

**THE ROLE OF DIETARY FATTY ACIDS FROM PLANT-BASED OILS
IN METABOLIC AND VASCULAR DISEASE**

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A Thesis submitted to the Faculty of Graduate Studies of the University of Manitoba

in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

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ABSTRACT

Dietary fat has long been implicated in the etiology of metabolic and cardiovascular disease, and both the amount of fat and the fatty acid composition of the diet play a role in disease progression. Although national health organizations have set guidelines for the recommended intake of dietary fats, questions remain regarding the optimal dietary lipid profile for maintaining health and improving disease conditions. Whether certain types of fatty acids from plant-based oils can improve metabolic and vascular disease has been studied and debated, but not fully determined. In this study, we investigated the role of dietary fatty acids from plant-based oils, and examined their effects on metabolic and vascular disease parameters.

Obese *fa/fa* Zucker rats were fed a diet containing flaxseed oil, which resulted in smaller adipocytes and decreased adipose tissue T-cell infiltration. Obese-prone Sprague Dawley rats were fed high-fat diets with different proportions of mono- and polyunsaturated fats. Changes were observed in adipose tissue levels of fatty acid synthase, adiponectin and fatty acid receptors GPR41 and GPR43, but other metabolic and inflammatory mediators in adipose tissue and serum remained stable.

A systematic review and meta-analysis on the impact of n3 fatty acids on major cardiovascular endpoints showed that little evidence exists to support their role in peripheral arterial disease. Then again, very few studies on this topic have been conducted. To address this research gap, a clinical trial was designed to investigate the effects of a dietary intervention on blood vessel properties in people with peripheral arterial disease. Participants in the Canola-PAD Study consumed 25 g/day of canola oil

or a Western diet oil mixture as part of their usual diet for 8 weeks. Although the intervention altered phospholipid fatty acids, vascular function, the lipid profile and inflammatory markers stayed relatively stable.

Overall, this research demonstrates that dietary fatty acids from plant-based oils can be immunomodulatory, but at the physiological doses tested they are not potent mediators of functional changes in obesity or vascular physiology.

ACKNOWLEDGEMENTS

My deepest gratitude and respect goes to my advisors, Drs Peter Zahradka and Carla Taylor, for guiding me through the work that has culminated in this dissertation. I hope that you know how much I appreciate all of your efforts on my behalf. There have been many opportunities for growth and learning, and lots of laughs and good times during my time at the lab. It has been a pleasure working in the positive environment you have cultivated here, and I feel very fortunate to have shared this part of my journey with you.

My examining committee members, Drs Peter Cattini, Jon McGavock, and Jeff Wigle, have provided me with much valuable advice, and I offer them my sincere thanks for their critical evaluation of my work over the last several years.

This work would not have been possible without the help of many people working in and associated with our laboratory at St. Boniface Research Centre. Thank you to current and former Zahradka/Taylor (Parla Zahraylor) lab students and staff, in particular, Leslee Tworek and Azadeh Yeganeh, who trained me and assisted me with many different lab techniques; Maria Baranowski and Danielle (Hanke) Perera, who managed the animal handling and feeding portions of the animal studies in which I was involved; Angela Park, whose assistance with qPCR experiments was very much appreciated; Raissa Perrault, Angela Wilson and Brendon Foot, who were instrumental in coordinating and conducting the Canola-PAD Study; and many others who were kind, supportive, knowledgeable, ridiculously entertaining, talented treat-makers, and overall

great people to get to know. Thank you also to our administrative staff at CCARM, Sue Zettler and Tracy Ewonchuk, and at the Department of Physiology, Gail McIndless and Judy Olfert, for your tireless efforts on my behalf.

I'm very grateful to the funding agencies that supported me financially while I was completing this work: the University of Manitoba's Faculty of Graduate Studies, the Manitoba Health and Research Council (now Research Manitoba), and the Natural Sciences and Engineering Research Council (NSERC) Collaborative Research and Training Experience Program.

And finally, thank you to my family and friends for your support and encouragement throughout my years in graduate school. Thanks to my parents and grandparents for instilling in me the importance of education and the ambition to shoot high. Thanks to my siblings for staying in school nearly as long as I did and making it seem normal. And thank you to my patient husband, Andrew, who is nearly as excited as I am to reach the end of this long process. Your love and support mean the world to me, and I couldn't have done it without you!

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ABBREVIATIONS

AA	Arachidonic acid
ABI	Ankle brachial index
ACLY	ATP-citrate lyase
Acn	Acetonitrile
AI	Adequate intake
ALA	Alpha-linolenic acid
ANOVA	Analysis of variance
BMI	Body mass index
CI	Confidence interval
CT	Computerized tomography
DAB	Diaminobenzidine
DEXA	Dual energy x-ray absorptiometry
DHA	Docosahexaenoic acid
DHET	Dihydroxyeicosatrienoic acid
DiHDoPE	Dihydroxydocosapentaenoic acid
DiHETE	Dihydroxyeicosatetraenoic acid
DiHODE	Dihydroxyoctadeca(di)enoic acid
DiHOME	Dihydroxyoctadeca(mono)enoic acid
DIO	Diet-induced obese/obesity
DPA	Docosapentaenoic acid
E	Energy
eEF2	Eukaryotic elongation factor 2
EEQ	Epoxyeicosatetraenoic acid
EET	Epoxyeicosatrienoic acid
EPA	Eicosapentaenoic acid
EpDoPE	Epoxydocosapentaenoic acid
EpODE	Epoxyoctadecadienoic acid
EpOME	Epoxyoctadecamonoenoic acid
faBASE	<i>fa/fa</i> baseline group
FABP	Fatty acid binding protein
faCTL	<i>fa/fa</i> control diet group
faFLAX	<i>fa/fa</i> flax diet group
GAPDH	Glyceradehyde 3-phosphate dehydrogenase
GC	Gas chromatography
GLA	Gamma-linolenic acid

HbA1c	Glycated hemoglobin
HDL	High-density lipoprotein
HDoHE	Hydroxydocosaheptaenoic acid
HEPE	Hydroxyeicosapentaenoic acid
HETE	Hydroxyeicosatetraenoic acid
HODE	Hydroxyoctadecadienoic acid
HOTE	Hydroxyoctadecatrienoic acid
HRP	Horseradish peroxidase
IC	Intermittent claudication
IFN- γ	Interferon-gamma
IL	Interleukin
KDoHE	Keto-docosaheptaenoic acid
KEGG	Kyoto Encyclopedia of Genes and Genomes
KEPE	Keto-eicosapentaenoic acid
KETE	Keto-eicosatetraenoic acid
KODE	Keto-octadecadienoic acid
KOTE	Keto-octadecatrienoic acid
LA	Linoleic acid
LC	Liquid chromatography
LDL	Low density lipoprotein
InCTL	Lean control diet group
LTA	Leukotriene
MAPK	Mitogen-activated protein kinase
MCP-1	Monocyte chemoattractant protein-1
MD	Mean difference
MI	Myocardial infarction
MMSE	Mini mental state exam
MRI	Magnetic resonance imaging
MS	Mass spectrometry
MUFA	Monounsaturated fatty acid
n3	Omega-3 (fatty acid)
n6	Omega-6 (fatty acid)
n9	Omega-9 (fatty acid)
ND	Not determined
NE	Not estimable
NMR	Nuclear magnetic resonance
OP	Obese prone
OR	Odds ratio

PAD	Peripheral arterial disease
PAI-1	Plasminogen activator inhibitor-1
PCA	Principal component analysis
PFWD	Pain-free walking distance
PGE	Prostaglandin
PL	Phospholipid
PUFA	Polyunsaturated fatty acid
RCT	Randomized controlled trial
RR	Risk ratio or relative risk
SD	Standard deviation
SDS-PAGE	Sodium dodecyl sulphate-polyacrylamide gel electrophoresis
SEM	Standard error of the mean
SFA	Saturated fatty acid
T2D	Type 2 diabetes
TLR4	Toll-like receptor 4
TNF- α	Tumour necrosis factor- α
TXA	Thromboxane

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CHAPTER 1: OVERVIEW

1.1 Introduction

Metabolic and vascular diseases have an enormous impact on the global health landscape. The prevalence of obesity, a metabolic disease characterized by excess or abnormal body fat accumulation that may impair health [1], has been steadily increasing for several decades, and is now considered a major public health issue worldwide [2]. Obesity is associated with an increased risk for cardiovascular disease and has been linked to many other disorders, including type 2 diabetes (T2D), hypertension and some types of cancer [3,4]. Vascular diseases, including peripheral arterial disease (PAD), are complex pathological conditions affecting the ability of blood vessels to regulate vascular tone [5]. Underlying atherosclerosis (hardening and stiffening of the arteries) in individuals with PAD greatly increases the risk for heart attack and stroke. PAD affects over 10 million people in North America while another estimated 10 million people are undiagnosed [6].

