
<tbody>
<tr>
<td>Co-Investigator:</td>
<td>Dr. Todd Rideout</td>
<td>Tel No.</td>
<td>204 480 1042</td>
</tr>
<tr>
<td>Study Physician:</td>
<td>Dr. Edward Kesselman</td>
<td>Tel No.</td>
<td>204 954 4486</td>
</tr>
</tbody>
</table>

For questions about your rights as a research subject, you may contact:

The Biomedical Research Ethics Board, University of Manitoba at 789-3389

Do not sign this consent form unless you have a chance to ask questions and have received satisfactory answers to all of your questions.

**Consent**
I agree to allow the study doctor to inform my family doctor that I am participating in this study or to obtain information regarding my medical history.

Yes ☐ No ☐

1. I have read and understood this Information and Consent Form, and I freely and voluntarily agree to take part in the clinical trial (research study) described above.

2. I understand that I will be given a copy of the signed and dated Information and Consent Form. I have received an explanation of the purpose and duration of the trial, and the potential risks and benefits that I might expect. I was given sufficient time and opportunity to ask questions and to reflect back my understanding of the study to study personnel. My questions were answered to my satisfaction.

3. I agree to cooperate fully with the study doctor and will tell him if I experience any side effects, symptoms or changes in my health.

December 3, 2010 Version 5
Subject initials______
Subject Consent Form: Canola and flax oils in modulation of vascular function and biomarkers of cardiovascular disease risk

4. I am free to withdraw from the study at any time, for any reason, and without prejudice to my future medical treatment.

5. I have been assured that my name, address and telephone number will be kept confidential to the extent permitted by applicable laws and/or regulations.

6. By signing and dating this document, I am aware that none of my legal rights are being waived.

Signature: ___________________________ Date/Time: _____________

Printed name of above: _____________________________________________

I confirm that I have explained the purpose, duration etc of this clinical trial, as well as any potential risks and benefits, to the subject whose name and signature appears above. I confirm that I believe that the subject has understood and has knowingly given their consent to participate by his/her personally dated signature.

Signature: ___________________________ Date/Time: _____________

Printed name of above: ___________________________ Study role: _____________

ALL SIGNATORIES MUST DATE THEIR OWN SIGNATURE

December 3, 2010 Version 5
Subject initials______
Subject genetic consent form

ADDITIONAL RESEARCH SUBJECT INFORMATION AND CONSENT FORM FOR GENETICS ANALYSIS

Title of Study:
Canola and flax oils in modulation of vascular function and biomarkers of cardiovascular disease risk

Investigator: Peter Jones, PhD
Richardson Centre Functional Foods and Nutraceuticals
University of Manitoba
196 Innovation Drive, Smartpark
Winnipeg, Manitoba R3T 6C5
Phone: (204) 474-9787

Sponsor: Canola Council of Canada, 400-167 Lombard Avenue, Winnipeg, Manitoba, R3B 0T6

You are being asked to participate in a research study. Please take your time to review this Information and Consent Form and discuss any questions you may have with the study staff. You may take your time to make your decision about participating in this clinical trial and you may discuss it with your regular doctor, friends and family. This consent form may contain words that you do not understand. Please ask the study doctor or study staff to explain any words or information that you do not clearly understand.

The study doctor and institution are receiving professional fees and financial support to conduct this study.

Nature and duration of procedure
From the blood drawn during the clinical study as outlined in the Research Subject Information and Consent Form, we would like to extract DNA and perform a genetic analyses using a laboratory technique that augments and recognizes specific genes to determine how your genetic makeup influences the efficiency of your body in converting dietary oil-derived fatty acids into longer chain fatty acids that are known to have health benefits. DNA is a molecule found in the

May 5, 2010
Version 2
cells of your body that is organized into genes that contain all of the information needed to make
the proteins that perform specific biological functions in your body.

Confidentiality and safekeeping of DNA samples
All of the information obtained about you and the results of the research will be treated
confidentially. We will protect your confidentiality by assigning your DNA sample a specific
code. This code will link you to your DNA sample and can only be decoded by the principal
researcher or an individual authorized by the latter. Samples of your DNA will be kept at the
Richardson Centre for Functional Foods and Nutraceuticals, University of Manitoba, under the
supervision of Dr. Peter Jones for a 5-year period following the end of the research project. After
this time, all samples will be destroyed. Your DNA samples will only be used for the purpose of
this research project.

Your participation and the results of the research will not appear in your medical record.
Although the results of this study may be published or communicated in other ways, it will be
impossible to identify you. Unless you have provided specific authorization or where the law
permits or a court order has been obtained, your personal results will not be made available to
third parties such as employers, government organizations, insurance companies, or educational
institutions. This also applies to your spouse, other members of your family and your physician.
However, for the purposes of ensuring the proper management of research, it is possible that a
member of an ethics committee, a Health Canada representative, or a representative from the
Richardson Center for Functional Foods and Nutraceuticals may consult your research data and
record. You can communicate with the research team to obtain information on the general
progress or the results of the research project. Project updates will be mailed at the end of the
project. However, we will not communicate any individual results to you.

Potential risks and/or benefits
Some known risks, although rare, are associated with placing a needle into a vein. These include
the possibility of infection, perforation or penetration of the needle through the vein, and
bleeding, pain, or bruising at the site. In case you feel any discomfort during the experimental
trial a physician, Dr. Kesselman, will be available to contact at any time. Dr. Kesselman can be
reached at 204 954 4486.

While there may be no direct benefits to you for taking part in these additional analyses, we hope
that these results will provide us with the information on genetic characteristics of people in the
which intake of plant sterols provided an enhanced or inferior cholesterol-lowering therapy.

Signature of participant
The content of the procedure and procedures associated with it have been fully explained to me,
and all experimental procedures have been identified. I have had the opportunity to ask
questions concerning any and all aspects of the project and procedures involved, and may
continue in the future to ask further questions at any time, as it is my right to do so. I am aware
that I may refuse to participate as well as withdraw my consent at any time. I acknowledge that
no guarantee or assurance has been given by anyone as to the results to be obtained and that my
participation in this study is completely voluntary. Confidentiality of records concerning my
ADDITIONAL RESEARCH SUBJECT INFORMATION AND CONSENT FORM FOR GENETIC ANALYSIS

involvement in this project will be maintained in an appropriate manner. Samples will not be utilized for any additional analyses, nor stored for any prolonged period, nor shared with any other group, other than is indicated in the protocol, without my specific consent.

I, ________________________, have read the above description. I have been made aware of all the procedures, advantages and disadvantages of the study, which have been explained to me.

Signature of Subject ____________________________ Date __________

I confirm that I have explained the purpose, duration etc of this clinical trial, as well as any potential risks and benefits, to the subject whose name and signature appears above. I confirm that I believe that the subject has understood and has knowingly given their consent to participate by his/her personally dated signature.

Signature: ____________________________ Date/Time: __________

Printed name of above: ____________________________ Study role: __________

5/6/2010
Version 2
Subject psychosocial consent form

ADDITIONAL RESEARCH SUBJECT INFORMATION AND CONSENT FORM FOR PSYCHOSOCIAL ANALYSIS

Title of Study:
Canola and flax oils in modulation of vascular function and biomarkers of cardiovascular disease risk

Investigator: Peter Jones, PhD
Richardson Centre Functional Foods and Nutraceuticals
University of Manitoba
196 Innovation Drive, Smartpark
Winnipeg, Manitoba R3T 6C5
Phone: (204) 474-9787

Sponsor: Canola Council of Canada, 400-167 Lombard Avenue, Winnipeg, Manitoba, R3B OT6

You are being asked to participate in a research study. Please take your time to review this Information and Consent Form and discuss any questions you may have with the study staff. The completion of the psychosocial questionnaires is optional and is not required in order to participate in the main study. You may take your time to make your decision about participating in this clinical trial and you may discuss it with your regular doctor, friends and family. This consent form may contain words that you do not understand. Please ask the study doctor or study staff to explain any words or information that you do not clearly understand.

The study doctor and institution are receiving professional fees and financial support to conduct this study.

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Version 1

____________________
Initials
ADDITIONAL RESEARCH SUBJECT INFORMATION AND CONSENT FORM FOR GENETIC ANALYSIS

Purpose of study

A wide variety of psychosocial factors have long been known to influence coronary heart disease (CHD) risk independent of biological risk factors. Our primary goal in this sub study is to look at psychosocial predictors of vascular function. The endothelial function, which is a measure of vascular function is the major endpoint measured in the main study. Therefore, the addition of psychosocial measures to the study will help correlate the psychosocial parameters and vascular function at minimal extra cost. Additional benefit would be the opportunity to examine whether dietary oils contribute to psychosocial well being. Some literature related to fish oil and psychosocial parameters already exist however, there is a dearth of literature in relation to canola and flax oils. Therefore, we propose the addition of psychosocial measures to the study: Canola and flax oils in modulation of vascular function and biomarkers of cardiovascular disease in order to better understand

• the psychosocial correlates that best predict Coronary Heart Disease risk factors, which serve as main endpoints in this study, and

• if the relationship between CHD risk factors and psychosocial determines changes based on consumption of differing oils (e.g. some research suggests that omega fatty acids can reduce both depressive scores and CHD risk factors).

Nature and duration of procedure

You will be asked to complete approximately 15 minutes of questionnaires regarding your immediate mood and recent sleep on 6 test days: one prior to starting the study and five during the last 3–4 days of each diet (these are the same test days you will receive vascular function [EndoPAT] tests). In addition, you will be asked to complete a set of questionnaires regarding your overall mood, social support and behaviors. You will complete this set of questions only once during the study. Completing these questions is estimated to take 30 minutes.

Confidentiality and safekeeping of psychosocial data

All of the information obtained about you and the results of the research will be treated confidentially. However, in spite of confidentiality measures, absolute confidentiality cannot be guaranteed. We will protect your confidentiality by assigning your questionnaire data a specific code. This code will link you to your data and can only be decoded by the principal researcher or an individual authorized by the latter. De-identified psychosocial data will be kept at the Richardson Centre for Functional Foods and Nutraceuticals, University of Manitoba, under the supervision of Dr. Peter Jones for a 5-year period following the end of the research project. After this time, all the data will be destroyed. Your psychosocial data will only be used for the purpose of this research project.

Your name, address and telephone number will remain inside the Richardson Centre for Functional Foods and Nutraceuticals and will not be disclosed to third Parties. Your de-identified data will be shared with Dr. Sheila West of the Pennsylvania State University, USA for the

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Initials
ADDITIONAL RESEARCH SUBJECT INFORMATION AND CONSENT FORM FOR GENETIC ANALYSIS

purpose of psychosocial analysis. Dr. Sheila West is a collaborating scientist in this study and she has a special expertise in psychosocial analysis. In an effort to easily transfer the survey data across sites Central Desktop (CD), an online project management site, also will be used as a means of storing psychosocial data. Specifically, a single confidential and password protected workspace will be designated to this study. The proposed surveys will be administered to participants in paper-pencil format. The survey will not contain identifiable information (such as name, date of birth, etc.). Only the specific study code will be written on each survey. Once complete the surveys will be uploaded onto CD in PDF format. No code break information will be stored with the data.

Additional notes regarding confidentiality of your data

Your participation and the results of the research will not appear in your medical record. Although the results of this study may be published or communicated in other ways, it will be impossible to identify you. Unless you have provided specific authorization or where the law permits or a court order has been obtained, your personal results will not be made available to third parties such as employers, government organizations, insurance companies, or educational institutions. This also applies to your spouse, other members of your family and your physician. However, for the purposes of ensuring the proper management of research, it is possible that a member of an ethics committee, a Health Canada representative, or a representative from the Richardson Center for Functional Foods and Nutraceuticals may consult your research data and record. You can communicate with the research team to obtain information on the general progress or the results of the research project. However, we will not communicate any individual results to you.