Although obesity and PAD manifest in very different ways, they have some features in common: they are chronic conditions that develop over the span of several decades and they share a major disease determinant or influencing factor, namely diet [7-9]. Dietary fat has long been implicated in the development of both obesity and cardiovascular disease [10,11]. However, not all types of dietary fat are the same. The term “fat quality” has been used to describe the fatty acid composition of the diet [12,13]. A diet containing *trans* fat and saturated fatty acids (SFAs) has low fat quality, because of strong associations between consumption of these fats and the risk of

disease. On the other hand, foods rich in polyunsaturated fatty acids (PUFAs), including the omega-3 (n3), omega-6 (n6), and omega-9 (n9) fatty acids, and monounsaturated fatty acids (MUFAs) are of high fat quality, due to demonstrated health benefits such as reducing the risk for cardiovascular disease [14] and, in the case of n3 fatty acids, exerting anti-inflammatory effects [15]. Examples of high fat quality foods include plant-based (vegetable) oils, such as canola oil and flaxseed oil.

The overall objective of this thesis is to investigate the role of fatty acids from plant-based oils, such as flaxseed oil and canola oil, which are rich in n3 fatty acids, in the development and progression of metabolic and vascular disease (obesity and PAD). Feeding studies using rodent models of obesity were employed to examine the effect of different proportions of SFAs, MUFAs and PUFAs in various plant-based oils on body weight, fat distribution, adipose tissue function and inflammatory status. In order to evaluate the role of n3 fatty acids in vascular disease, we conducted a systematic review and meta-analysis of the literature on the effects of n3 fatty acids on major cardiovascular endpoints in PAD patients. A clinical trial was conducted to investigate the effects of a diet enriched in canola oil on blood vessel properties and other functional parameters in people with PAD.

The findings from this program of research provide insight into the actions of plant-based oils rich in n3 fatty acids, and compare their effects to other dietary fatty acid profiles in the context of metabolic and vascular disease. Chapter 2 reviews pertinent literature to provide context for the studies conducted. Chapter 3 outlines the rationale, hypotheses and objectives of the research. Chapters 4-7 (which each comprise

a peer-reviewed publication) and Chapter 8 describe the studies and data analyses conducted. Chapter 9 discusses the implications of the research findings and provides direction for future research. These studies represent the joint efforts of several people. As such, Chapters 4-8 are each prefaced by an author contribution statement, detailing the contribution I made to the work presented and listing the other people involved in the study.

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CHAPTER 2: LITERATURE REVIEW

2.1 Dietary Fats

Three main classes of lipids (fats) will be discussed in this dissertation, although the first of these will receive the most attention. 1) Triglycerides (also called neutral fats) are composed of a glycerol “backbone” joining three fatty acids (Figure 2.1). The vast majority of dietary fats are triglycerides. 2) Phospholipids are formed from a glycerol molecule and two fatty acids. The third carbon of the glycerol molecule is linked through a phosphate group to a polar molecule such as ethanolamine, serine or choline. 3) Sterols are organic molecules that contain a characteristic four-ring structure consisting of three 6-carbon rings and one 5-carbon ring. Although they bear no structural similarity to fatty acid-derived lipids, sterols are classified as lipids due to their hydrophobicity.

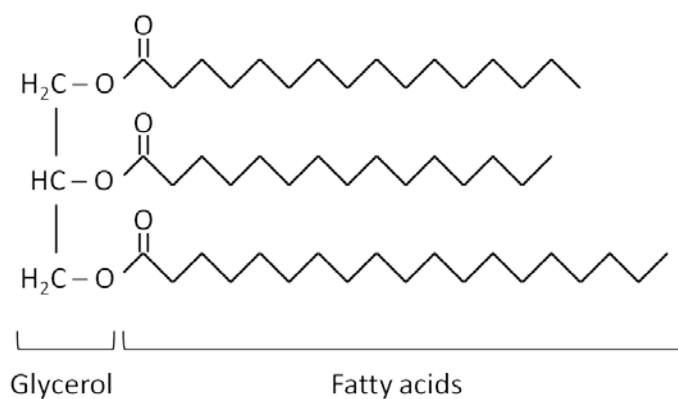


Figure 2.1 Structure of a triglyceride. Glycerol forms a “backbone” for three fatty acid “tails” that may or may not be the same.

2.1.1 Fatty Acid Nomenclature

Fatty acids are the fundamental building blocks of triglycerides, and the properties of any specific triglyceride molecule depend on its fatty acid composition. Fatty acids can be classified by hydrocarbon chain length, degree of saturation and orientation of double bonds [1]. The carbon next to the carboxyl end is designated C1, and the numbers increase sequentially until the terminal methyl group (or omega [ω] carbon) is reached. SFAs have each carbon atom in the hydrocarbon chain bonded to as many hydrogen atoms as possible. Unsaturated fatty acids contain double bonds within the carbon chain. Fatty acids with only one double bond are monounsaturated, while those with more than one double bond are polyunsaturated. MUFAs and PUFAs are additionally classified by the position of their double bonds: counting from the omega end, PUFAs can be categorized as, for example, n3, n6, or n9, with the numbers 3, 6, and 9 referring to the carbon where the first double bond occurs. In unsaturated fatty acids, the orientation of the hydrogen atoms relative to the double bond can be *cis* (same side of the double bond) or *trans* (opposite side of the double bond). In terms of hydrocarbon chain length, short-chain fatty acids have fewer than 6 carbons, medium-chain fatty acids have 6-12 carbons, long-chain fatty acids have 13-21 carbon atoms, and very long-chain are 22 carbons or longer. Examples of these different fatty acid types are depicted in Figure 2.2. Fatty acids can also be described using the lipid numbers nomenclature system. Lipid numbers take the form $C:D(n)$, where C is the number of carbon atoms, D is the number of double bonds and n denotes the position of the double bond closest to the omega end of the fatty acid [1].