Risks and discomforts
Some of the questions you will be asked to answer are personal and might cause discomfort. Answering questions about recent stressful life events or recent mood may bring to mind troubling events or feelings. If you wish to seek assistance for troubling thoughts, stress, or negative emotion as the result of participating in the research are encouraged to contact the experimental trial physician, Dr. Kesselman, who will be available to contact at any time. Dr. Kesselman can be reached at 204 954 4486.

Benefits
While there may be no direct benefits to you for taking part in these additional analyses, we hope that these results will provide us with the information on psychosocial characteristics that affect the vascular function and whether the treatment oils can influence these psychosocial characteristics.

Voluntary participation/withdrawal from the study
Your decision to take part in this study is voluntary. You may refuse to participate or you may withdraw from the study at any time. Your decision to not participate or to withdraw from the study will not affect your other medical care.

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Initials
ADDITIONAL RESEARCH SUBJECT INFORMATION AND CONSENT FORM FOR GENETIC ANALYSIS

Your participation in this study may be terminated without your consent by the study coordinators, physician or principal investigator. The study staff will withdraw you if he/she feels that participation is no longer in your best interest, or if you fail to follow the directions of the study staff.

If you decide to participate, you will agree to cooperate fully with the study visit schedule, and will follow the study staff’s Instructions.

We will tell you about new information that may affect your health, welfare, or willingness to stay in this study.

Should you wish to withdraw your participation from the study, you must inform the study coordinators so that your file can be officially closed.

Questions
You are free to ask any questions that you may have about your treatment and your rights as a research subject. If any questions come up during or after the study or if you have a research-related injury, contact the study doctor and the study staff.

| Investigator: | Dr. Peter Jones | Tel No. | 204 474 9787 |
| Study Physician: | Dr. Edward Kesselman | Tel No. | 204 954 4486 |

For questions about your rights as a research subject, you may contact:

The Biomedical Research Ethics Board, University of Manitoba at 789-3389

Do not sign this consent form unless you have a chance to ask questions and have received satisfactory answers to all of your questions.

Signature of participant
The content of the procedure and procedures associated with it have been fully explained to me, and all experimental procedures have been identified. I have had the opportunity to ask questions concerning any and all aspects of the project and procedures involved, and may continue in the future to ask further questions at any time, as it is my right to do so. I am aware that I may refuse to participate as well as withdraw my consent at any time. I acknowledge that no guarantee or assurance has been given by anyone as to the results to be obtained and that my participation in this study is completely voluntary. Confidentiality of records concerning my involvement in this project will be maintained in an appropriate manner. The data obtained will not be utilized for any additional analyses, nor stored for any prolonged period, nor shared with any other group, other than is indicated in the protocol, without my specific consent. By signing and dating this document, I am aware that none of my legal rights are being waived.

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Version 1

Initials
ADDITIONAL RESEARCH SUBJECT INFORMATION AND CONSENT FORM FOR GENETIC ANALYSIS

I, ______________________, have read the above description. I have been made aware of all the procedures, advantages and disadvantages of the study, which have been explained to me.

_________________________________________  ______________________
Signature of Subject                          Date

I confirm that I have explained the purpose, duration etc of this clinical trial, as well as any potential risks and benefits, to the subject whose name and signature appears above. I confirm that I believe that the subject has understood and has knowingly given their consent to participate by his/her personally dated signature.

Signature: ___________________________________  Date/Time: ____________

Printed name of above: ________________________  Study role: ______________

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Version 1

Initials  5
RCFFN preliminary trial screening consent

You have expressed an interest in participating in a study at the Richardson Center for Functional Foods and Nutraceuticals. You have been invited to have your health assessed to determine if you meet the requirements of the study.

The clinical coordinator team will assess your cholesterol level, medical history, body measurements, and your availability over the next 2 years. Depending on your results you will be offered the opportunity to participate in a study.

To allow the necessary information to be obtained, you agree to provide fasting blood samples (approximately 10 ml or 2 teaspoons) for the measurement of blood cholesterol, blood count, and iron levels.

Prior to taking part in any study, you will be given the specific study consent form to read and sign if you are still interested in participating.

The blood is taken from a vein in the forearm, as is usually done during a blood test. Some known risks, although rare, are associated with placing a needle into a vein. These include the possibility of infection, perforation or penetration of the needle through the vein, and bleeding, pain, or bruising at the site.

I understand I can withdraw from this process at any time at my discretion.

Participant’s Signature  
Participant’s Name (please print)  
Date

Investigator’s Signature  
(or Clinical Coordinator)  
Investigator’s Name (please print)  
(position)  
Date

Page 1 of 1
08/17/2010
**Blood sign-up sheet**

**Screening Sign-up Sheet – Wednesday, January 19**

<table>
<thead>
<tr>
<th>Time</th>
<th>Name</th>
<th>Phone</th>
<th>Email</th>
</tr>
</thead>
<tbody>
<tr>
<td>7:30</td>
<td></td>
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<tr>
<td>7:40</td>
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<tr>
<td>7:50</td>
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<td>8:00</td>
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<td>8:20</td>
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<td>9:10</td>
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<tr>
<td>9:20</td>
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<td></td>
<td></td>
</tr>
</tbody>
</table>
COMIT

Blood Draw – January 10, 2011

Instructions

2 X 6ml serum tubes (red/grey) – invert 5 times, leave at room temp

2 X 6 ml plasma EDTA (purple top) - invert 8 times, *place on ice*

2 X 4 ml plasma heparin (green top) – invert 8 times, *place on ice*

15 Subjects:

<table>
<thead>
<tr>
<th>Subject code</th>
<th>Check when done</th>
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<tbody>
<tr>
<td>MD109</td>
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<tr>
<td>JG119</td>
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<tr>
<td>NG121</td>
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</tr>
<tr>
<td>CA122</td>
<td></td>
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<tr>
<td>WW123</td>
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<td>MQ125</td>
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<td>MO127</td>
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<td>SR129</td>
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<td>DK124</td>
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<td>PD130</td>
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<td>ND131</td>
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<td>EE132</td>
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<td>NG133</td>
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<tr>
<td>MB134</td>
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</table>

THANKS!!
Weight monitoring recording form

COMIT Study
Subject Code: SM:101
Phase: 2

<table>
<thead>
<tr>
<th>Date</th>
<th>Weight (Lbs)</th>
<th>Date</th>
<th>Weight (Lbs)</th>
</tr>
</thead>
<tbody>
<tr>
<td>10-Feb</td>
<td></td>
<td>25-Feb</td>
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</tr>
<tr>
<td>11-Feb</td>
<td>X</td>
<td>26-Feb</td>
<td>X</td>
</tr>
<tr>
<td>12-Feb</td>
<td>X</td>
<td>27-Feb</td>
<td>X</td>
</tr>
<tr>
<td>13-Feb</td>
<td>X</td>
<td>28-Feb</td>
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</tr>
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<td>14-Feb</td>
<td></td>
<td>1-Mar</td>
<td></td>
</tr>
<tr>
<td>15-Feb</td>
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<td>2-Mar</td>
<td></td>
</tr>
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<td>16-Feb</td>
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<td>3-Mar</td>
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</tr>
<tr>
<td>17-Feb</td>
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<td>4-Mar</td>
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</tr>
<tr>
<td>18-Feb</td>
<td></td>
<td>5-Mar</td>
<td>X</td>
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<tr>
<td>19-Feb</td>
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<td>6-Mar</td>
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</tr>
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<td>20-Feb</td>
<td>X</td>
<td>7-Mar</td>
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<td>21-Feb</td>
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<td>8-Mar</td>
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</tr>
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<td>22-Feb</td>
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</tr>
<tr>
<td>24-Feb</td>
<td></td>
<td>11-Mar</td>
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</table>
Medical examination form

Canola and flax oils in modulation of vascular function and biomarkers of cardiovascular disease risk - 2010
Medical Examination Form

<table>
<thead>
<tr>
<th>Phase</th>
<th>Pre-study</th>
<th>Study Physician</th>
<th>Study Physician</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Dr. Edward Kesselman</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Date of Visit</th>
<th>Investigator</th>
<th>Subject Code</th>
</tr>
</thead>
<tbody>
<tr>
<td>MM DD YR</td>
<td>Dr. Peter Jones</td>
<td>WW:123-CO</td>
</tr>
</tbody>
</table>

**COMPLETE PHYSICAL EXAMINATION**

**A. Vital Signs**

<table>
<thead>
<tr>
<th>Body Weight:</th>
<th>67.4</th>
<th>kg</th>
</tr>
</thead>
<tbody>
<tr>
<td>Height:</td>
<td>158.0</td>
<td>cm</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Respiration:</th>
<th></th>
</tr>
</thead>
</table>

<table>
<thead>
<tr>
<th>Blood Pressure (seated):</th>
<th>Heart Rate:</th>
</tr>
</thead>
<tbody>
<tr>
<td>systolic</td>
<td>diastolic</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Race/Ethnic Origin:</th>
</tr>
</thead>
<tbody>
<tr>
<td>Caucasian</td>
</tr>
</tbody>
</table>

**B. Body Systems**

(Check the appropriate box if organ system was examined. If not done, write N/D in the box)

<table>
<thead>
<tr>
<th>Normal</th>
<th>Abnormal</th>
<th>*Details of abnormal finding</th>
</tr>
</thead>
</table>

1) Ears, Nose, Throat
2) Eyes
3) Dermatological
4) Musculoskeletal
5) Lymph Nodes
6) Neurological
7) Cardiovascular
8) Respiratory
9) Endocrine
10) Urogenital
11) Gastrointestinal (complete section C)

**C. Gastrointestinal Cont...**

<table>
<thead>
<tr>
<th>Bowel Habits:</th>
<th>Frequency</th>
<th>Urination:</th>
<th>Frequency</th>
</tr>
</thead>
<tbody>
<tr>
<td>Consistency</td>
<td>/Day</td>
<td>Nocturia</td>
<td>/Night</td>
</tr>
</tbody>
</table>
Canola and flax oils in modulation of vascular function and biomarkers of cardiovascular disease risk 2010
Medical Examination Form

<table>
<thead>
<tr>
<th>Medications:</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Hospitalizations:</td>
<td></td>
</tr>
<tr>
<td>Family History:</td>
<td></td>
</tr>
</tbody>
</table>

### D. Medical History

<table>
<thead>
<tr>
<th>Question</th>
<th>YES</th>
<th>NO</th>
</tr>
</thead>
<tbody>
<tr>
<td>Have you taken a glucose lowering medication or a medication affecting lipid metabolism (cholestryamine, colestipol, niacin, colchicine, gemfibrozil, probucol, HMG-CoA reductase inhibitors, and high-dose dietary supplements, plant sterols or fish oil capsules) within the past 3 months?</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Do you take systemic aspirin, NSAIDS, antibodies, corticosteroids, androgens or phenytoin within the past 3 months?</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Are you on anticoagulant therapy?</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Do you smoke?</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Do you consume large amounts of alcohol? (more than 2 drinks per day or 12 drinks per week)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Do you follow a specific diet?</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Do you have major food allergy?</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Do you have lactose intolerance?</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Have you had major surgery in the last 6 months?</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Do you have diabetes mellitus?</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Do you have kidney disease?</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Do you have liver disease?</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Do you have heart disease?</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Do you have gastrointestinal, pancreatitis or biliary disease (onset within past three months)?</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Canola and flax oils in modulation of vascular function and biomarkers of cardiovascular disease risk - 2010
Medical Examination Form