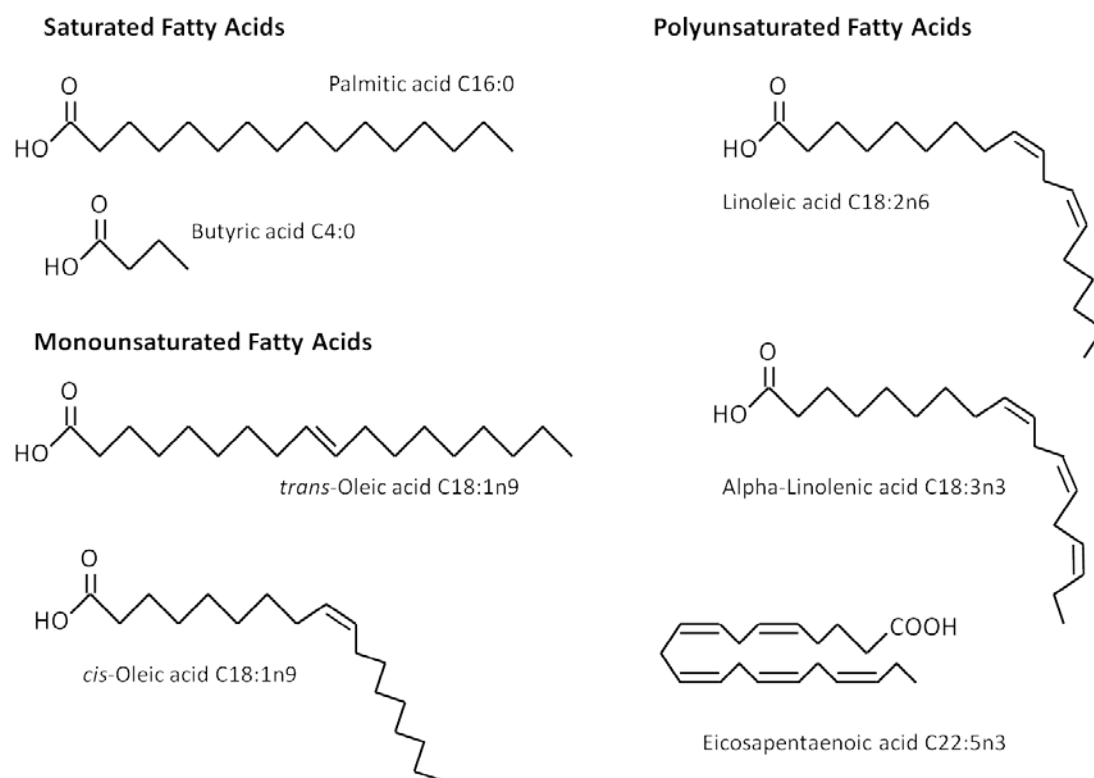


Figure 2.2 Examples of different types of fatty acids: saturated fatty acids, monounsaturated fatty acids with *cis*- and *trans*-oriented double bonds, and n3 and n6 polyunsaturated fatty acids.

2.1.2 Digestion, Absorption and Metabolism of Fatty Acids

Fatty acids enter the body primarily in the form of triglycerides [2,3]. In the small intestine, triglycerides are hydrolyzed to monoglycerides and free fatty acids, a process aided by emulsifying bile salts that increase the surface area where water-soluble pancreatic lipases can work [4]. The free fatty acids and monoglycerides enter the intestinal enterocytes by diffusing across the brush-border membrane via “flip-flop” of protonated fatty acids down a favourable concentration gradient [5] or via fatty acid transport proteins FATP4 and CD36 [6,7] with the help of fatty acid binding protein (FABP) [5]. The more water-soluble short-chain and medium-chain fatty acids enter the venous blood in the form of free fatty acids, while the monoglycerides and long-chain fatty acids are resynthesized into triglycerides in the enterocyte [8]. The newly formed triglycerides are packaged into chylomicrons along with cholesterol, phospholipids and fat-soluble vitamins, and released into the lymph by exocytosis. The chylomicrons enter the bloodstream at the thoracic duct and travel in the circulation to deliver triglycerides to the body’s tissues. Free fatty acids travel in the bloodstream bound to albumin. The insulin-responsive enzyme lipoprotein lipase is attached to the luminal surface of endothelial cells lining the capillaries of adipose tissue, skeletal muscle, heart and other tissues, and is responsible for releasing fatty acids from the triglycerides in lipoproteins (including chylomicrons) [9]. The fatty acids are then absorbed by the target tissue via the action of fatty acid transporters [10]. Inside the cells, the fatty acids are re-esterified to another glycerol molecule to form triglycerides that are stored in lipid droplets. The chylomicron remnants are taken up by the liver for recycling [11]. Some fatty acids can

be synthesized *de novo*; this occurs primarily in the liver [12]. Palmitic acid C16:0 is synthesized by the multimeric enzyme complex fatty acid synthase, which catalyzes recurring reactions (adding 2-carbon units) using acetyl-CoA and malonyl-CoA (from the breakdown of glucose) as substrates (Figure 2.3). Modification of palmitate leads to the formation of other fatty acids [13]. For example, further 2-carbon units can be added to elongate the fatty acid, or desaturase enzymes can insert double bonds to form unsaturated fatty acids.

Essential Fatty Acids and their Derivatives

Essential fatty acids are fatty acids containing a double bond at positions beyond C9-C10. Humans lack the specific desaturases required to introduce double bonds at carbon atoms beyond C9-C10 in the hydrocarbon chain; therefore, essential fatty acids must be obtained in the diet [14]. Two fatty acids are known to be essential for human health: alpha-linolenic acid (C18:3n3; ALA) and linoleic acid (C18:2n6; LA). ALA is the parent fatty acid of the n3 family, and has two main long-chain derivatives, eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) (Figure 2.4). Although there is some controversy about the conversion efficiency of dietary ALA to EPA and DHA [15], we have demonstrated that dietary ALA from plant sources increases the concentration of these long-chain fatty acids in various tissues [16,17]. The health benefits of n3 fatty acids, particularly EPA and DHA, have been well studied. They play important roles in energy homeostasis by binding the nuclear receptor peroxisome

proliferator activated receptor (PPAR)- α and regulating the activity of enzymes involved in lipid transport and metabolism [18], leading to their designation as cardioprotective [19]. They also have anti-inflammatory properties related to their ability to suppress the activation of T-cells [20]. LA is the parent fatty acid of the n6 PUFA family, which also includes gamma-linolenic acid (GLA) and arachidonic acid (AA). Their metabolic effects, exerted primarily through the nuclear receptor PPAR- γ , are focused on modulating fat distribution and storage with the end result of increasing insulin sensitivity [14,21]. AA is also an important molecule for the biosynthesis of inflammatory molecules [14].

The essential fatty acid families serve as precursors to a class of oxygenated PUFAs called oxylipins (Figure 2.4). Oxylipins are produced through the actions of the enzymes cyclooxygenase, lipoxygenase and cytochrome p450 on PUFAs in the n3 and n6 fatty acid families, or through the interaction of reactive oxygen species with AA [22]. They can act locally as paracrine or autocrine cell signalling mediators [23]. The eicosanoids are a 20-carbon subgroup of oxylipins that include prostaglandins, thromboxanes and leukotrienes, as well as a number of newly recognized molecules [23]. Eicosanoids exert complex control over the inflammatory response and immunity [24], and are involved in regulating endothelial function in the development of atherosclerosis [23]. In general, the eicosanoids derived from n3 fatty acids are less inflammatory, or even anti-inflammatory and pro-resolving [24], compared to eicosanoids stemming from n6 fatty acids [25].