<table>
<thead>
<tr>
<th>Question</th>
<th>Answer</th>
</tr>
</thead>
<tbody>
<tr>
<td>Have you had cancer? If yes, occurrence of therapy within past 1 year?</td>
<td></td>
</tr>
<tr>
<td>Do you have anemia, bleeding disorder or significant blood loss/donation?</td>
<td></td>
</tr>
<tr>
<td>Do you have uncontrolled thyroid disease or hypertension? (Subject will be accepted if she is on a stable dose of a thyroid or blood pressure medication that has no known effects on blood lipid metabolism.)</td>
<td></td>
</tr>
<tr>
<td>Do you have a history of eating disorders?</td>
<td></td>
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</table>

E. Additional Physician Notes

Based on the medical examination and medical history, is the subject eligible to participate in the study protocol (circle one):

<table>
<thead>
<tr>
<th>YES</th>
<th>NO</th>
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Physician’s Signature: ________________________________

Date: ________________________________
### COMIT Study Schedule, Phase

<table>
<thead>
<tr>
<th>Sun</th>
<th>Mon</th>
<th>Tue</th>
<th>Wed</th>
<th>Thurs</th>
<th>Fri</th>
<th>Sat</th>
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<td>1</td>
<td>2</td>
<td>3</td>
<td>4</td>
<td>5</td>
<td>6</td>
</tr>
<tr>
<td></td>
<td>• Breakfast at RCFFN</td>
<td>• No alcohol</td>
<td>• 12 h fast</td>
<td>• Breakfast at RCFFN</td>
<td>• Blood draw</td>
<td>• Breakfast at RCFFN</td>
</tr>
<tr>
<td>7</td>
<td>8</td>
<td>9</td>
<td>10</td>
<td>11</td>
<td>12</td>
<td>13</td>
</tr>
<tr>
<td>• Breakfast at RCFFN</td>
<td>• Breakfast at RCFFN</td>
<td>• Breakfast at RCFFN</td>
<td>• Breakfast at RCFFN</td>
<td>• Breakfast at RCFFN</td>
<td>• Breakfast at RCFFN</td>
<td>• Breakfast at RCFFN</td>
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<td>16</td>
<td>17</td>
<td>18</td>
<td>19</td>
<td>20</td>
</tr>
<tr>
<td>• Breakfast at RCFFN</td>
<td>• Breakfast at RCFFN</td>
<td>• Breakfast at RCFFN</td>
<td>• Breakfast at RCFFN</td>
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<td>22</td>
<td>23</td>
<td>24</td>
<td>25</td>
<td>26</td>
<td>27</td>
</tr>
<tr>
<td>• Breakfast at RCFFN</td>
<td>• Breakfast at RCFFN</td>
<td>• Breakfast at RCFFN</td>
<td>• Breakfast at RCFFN</td>
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</tr>
<tr>
<td>28</td>
<td>29</td>
<td>30</td>
<td>1</td>
<td>2</td>
<td>3</td>
<td>4</td>
</tr>
<tr>
<td>• Breakfast at RCFFN</td>
<td>Breakfast at RCFFN</td>
<td></td>
<td></td>
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<td></td>
<td></td>
</tr>
</tbody>
</table>
COMIT Study: Instructions for day 1 & 2 (Phase 2)

**Monday, October 18**

12 hour overnight fasting

**STEP 1 (6:15am)**
Enter from front door and proceed to room on right

**STEP 2**
Meet Meriam for screening forms and weight, height, waist/hip & BP measurements.

**STEP 3**
Meet Vijitha for EndoPat in main office area

**STEP 4**
Proceed back to DXA room for DXA scan by Meriam

**STEP 5**
Proceed to 2nd level for blood sample

**STEP 6**
Meet Hanja/Maggie in cafeteria for labelled water & breakfast

**Tuesday, October 19**

12 hour overnight fasting

**STEP 1**
Proceed to 2nd level for blood draw

**STEP 2**
Complimentary breakfast
Phase 1 Complete
Sampling collection form

COMIT DATA COLLECTION AND MONITORING FORMS

Subject Code: __________ Phase: _____

Subject Code __________
Study Phase __________ Treatment ______
Start Date ________ End Date ________

SECTION 1: START AND END WEIGHT
Day 1: Weight (lbs) ________ Staff Initials ________
Day 2: Weight (lbs) ________ Staff Initials ________
Day 29: Weight (lbs) ________ Staff Initials ________
Day 30: Weight (lbs) ________ Staff Initials ________

SECTION 2: WAIST CIRCUMFERENCE
Day 1: First measurement (cm) ________ Second measurement (cm) ________
Day 30: First measurement (cm) ________ Second measurement (cm) ________

SECTION 3: SEATED BLOOD PRESSURE

Has the subject consumed caffeine, used any medication or eaten any food in the last 12 hours and/or exercised in the past 2 hours? □ Yes □ No
If yes, subject needs to be rescheduled for a blood pressure measurement.

Instructions: Apply cuff to left dominant. After applying the cuff, the subject must be quiet and remain continuously seated without legs crossed for 5 minutes. Instruct the subject not to talk during the reading. Wait 1 minute after each reading before taking the next reading.

Day 1 or 2:

a. First blood pressure measurement: __ __ / __ __ (SBP/DBP)
b. Second blood pressure measurement: __ __ / __ __
c. Third blood pressure measurement: __ __ / __ __
d. Average (2nd and 3rd) blood pressure measurement: __ __ / __ __
COMIT DATA COLLECTION AND MONITORING FORMS

Day 29 or 30:

a. First blood pressure measurement: ___ ___ / ___ ___ (SBP/DBP)
b. Second blood pressure measurement: ___ ___ / ___ ___
c. Third blood pressure measurement: ___ ___ / ___ ___
d. Average (2nd and 3rd) blood pressure measurement: ___ ___ / ___ ___

SECTION 4: BLOOD COLLECTION

DAY 1

Date (DD/MMM/YY) _________  Time (HH:MM) _________

Did the subject fast? Yes ___ / No ___  Feeling Ill?  ________________

Caffeine in last 24hrs? Yes ___ / No ___  If yes, when ____________

Alcohol in last 24hrs? Yes ___ / No ___  If yes, when ____________

Isotope tracer (13C-ALA) provided?*** _____  **Only required on day 28 of each phase

Arm   Right ___ / Left___

Collect:  
2 X 6 ml serum (red/grey SST tube) ___ (day 1, 2, 29, 30)
2 X 6 ml plasma heparin (green top) ___ (day 1, 2, 29, 30)
2 X 6 ml plasma EDTA (purple top) ___ (day 1, 2, 29, 30)
1 X 2.5 ml whole blood (Paxgene tube) ___ (day 29, 30)

Have all blood samples been obtained? Yes ___ / No** ___

** If No, please state reasons why: ________________________________

Blood Draw: Excellent ___  Good ___  Difficult ___  Very Difficult ___

Comments: _______________________________________________________

Blood Taker: ______________________

Subject Code: __________  Phase: ___
DAY 2

Date (DD/MMM/YY) __________  Time (HH:MM) __________

Did the subject fast? Yes ___ / No ___  Feeling ill? ________________

Caffeine in last 24hrs?  Yes ___ / No ___  If yes, when __________

Alcohol in last 24hrs?  Yes ___ / No ___  If yes, when __________

Isotope tracer (^13C-ALA) provided?** ______  **Only required on day 28 of each phase

Arm  Right ___ / Left ___

Collect:  
2 X 6 ml serum (red/grey SST tube)  ___ (day 1, 2, 29, 30)
2 X 6 ml plasma heparin (green top)  ___ (day 1, 2, 29, 30)
2 X 6 ml plasma EDTA (purple top)  ___ (day 1, 2, 29, 30)
1 X 2.5 ml whole blood (Paxgene tube)  ___ (day 29, 30)

Have all blood samples been obtained?  Yes ___ / No** ___

** If No, please state reasons why: _______________________________

Blood Draw:  Excellent ___  Good ___  Difficult ___  Very Difficult ___

Comments: ______________________________________________________

Blood Taker: ____________________
DAY 29

Date (DD/MM/YY) __________  Time (HH:MM) __________

Did the subject fast? Yes ___ / No ___  Feeling ill? ______________

Caffeine in last 24hrs? Yes ___ / No ___  If yes, when___________

Alcohol in last 24hrs? Yes ___ / No ___  If yes, when___________

Isotope tracer (^{13}C-ALA) provided?** _____  **Only required on day 28 of each phase

Arm  Right ___ / Left___

Collect:  
2 X 6 ml serum (red/grey SST tube) ___ (day 1, 2, 29, 30)
2 X 6 ml plasma heparin (green top) ___ (day 1, 2, 29, 30)
2 X 6 ml plasma EDTA (purple top) ___ (day 1, 2, 29, 30)
1 X 2.5 ml whole blood (FAXgene tube) ___ (day 29, 30)

Have all blood samples been obtained? Yes ___ / No** ___

** If No, please state reasons why: __________________________

Blood Draw:  Excellent ___  Good ___  Difficult ___  Very Difficult ___

Comments: ________________________________

Blood Taker: ____________________________
COMIT DATA COLLECTION AND MONITORING FORMS

DAY 30

Date (DD/MM/YY) __________  Time (HH:MM) __________

Did the subject fast? Yes ___ / No ___  Feeling ill? ________________

Caffeine in last 24hrs?  Yes ___ / No ___  If yes, when __________

Alcohol in last 24hrs?  Yes ___ / No ___  If yes, when __________

Isotope tracer (\(^{13}\)C-ALA) provided? ***  **Only required on day 28 of each phase

Arm  Right ___ / Left___

Collect:  2 X 6 ml serum (red/grey SST tube) ___ (day 1, 2, 29, 30)
         2 X 6 ml plasma heparin (green top) ___ (day 1, 2, 29, 30)
         2 X 6 ml plasma EDTA (purple top) ___ (day 1, 2, 29, 30)
         1 X 2.5 ml whole blood (Paxgene tube) ___ (day 29, 30)

Have all blood samples been obtained?  Yes ___ / No___

*** If No, please state reasons why: __________________________

Blood Draw:  Excellent ___ Good ___ Difficult ___ Very Difficult ___

Comments: ___________________________________________________

Blood Taker: ___________________
SECTION 5: DXA

DAY 1 or 2

Date (DD/MM/MM/YY): _______________ Time of scan (HH:MM): _______________

Since screening has the volunteer had any radiation exposure (e.g. x-rays, radiotherapy, CT scan)? Yes ___ / No ___

Date & details: __________________________

FEMALES ONLY:
Possibility of being pregnant Yes ___ / No ___

If there is a possibility and/or they are pre-menstrual and not using contraceptives, has a pregnancy test been offered? Yes ___ / No ___

Has a test been taken? Yes ___ / No* ___ If yes, what was the result? - / +

*If no, why? ________________

If pregnant DO NOT undergo DEXA.

Check:
Jewelry removed (including glasses): Yes ___ / No ___

Spine straight: Yes ___ / No ___

Comments: ____________________________

Investigator Initials:____________________
DAY 29 or 30

Date (DD/MMM/YY): ________________ Time of scan (HH:MM): ____________

Since screening has the volunteer had any radiation exposure (e.g. x-rays, radiotherapy, CT scan)? Yes ___ / No ___

Date & details: __________________________________________

FEMALES ONLY:
Possibility of being pregnant Yes ___ / No ___

If there is a possibility and/or they are pre-menstrual and not using contraceptives, has a pregnancy test been offered? Yes ___ / No ___

Has a test been taken? Yes ___ / No* ___ If yes, what was the result? - / +
*If no, why? _______________________

If pregnant DO NOT undergo DEXA.