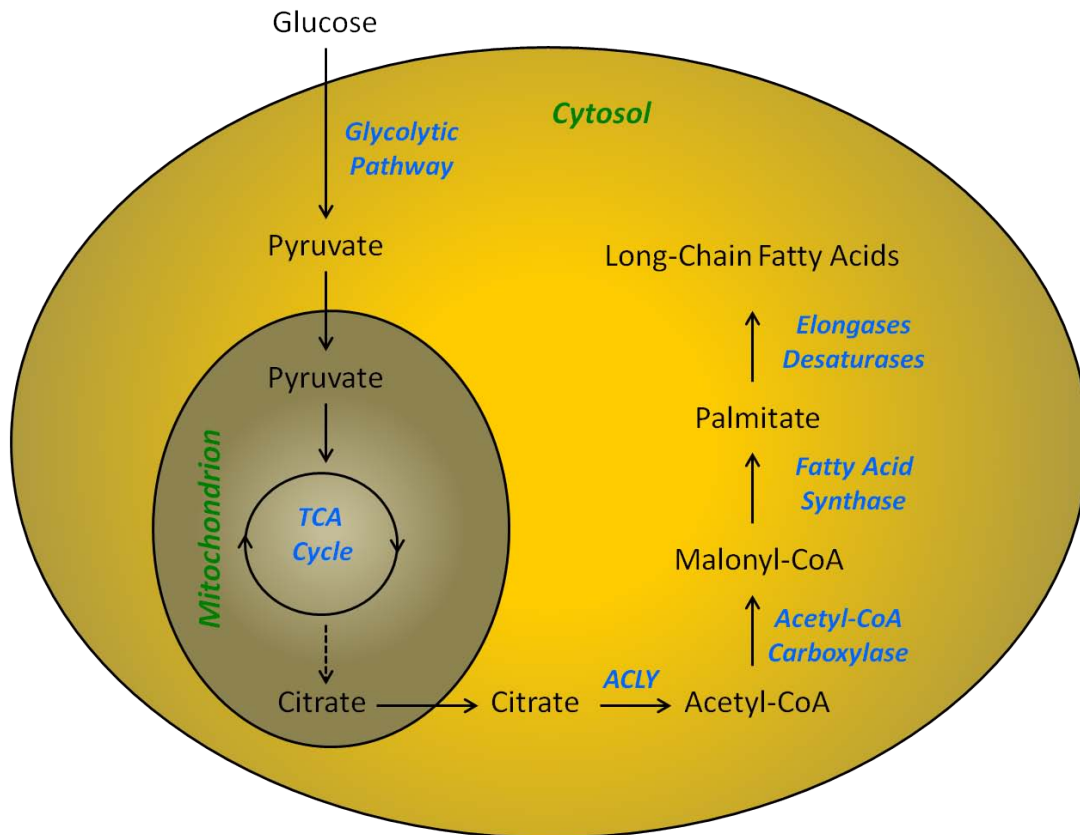


Figure 2.3 Overview of *de novo* fatty acid synthesis. Glucose is taken into the cell via a glucose transporter, and enters the glycolytic pathway to produce pyruvate. Pyruvate enters the mitochondria and is converted to acetyl-CoA via the TCA, which yields citrate. When there is an excess of citrate in the TCA cycle, citrate moves to the cytosol and is converted back to acetyl-CoA by ATP-citrate lyase (ACLY), from which malonyl-CoA is formed by acetyl-CoA carboxylase. Malonyl-CoA is used as a substrate by fatty acid synthase to produce the 16-carbon fatty acid palmitate, which serves as a precursor for more complex fatty acids. Adapted from [12].

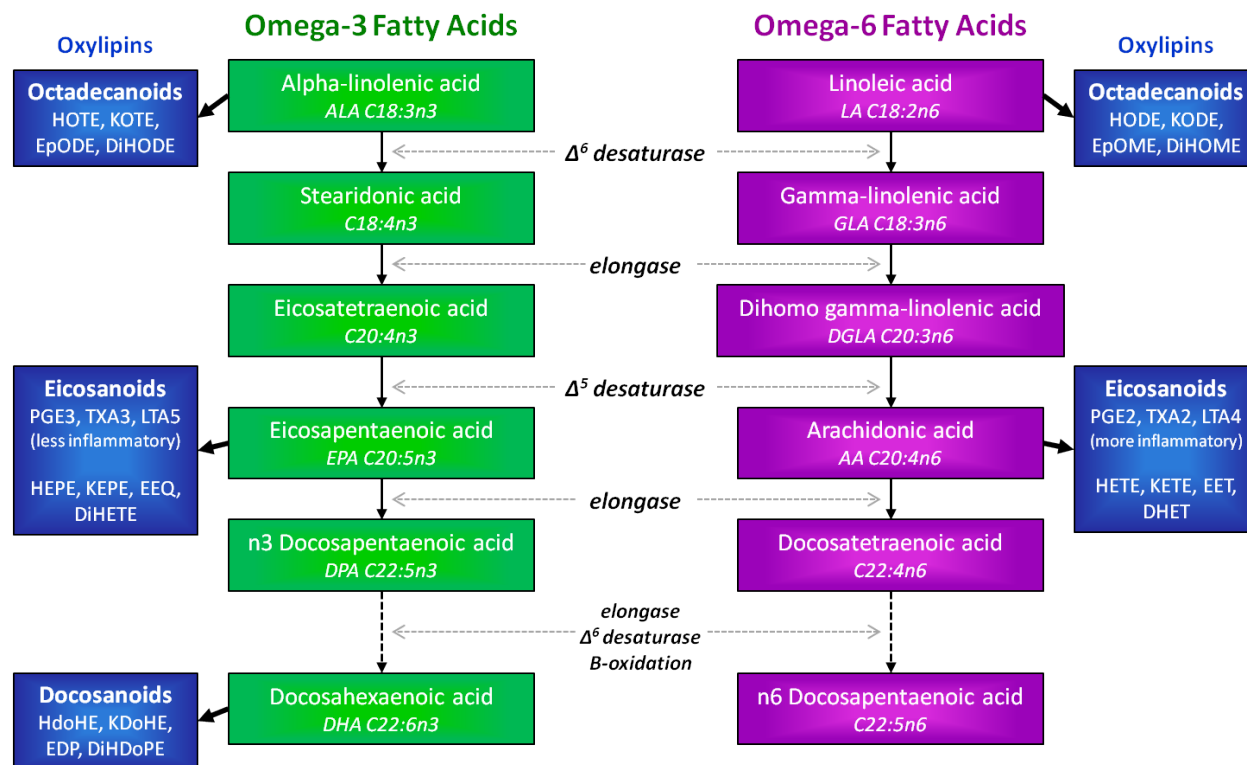


Figure 2.4 Oxylipins derived from members of the n3 and n6 fatty acids families at various degrees of desaturation and elongation. The derivatives of n3 fatty acids are less inflammatory, whereas the derivatives of the n6 fatty acid family drive inflammatory cascades. LTA: leukotriene; PGE: prostaglandin; TXA: thromboxane. All other abbreviations are defined in the Abbreviations section starting on page xi. Adapted from [23,26,27].

2.1.3 Physiological Functions of Fatty Acids

Fuel: Fatty acids are important energy substrates. When hormones (e.g., epinephrine and glucagon) signal the need for metabolic energy, fatty acids are mobilized from cellular storage depots and transported to tissues to be degraded by β -oxidation [11]. The oxidation yield of fatty acids is 9 kcal/g, more than twice the yield of energy from protein or carbohydrates (4 kcal/g) [11].

Structural Elements: Fatty acids are major components of the phospholipids and glycolipids that constitute the lipid bilayer of cell membranes. Fatty acid structure modulates membrane fluidity and the behaviour of membrane-bound receptors and transporters. The melting point of fatty acids increases with the length of the hydrocarbon chain, and decreases with the number of double bonds [28]. Unsaturated fatty acids are bulkier than SFAs; thus, the lipid bilayer is less tightly packed and membrane fluidity is increased. This has a direct impact on the number and affinity of cell surface receptors. For example, membranes with higher SFA content (which are more rigid) demonstrate a reduction in insulin receptors and less affinity of insulin for its receptor, and this may contribute to insulin resistance [29].

Signalling Molecules: Fatty acids serve as mediators of signalling pathways in several different ways (Figure 2.5): i) Dietary PUFAs are rapidly incorporated into cell membranes [30], where they act as precursors to a variety of second-messengers that function locally to modulate a broad array of cellular functions [31]; ii) long-chain PUFAs and their metabolites are ligands of nuclear receptors that regulate the synthesis of transcription factors and gene products [32]; and iii) free fatty acids can bind directly to

G-protein coupled receptors to regulate energy homeostasis in metabolically important tissues [33-35].