Check:
Jewelry removed (including glasses): Yes ___ / No ___

Spine straight: Yes ___ / No ___

Comments:
________________________________________________________

Investigator Initials: ____________________________
SECTION 6 – ENDOPAT ANALYSIS

DAY 1 or 2 (phase 1 only)

In the last 12 hours, have you consumed any food or drink besides water? Yes No

if yes, note the time they ate/drank (_._._._ am/pm) and reschedule test.

Have you consumed alcohol or alcohol containing products in the last 48 hours? Yes No

if yes, note time (_._._._ am/pm) and reschedule test.

1. Since you woke up, have you exercised vigorously? Yes No

if you, not time they exercised (_._._._ am/pm) and reschedule test.

2. If a Premenopausal Female: How many days has it been since you last period began? _____

*The participant should be on day 1 through day 7 of menstruation, if not reschedule the test*

*Please make sure the subject understands that Day 1 is the first day of menstruation*

3. What medications do you take regularly? (List name, dose, frequency)

4. In the last 12 hours, have you taken any medication, prescription or over the counter? Yes No

*Reschedule if participant has taken any antibiotics, meds with a decongestant component (Ephedrine), NSAIDS (Advil, Motrin, Aleve), steroids, or used an inhaler*

5. Do you take vitamins or herbal supplements? Yes No

6. Blood Pressure Measurements after a 5 min rest (1 min apart): _._._._ (1st reading)

   _._._._ (2nd reading)

   Enter into EndoPAT Program: _._._._ (Avg reading)

7. Weight: ___(lbs)___(kg)

8. Height: ___(ft.in)___(cm)

   Room Temp: _____ Gender: _____ Age: _____ Cuff setpoint: _____ cm from crease: _____

Begin Rest (yellow) _._._._ am

Begin Baseline (green) _._._._ am

Cuff Inflation _._._._ am

Cuff Deflation _._._._ am

End Study (red) _._._._ am

Rest = 10min | Baseline = 5min | Occlusion = 5min | Deflation 5min

RHI: _____ AI: _____ AI@75bpm: _____ HR: _____

Comments/Problems:
DAY 29 or 30 (phase 1 only)

In the last 12 hours, have you consumed any food or drink besides water? Yes No
*If yes, note the time they ate/drank (___:_._ am/pm) and reschedule test.

Have you consumed alcohol or alcohol containing products in the last 48 hours? Yes No
*If yes, note time (___:_._ am/pm) and reschedule test.

9. Since you woke up, have you exercised vigorously? Yes No
   *If yes, not time they exercised (___:_._ am/pm) and reschedule test.

10. If a Premenopausal Female: How many days has it been since you last period began? ______
    *The participant should be on day 1 through day 7 of menstruation, if not reschedule the test*
    *Please make sure the subject understands that Day 1 is the first day of menstruation*

11. What medications do you take regularly? (List name, dose, frequency)

12. In the last 12 hours, have you taken any medication, prescription or over the counter? Yes No
   *Reschedule if participant has taken any antibiotics, meds with a decongestant component (Ephedrine),
   NSAIDS (Advil, Motrin, Aleve), steroids, or used an inhaler*

13. Do you take vitamins or herbal supplements? Yes No

14. Blood Pressure Measurements after a 5 min rest (1 min apart): ___/___ (1st reading)
    ___/___ (2nd reading)
    Enter into EndoPAT Program: ___/___ (Avg reading)

15. Weight: _______ (lbs) _______ (kg)
16. Height: _______ (ft.in) _______ (cm)

Room Temp: _____  Gender:_____  Age:______  Cuff setpoint: ____  cm from crease:_____

Begin Rest (yellow) ___ / ___ am  Begin Baseline (green) ___ / ___ am  Cuff Inflation ___ / ___ am  Cuff Deflation ___ : ___ am  End Study (red) ___ : ___ am

Rest = 10min  Baseline = 5min  Occlusion = 5min  Deflation 5min

RHI: _____  AI: _____  AI@75bpm: _____  HR: _____

Comments/Problems:
To be completed by study staff:

<table>
<thead>
<tr>
<th>Diet Period</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>Week</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
</tr>
</thead>
</table>

1. In the past week has your exercise level changed?  □ Yes  □ No
   If Yes, was it:
   - □ More Active
   - □ Less Active
   - □ No Exercise

   Please remember to keep your exercise level constant throughout the study.

2. Have you taken any prescription or non-prescription drugs in the past week?  □ Yes  □ No
   If Yes, specify:  
   - description
   - amount

3. Have you taken any vitamins, minerals or other supplements  □ Yes  □ No
   If Yes, in the past week:
   - description
   - amount

4. Have you been ill in the past week?  □ Yes  □ No
   If Yes, describe illness: ____________________________

5. If you were ill in the past week, did your eating change as a result?  □ Yes  □ No
   If Yes, describe: ____________________________
### ADVERSE EVENTS

<table>
<thead>
<tr>
<th>Description</th>
<th>Start Date</th>
<th>Intensity*</th>
<th>Outcome</th>
<th>Date Ended</th>
<th>Diet Related?</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
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</table>

*Mild: An event that is easily tolerated by the volunteer, causing minimal discomfort and not interfering with everyday activities
Moderate: An event that is sufficiently discomforting to interfere with normal everyday activities
Severe: An event which is incapacitating and prevents normal everyday activities
### STUDY COMMENTS & PROTOCOL DEVIATIONS

<table>
<thead>
<tr>
<th>Date (DD/MMM/YY)</th>
<th>Comments</th>
<th>Study Personnel Initials</th>
</tr>
</thead>
<tbody>
<tr>
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</tbody>
</table>
STATUS SUMMARY

- Volunteer completed the study
- Volunteer withdrew from the study  Date of withdrawal: ___________

REASON FOR WITHDRAWAL. Tick appropriate box(es):

- Informed consent withdrawn by the volunteer
- Failure to comply with study requirements
- Investigator decision to withdraw volunteer

Provide rationale for withdrawal (if applicable):

________________________________________________________________________
________________________________________________________________________
________________________________________________________________________
________________________________________________________________________