A series of G-protein coupled receptors have been identified as responsive to free fatty acids and their derivatives; five of these have received considerable attention as a result of their role in various physiological processes. Free fatty acid receptor 1 (FFA1 or GPR40) and GPR120 are activated by medium- and long-chain fatty acids, while GPR84 binds medium-chain fatty acids, and FFA2 (GPR43) and FFA3 (GPR41) are activated by short-chain fatty acids [36,37]. GPR43 and GPR41 are expressed in adipose tissue where they play a role in regulating energy homeostasis by suppressing lipolysis and release of fatty acids from adipocytes, and controlling the production and release of leptin and adiponectin [35]. Most of these fatty acid-binding receptors are also expressed in the intestine, where most of the research efforts in this field have been focused to date [35].

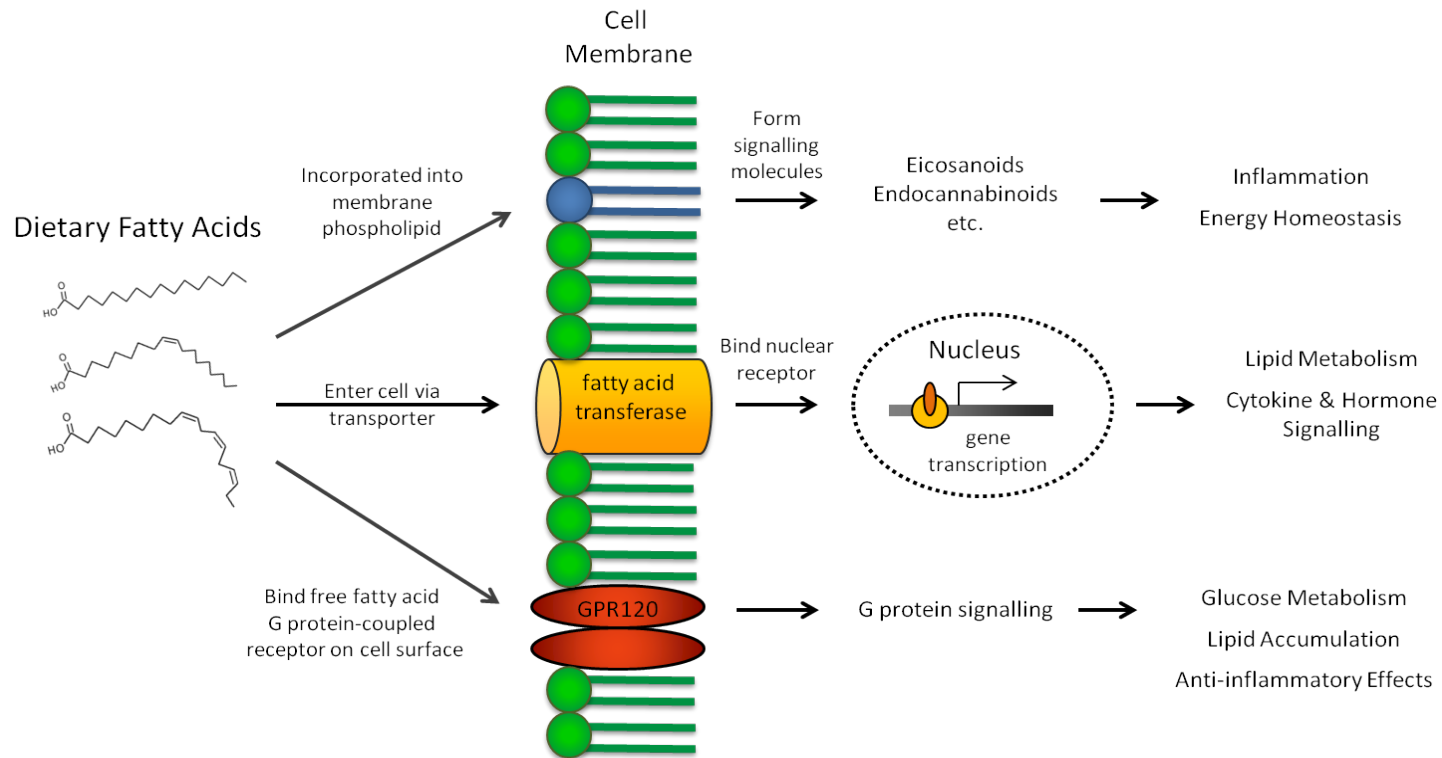


Figure 2.5 Dietary fatty acids can act as signalling molecules in several different ways. They can be incorporated into membrane phospholipids, where they can serve as mediators of signalling cascades. They can bind nuclear receptors, regulating gene transcription and the production of various signalling molecules. They can also bind directly to free fatty acid G protein-coupled receptors in the cell membrane, thereby initiating signalling pathways that regulate critical cellular processes. Adapted from [37-39].

2.1.4 Dietary Sources of Fatty Acids

Dietary fatty acids are present in a wide variety of foods, including animal sources such as meat, fish and dairy, and plant sources such as various nuts and vegetable oils. A comprehensive review of dietary fat sources is beyond the scope of this work, and so the focus here will be on vegetable oils commonly consumed in North America. Figure 2.6 depicts the fatty acid proportions in a number of vegetable oils relevant to this work. Among these, flaxseed oil and conventional canola oil have the highest proportions of ALA, but while flaxseed oil is generally unsuitable for cooking or frying due to its low smoke point, canola oil is widely consumed in Canada and may represent a significant source of ALA in the diet [40]. High-oleic canola oil is commonly used in commercial food production and food service because it replaces hydrogenated fats containing *trans* fatty acids in commercial food products, and it has greater stability under high heat conditions than conventional canola oil [41]. The content of ALA in high-oleic canola oil is considerably lower than in conventional canola oil, but both contain high amounts of MUFAs. The use of n6 PUFA-rich corn oil is more common in the USA than in Canada, but both countries rely heavily on soybean oil for the production of food products (for example, margarine, salad dressing, potato chips, and baked goods) [42]. Soybean oil has a predominance of n6 PUFAs and an otherwise relatively balanced fatty acid profile, with an ALA content slightly lower than canola oil.

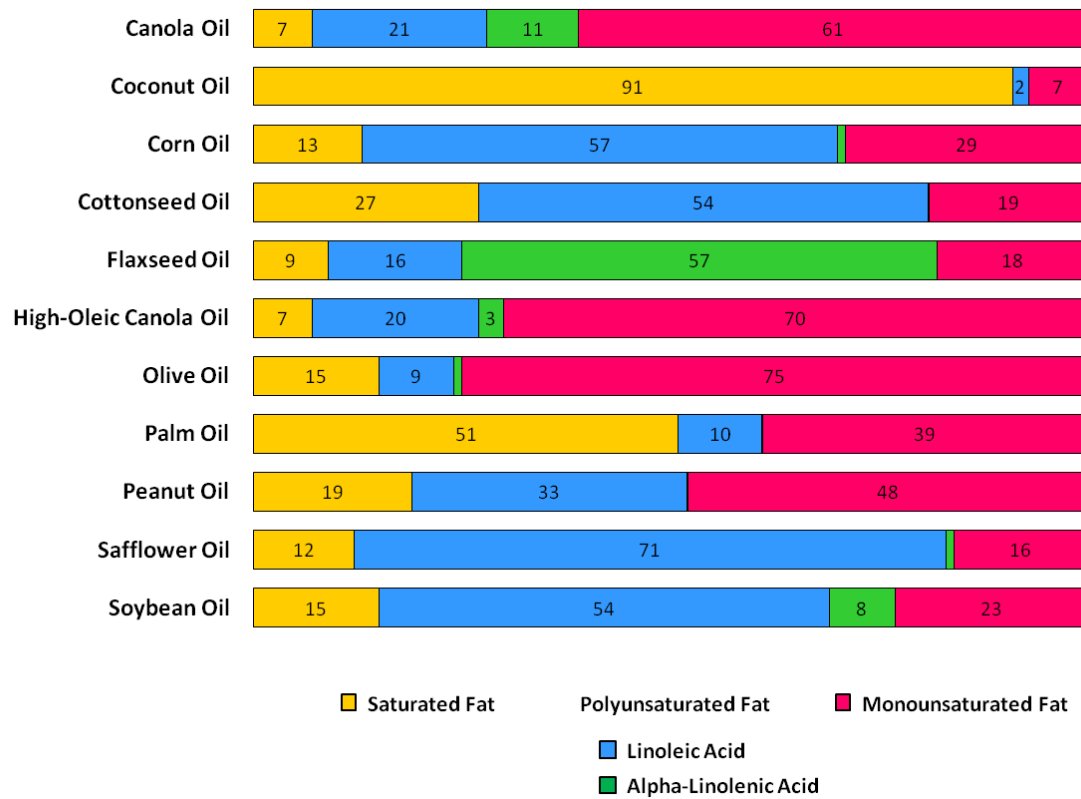


Figure 2.6 The fatty acid composition (%) of commonly consumed vegetable oils. Adapted from [43].