- Volunteer’s final results sent
- Statement supplier form completed

Investigator Initials _______
<table>
<thead>
<tr>
<th>Diet sheet</th>
<th>3000 KCAL</th>
<th>Menu 1</th>
</tr>
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<tbody>
<tr>
<td><strong>PARTICIPANT</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>BREAKFAST</strong></td>
<td>gm</td>
<td></td>
</tr>
<tr>
<td>Canola Shake</td>
<td>328</td>
<td></td>
</tr>
<tr>
<td>English Muffin</td>
<td>65</td>
<td></td>
</tr>
<tr>
<td>Egg Substitute, liquid</td>
<td>75</td>
<td></td>
</tr>
<tr>
<td>American cheese</td>
<td>28</td>
<td></td>
</tr>
<tr>
<td><strong>LUNCH</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Healthy Choice deli cooked roast beef, sliced</td>
<td>84</td>
<td></td>
</tr>
<tr>
<td>Tomato, sliced</td>
<td>35</td>
<td></td>
</tr>
<tr>
<td>Romaine lettuce</td>
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<td></td>
</tr>
<tr>
<td>Whole wheat hamburger bun</td>
<td>60</td>
<td></td>
</tr>
<tr>
<td>Reduced fat mayonnaise</td>
<td>10</td>
<td></td>
</tr>
<tr>
<td>Sun chips, (1 bag=28g)</td>
<td>28</td>
<td></td>
</tr>
<tr>
<td>Reduced fat provolone cheese</td>
<td>28</td>
<td></td>
</tr>
<tr>
<td>Baby carrots, raw</td>
<td>60</td>
<td></td>
</tr>
<tr>
<td>Apple (1=138g)</td>
<td>138</td>
<td></td>
</tr>
<tr>
<td><strong>Unit Foods</strong></td>
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</tr>
<tr>
<td><strong>DINNER</strong></td>
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</tr>
<tr>
<td>Ground beef, lean (10% fat), cooked</td>
<td>84</td>
<td></td>
</tr>
<tr>
<td>Traditional pasta sauce</td>
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<td></td>
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<tr>
<td>Spaghetti, cooked</td>
<td>250</td>
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<tr>
<td>Parmesan cheese, grated</td>
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<tr>
<td>Green beans, frozen</td>
<td>135</td>
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<tr>
<td>Whole wheat dinner roll</td>
<td>57</td>
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<tr>
<td>Margarine</td>
<td>10</td>
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<tr>
<td><strong>SNACK</strong></td>
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</tr>
<tr>
<td>Canola Shake</td>
<td>328</td>
<td></td>
</tr>
<tr>
<td>Graham crackers</td>
<td>45</td>
<td></td>
</tr>
</tbody>
</table>

Menu 1
Total calories: 3003
Protein: 114g (15%)
Carbs: 375g (50%)
Fat: 119g (35%)
Appendix III: Article publication of thesis relevance

Using stable isotopes to trace diet-induced shifts in pathways of lipid metabolism

Shuishu Pu, Haifeng Yang, Peter J.H. Jones
University of Manitoba, Winnipeg, Manitoba, Canada; E-mail: peter_jones@umanitoba.ca

Summary
Advances in the use of stable isotopes in tracer methodology have clearly improved our capacity to investigate in vivo lipid metabolism in humans. The introduction of the isotope ratio mass spectrometry (IRMS) provides a new mechanism to track shifts in diet on the flux of tracers through metabolic pathways. Novel applications using stable isotopes including $^{13}C$, $^{15}N$, and $^{18}O$ have allowed important discoveries to be made in areas of fat and cholesterol metabolism in ways that have assisted in understanding disease relationships.

Introduction
Isotopes have long been an important tool for metabolic studies in humans. One of the earliest uses of isotopes predominately involved radioactive isotopes due to their sensitivity and accuracy, as well as availability of methods for measurement. Combined with an improvement in traditional mass spectrometry and related instrumentation, stable isotope tracers have gradually emerged as viable and more useful alternatives to radioactive tracers in biological studies over the past few decades [1].

In humans, dietary carbohydrates, proteins, and other components can be converted to fatty acids and stored in the form of triglycerides. De novo fat synthesis, the formation of fatty acids in humans, occurs primarily in the liver and lactating mammary glands, and to a lesser extent, in adipose tissue. Generally, the process commences with acetyl-CoA and incorporates the carbons into the existing fatty acid chain, using energy from adenosine triphosphate (ATP) and nicotinamide adenine dinucleotide phosphate (NADPH). In contrast, the essential fatty acids linoleic acid (LA, C18:2n-6) and $\alpha$-linolenic acid (ALA, C18:3n-3), serve as precursors for other omega-6 and omega-3 fatty acids through metabolic pathways, primarily the Sprecher pathway (Figure 1). LA and ALA, as dietary essential fatty acids in humans, must be obtained from our diets because they cannot be synthesized endogenously. Since the metabolic pathways share the same group of desaturation and elongation enzymes, it is believed that dietary intake of LA may influence omega-3 metabolism which includes the relatively important omega-3 long chain fatty acids eicosapentaenoic acid (EPA, C20:5n-3) and docosahexaenoic acid (DHA, C22:6n-3). Over past decade, numerous research projects have focused on omega-3 metabolic pathways, with the question of the rate of conversion of dietary ALA to EPA/DHA remaining debatable. Therefore, these investigations require accurate and reliable methodologies to answer such questions.

Elevated plasma cholesterol levels in humans are considered as a risk factor for developing cardiovascular disease and its associated complications in humans [2]. The total body pool of cholesterol is maintained by endogenous cholesterol synthesis as well as by cholesterol absorption from dietary and biliary sources. When ingested cholesterol is mixed with exogenous cholesterol from bile in the intestinal tract, ATP-binding cassette (ABC) transport protein interacts with cholesterol in the intestine, promoting efflux of cholesterol into the intestinal lumen. Through the lymph circulation, cholesterol is transported by chylomicrons to the liver and then to the circulation. Therefore, it is cri-