2.1.5 Dietary Recommendations for Fat Consumption

Ever since an association between dietary fat and cardiovascular disease was first hypothesized in the 1950s [44,45], health organizations have aimed to translate growing evidence linking diet and chronic disease into guidelines for consumption of fat and fatty acids with the objectives of preventing clinical deficiencies, providing optimal health and reducing the risk of developing chronic disease [46].

Total Fat: Most sources list the recommended intake for total fat in adults as 20-35%E (energy) [46], although some recommend the lower value of 15%E [47]. There is limited evidence linking total fat intake to increased weight [48-50], cancer [51] and T2D [52].

Saturated Fat: The recommended intake for SFAs is below 10%E, or in some reports, as low as possible [46]. The reports cite evidence regarding a positive association between low density lipoprotein (LDL)-cholesterol levels and cardiovascular disease risk [53-56], and a reduction in cardiovascular disease risk when SFAs are replaced with PUFAs, fruit and vegetables [57], but not with simple carbohydrates [58].

Monounsaturated Fat: Most guidelines do not include a recommended intake for MUFAs, but instead suggest MUFA intake should be calculated by difference, based on recommendations for other fat types, i.e. subtracting SFAs, PUFAs and *trans* fatty acids from total fat intake. There is evidence that substituting carbohydrates with MUFAs lowers LDL-cholesterol, increases high-density lipoprotein (HDL)-cholesterol, and may improve insulin sensitivity [57,59,60].

Polyunsaturated Fat: PUFAs include the essential fatty acids LA (n6) and ALA (n3). The minimum intake levels for essential fatty acids are 2.5%E for LA and 0.5%E for ALA to prevent deficiency symptoms in adults [47,61]. The acceptable range of dietary intake for all n6 PUFAs is 2.5-9%E [47], based on the evidence for LDL-cholesterol and triglyceride lowering, and therefore a decreased risk for coronary heart disease [47]. However, some controversy exists due to uncertainty about whether the positive effects seen with increased LA intake could in part be attributed to a corresponding increase in n3 PUFAs and reduction in *trans* fat and SFA intake in some studies [62]. Some researchers have also raised concerns that n6 PUFA intake could increase lipid peroxidation and the production of proinflammatory eicosanoids via AA [63,64]. A recent systematic review of randomized controlled trials (RCTs) found no evidence of increased biological markers of inflammation in people consuming LA [65]. However, there is a need for further fatty acid-specific research to determine the optimal intake of n6 PUFAs [66].

Recommended intakes of n3 PUFAs are set for the n3 fatty acids ALA, EPA and DHA [67]. The adequate intake (AI) value for all population groups is set at 0.5%-2%E for ALA, and for EPA + DHA the AI is 0.250-2 g/day. Recommendations for primary prevention of coronary disease are based on evidence from clinical trials [46], and vary between 250-660 mg/day for the long-chain n3 PUFAs EPA and DHA. The recommended upper limit for n3 PUFA intake is 3 g/day [49]. The evidence supporting these recommendations for adults is mostly related to primary and secondary prevention of cardiovascular disease, although the results of clinical trials are not always consistent.

Physiological benefits of n3 PUFA consumption include reductions in all-cause mortality, cardiac and sudden death, and stroke [68]. Three large (n=2000-18000) RCTs using n3 PUFA interventions [69-71] reported improvements in patients with established coronary heart disease. Other studies [72-74] have not demonstrated a beneficial effect, although these trials have been criticized for failure to take into account baseline n3 PUFA intake in their populations. The debate over the role of n3 PUFAs as a preventative and/or therapeutic agent continues.

The n6:n3 PUFA ratio: One of the main considerations in meeting recommendations for n3 and n6 PUFA intake is the ratio between these two types of fatty acids, which share some of the same metabolic pathways and enzymes. The current dietary pattern in North America is characterized by a high content of fat, including a high proportion of saturated fat (>10%) and n6 PUFAs, and a low proportion of n3 PUFAs, resulting in an n6:n3 ratio of approximately 20:1. This is very different from the n6:n3 ratio humans consumed 100-150 years previously [75], which was closer to 1:1. The changes are attributed to the advent of the industrial revolution, which altered agricultural and livestock feeding practices and food production [75,76]. Figure 2.4 depicts the metabolic pathways by which LA and ALA are transformed to their principle derivatives, AA, EPA and DHA. The substrates in the two pathways compete for the same set of catalytic enzymes, and although the enzyme Δ^6 desaturase has greater affinity for ALA than for LA, the conversion efficiency of ALA to EPA and DHA is limited, due to the overabundance of LA in the diet [77]. In this context, the experimental evidence indicates that the optimum ratio between LA and ALA should be close to 5:1,

and should not exceed 10:1 [78]. Since all of the PUFA derivatives in these pathways are important for maintaining homeostasis, an imbalance in n6:n3 PUFA availability may have wide-reaching effects on various physiological processes, which in turn impact the development of chronic disease [77,79].

Trans fats: Overall, the recommendation for synthetic trans fatty acids is to keep intake as low as possible. Evidence for this guideline is based on the adverse effects of these *trans* fatty acids on serum lipids, LA and ALA balance, and cardiovascular disease risk [80-83]. Small amounts of natural *trans* fatty acids (e.g., vaccenic acid, conjugated linoleic acid) occur in beef and dairy products, and their metabolic effects are distinguishable from synthetic trans fats in that they demonstrate none of the adverse properties associated with the latter [62,84].

In summary, current dietary recommendations are to decrease SFAs and *trans* fatty acid intake, and to emphasize unsaturated fat (both MUFAs and PUFAs) intake. The type of fat in the diet is an important factor in maintaining metabolic balance, since what is consumed determines which fatty acids are available to the body [85]. The term “fat quality” has been used to describe the fatty acid composition of the diet [62,86]. A diet containing *trans* and higher amounts of SFAs has low fat quality, because of strong associations between consumption of these fats and the risk of heart disease. On the other hand, foods rich in PUFAs (including the n3 and n6 fatty acids) and MUFAs are of high fat quality, due to demonstrated health benefits such as reducing the risk for

cardiovascular disease [87,88]. Thus, dietary fat choices can affect the metabolic health of the whole body.

2.2 Obesity and Related Metabolic Disease

Obesity is defined as the accumulation of adipose tissue to the extent that it presents a risk to an individual's health [89,90]. Obesity is primarily an imbalance of energy metabolism, and results when energy ingested in food exceeds energy expenditure [91]. The excess energy is stored as fat in the adipose tissue. Obesity is considered a type of metabolic disease, because of its influence on energy homeostasis.

2.2.1 Epidemiology of Obesity

In Canada, more than one in four adults are obese and the prevalence of obesity has roughly doubled over the past two decades [92]. Obesity is associated with a broad spectrum of cardiovascular and metabolic disorders, including accelerated atherosclerosis, hypertension and T2D [93,94], and it contributes to increased morbidity and mortality in these conditions [95]. Obesity continues to have an enormous impact on the health and well-being of the population, and places a large economic burden on the health care system due to direct health care expenditures, obesity-related injury and illness, or pre-mature death [96].

Measuring Obesity: At the population level, obesity is usually assessed using the body mass index (BMI), which is calculated by dividing an individual's weight (kg) by height in metres (m) squared. A BMI < 18.5 kg/m² is considered underweight, a BMI of 18.5-24.9 kg/m² is within the normal range, a BMI of >25-30 kg/m² is overweight, and a BMI >30 kg/m² is obese. Obesity is further classified as severe obesity (BMI 35-40

kg/m²), morbid obesity (≥ 35 kg/m² and experiencing obesity-related health problems, or >40 - 45 kg/m²), and super obesity (>50 kg/m²) [90]. The advantages of using BMI are that it is simple to calculate, non-invasive and a strong predictor of chronic disease risk [97]. However, it is also a fairly crude measure that does not account for differences in body frame size or the distribution of lean mass and adipose tissue, and it tends to overestimate adiposity in those with more lean body mass (e.g., athletes). For this reason, additional measurements such as waist circumference, waist-to-hip ratio, or bioelectrical impedance can be taken in the laboratory or clinic to provide a more detailed interpretation of overweight or obesity. Another device used to assess obesity is the BOD POD®, a chamber that uses air displacement plethysmography to calculate whole-body density and estimate body composition [98]. Imaging methods such as dual energy X-ray absorptiometry (DEXA), magnetic resonance imaging (MRI), or computerized tomography (CT scan) can be used to accurately determine fat mass and fat distribution, although these tend to be more costly and are typically not available in the laboratory setting [97].

Determinants of Obesity: Research has identified several determinants of obesity, some of which are non-modifiable (for example, genetic predisposition) and some of which are modifiable factors. The two most important and best-studied modifiable determinants are physical activity level and diet. The main cause of the widespread increase in obesity rates is a change in lifestyle habits and environment: easy access to and overconsumption of energy-dense, highly processed foods, coupled with reduced physical activity and lack of vigorous exercise [99,100]. Consumption of a

balanced diet is one of the most critical components in maintaining a healthy weight [101,102].

Consequences of Obesity: It is well established that obesity is a cornerstone of many major metabolic disorders, including T2D, cardiovascular disease, hypertension, and fatty liver disease [103,104]. There is growing evidence linking obesity to other debilitating disorders, including neurodegenerative disease [105], airway disorders [106], and cancer [107], all of which contribute to the staggering morbidity and mortality associated with obesity.

2.2.2 Adipose Tissue Physiology

Adipose tissue is the main storage site for fat in the human body and plays an important endocrine role in maintaining energy homeostasis [108]. Two types of adipose tissue are present: white fat and brown fat, although white predominates in adult humans [109] and will be the subject of this review. Adipose tissue is composed of pre-adipocytes and mature adipocytes, vascular cells, fibroblasts, and a resident population of immune cells, including macrophages and T-cells [110]. Adipocytes are derived from mesenchymal stem cells [111], and in their mature form are nucleated, modified fibroblasts. Mature adipocytes store almost pure triglycerides in a single large lipid droplet that can occupy up to 95% of the cell volume. The remaining organelles are contained in a thin slice of cytoplasm displaced to the periphery of the cell (Figure 2.7). Adipose tissue is found in several depots throughout the body: the abdominal depot

includes visceral, omental and retroperitoneal adipose tissue; perirenal adipose tissue surrounds the kidneys; epicardial and perivascular adipose tissue frame the heart and the major blood vessels, respectively; and subcutaneous adipose tissue forms a layer under the skin.

Lipid Storage Function: Adipocytes serve as a storage depot for energy in the form of triglycerides. After a meal (when there is a positive energy balance), circulating triglycerides bound in lipoproteins are hydrolysed to glycerol and free fatty acids by the enzyme lipoprotein lipase at the luminal surface of the endothelium [2]. Fatty acid binding protein and fatty acid translocase aid in transporting the free fatty acids into the cytoplasm of adipocytes, where they are re-esterified with glycerol into triglycerides and stored in lipid droplets. The droplets are coated with the protein perilipin, which serves as a lipid droplet gatekeeper and messenger, and interacts with other molecules involved in lipid storage [112]. Insulin signalling enhances lipid storage by stimulating triglyceride synthesis and inhibiting breakdown of stored triglycerides [113]. When energy is required (e.g., during starvation or exercise), lipolysis occurs to break down stored triglycerides to glycerol and free fatty acids. Three key lipases control lipolysis: i) adipose tissue triglyceride lipase (which catalyses the hydrolysis of triglycerides into diacylglycerides); ii) hormone-sensitive lipase (which mainly breaks down diacylglycerides to monoacylglycerides, due to its high affinity for diacylglycerides); and iii) monoacylglyceride lipase, which hydrolyses monoacylglycerides to free fatty acids and glycerol [114-117]. In this manner, adipocytes act as a lipid buffer for the other

organs in the body, protecting them from excess lipid exposure and lipotoxicity, and also as an easily accessible storage depot for energy when needed.

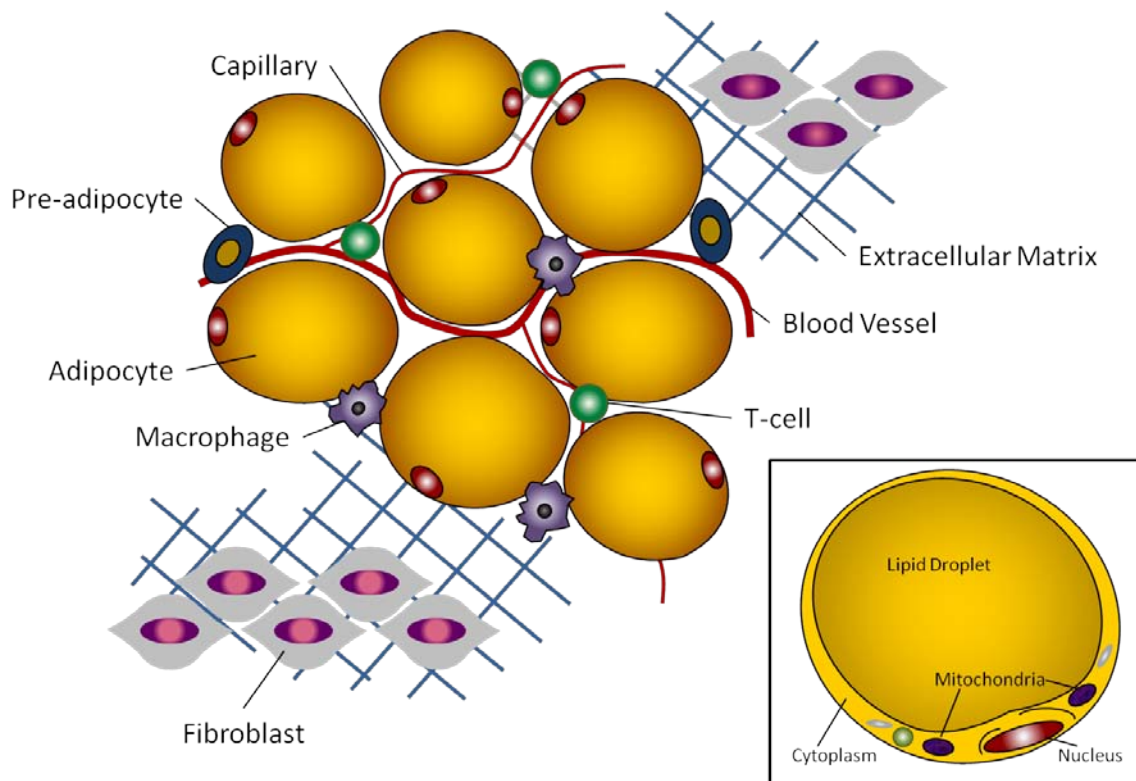


Figure 2.7 Overview of adipose tissue constituents and adipocyte structure. Mature adipocytes are primarily formed by a single large lipid droplet containing stored triglycerides. The adipose tissue also is also composed of pre-adipocytes (the precursors to adipocytes), a resident population of immune cells, including T-cells and macrophages, fibroblasts, and endothelial cells (blood vessels). Adapted from [118].

Endocrine Function: Adipose tissue is also recognized as an endocrine organ that produces and secretes a variety of bioactive molecules. Adipokines, or adipocytokines, are a diverse group of molecules consisting of over 100 different peptides, hormone-like factors and cytokines. The best-studied of these include leptin, adiponectin, resistin and visfatin. Several other metabolically important signalling molecules are produced and released by adipose tissue, such as cytokines (e.g., tumour necrosis factor (TNF)-alpha, interleukins (ILs)), cytokine-related proteins, fibrinolytic proteins, and proteins of the renin-angiotensin system [119,120].

Adipokines play a critical role in maintaining an energy balance in the body by mediating crosstalk between the adipose tissue and other key metabolic organs, including brain, liver, pancreas and skeletal muscle. For example, the adipokine leptin enters the circulation and binds its receptor in the hypothalamus to suppress appetite. Leptin is produced in proportion to the number of adipocytes in the body; thus, in people who are obese, leptin levels are high, but leptin resistance often occurs, diminishing the anorexigenic effect [121]. Another adipokine, adiponectin, acts through its receptors in skeletal muscle and liver to increase glucose uptake and reduce glucose output, respectively [122,123]. Adiponectin also has anti-inflammatory, cardioprotective, and vasoactive properties [124,125]. Serum levels of many adipokines are closely related to degree of adiposity [126,127], indicating that the synthesis and secretion of these molecules is dynamic and modifiable. It follows that dysfunctions in adipokine pathways can result in impaired organ communications and metabolic abnormalities, thereby constituting a critical pathological component in the

development of metabolic disease [128]. Table 2.1 lists several metabolically important adipokines and describes their roles in energy homeostasis.

Table 2.1 Adipokines and their Roles in Energy Homeostasis

Adipokine	Action	Response to Obesity	Reference
Leptin	Inhibits appetite, regulates hepatic lipogenesis	up	[129]
Resistin	Regulates inflammation, induces insulin resistance and endothelial dysfunction, promotes atherosclerosis	up	[130]
Adipsin	Activates complement pathway (inflammatory mediator)	up	[131]
PAI-1	Inhibits plasminogen activator (blocks fibrinolysis and break down of blood clots)	up	[132]
MCP-1	Induces insulin resistance and liver steatosis	up	[133]
TNF-α	Interferes with insulin signalling, induces inflammatory response, stimulates free fatty acid release	up	[134]
IL-1, IL-6, IL-8, IL-18	Interfere with insulin signalling, induce inflammatory response, promote atherosclerosis	up	[135]
Visfatin	Insulin-mimetic effects (improves glucose clearance from circulation)	up	[136]
Adiponectin	Regulates glucose and lipid metabolism, enhances insulin action, exerts cardioprotective effects	down	[137,138]
IL-10	Anti-inflammatory properties, improves insulin sensitivity	down	[135]

IL: interleukin; MCP: monocyte chemoattractant protein; PAI: plasminogen activator inhibitor; TNF: tumour necrosis factor

2.2.3 Pathophysiology of Obesity

Adipose Tissue Dysfunction: The chronic weight gain that characterizes obesity has pathophysiological effects on the body, and particularly the adipose tissue. In obesity, adipocytes undergo morphological changes, becoming enlarged (hypertrophy) and increasing in number (hyperplasia), in an attempt to cope with the higher demand for triglyceride storage [139]. Both hypertrophy and hyperplasia contribute to the increase in adipose tissue mass. The development of obesity requires a high degree of plasticity at the cellular and tissue level, and includes remodelling of the extracellular matrix and vasculature as the body tries to compensate for a greater need for nutrient and oxygen exchange. As the storage capacity of adipocytes reaches its maximum, serum levels of free fatty acids become elevated, which can lead to other metabolic complications [140]. In most subjects, obesity is associated with marked changes in the secretory function of visceral adipocytes, macrophages and other cells of the adipose tissue, and with chronic low-grade inflammation (Table 2.1) [139]. These processes form the basis of adipose tissue dysfunction, a state of dysregulated interaction between the adipose tissue and environmental factors in response to the stress of excess lipid storage.

Adipose tissue actively participates in inflammation and immunity through the production and release of proinflammatory molecules (e.g., leptin, TNF- α , interferon- γ , IL-6, IL-18) and anti-inflammatory molecules (e.g., adiponectin, IL-10). Both the resident cells of adipose tissue and infiltrating immune cells (T-cells and macrophages) produce these bioactive substances. T-cells are early mediators of the inflammatory response in

adipose tissue, stimulating pre-adipocytes to recruit macrophages via release of chemotactic factors such as monocyte chemoattractant protein (MCP)-1 [141]. The number of macrophages present in adipose tissue is positively associated with increasing body weight, BMI and total body fat [142]. This is important, since macrophages have been identified as a major source of proinflammatory IL-6 and TNF- α [143,144], which contribute to the chronic systemic inflammation and insulin resistance observed in obesity. Macrophages may modulate the phenotype of adipocytes, altering their secretory functions [144,145], and further contributing to the dysregulation of this tissue.

Insulin Resistance and T2D: Insulin resistance, i.e., the blunting of the normal cellular response to insulin, develops in response to obesity-driven metabolic disturbances [146]. For example, high serum levels of free fatty acids induce increased hepatic lipid and glucose synthesis, and contribute to insulin resistance in the liver and peripheral tissues [147,148]; free fatty acids, such as the SFA palmitate, also serve as ligands for toll-like receptor 4 (TLR4) [149] and stimulate cytokine production by adipose tissue macrophages [150], thereby driving local and systemic inflammation; and some adipokines that are up-regulated in obesity activate molecular signalling pathways with direct effects on insulin resistance, including TNF- α , which interferes with the insulin signalling pathway [151], and transforming growth factor- β , which links obesity to insulin resistance through the Smad3 signalling pathway in liver and adipose tissue [152]. Obesity and insulin resistance are major predictors for the development of T2D [153]. T2D is characterized by hyperglycemia and hyperinsulinemia in the early stages of

the disease, progressing to pancreatic β -cell exhaustion and dysfunction, with subsequent hypoinsulinemia in later stages [154]. A myriad of complications are associated with T2D, including increased risk of cardiovascular disease (coronary heart disease, stroke and atherosclerosis), blindness, kidney failure, and lower limb amputation [154].

Cardiovascular Disease: Cardiovascular disease is a general term for pathologies of the heart and the vasculature. Obesity and obesity-related complications are important determinants for cardiovascular disease [155]. Several decades of research demonstrate that obesity is an independent predictor of coronary death, coronary heart disease, heart failure and stroke [156-161], and that it accelerates the progression of atherosclerosis [162]. Obesity is also linked to hypertension [163], which together with other deleterious hemodynamic effects on the heart increases the risk for congestive cardiac failure [157]. In patients with established vascular disease, obesity is an independent risk factor for major cardiovascular events [164].

2.2.4 Animal Models of Obesity

A number of animal and cell culture models are available to researchers for the study of the pathophysiology of obesity. Only the models relevant to this work will be described here, although there are several other widely used models that have contributed significant findings to the area of obesity research, details of which have been the subject of recent reviews [165,166].

The Obese (fa/fa) Zucker Rat

The obese (*fa/fa*) Zucker rat is a monogenic model of obesity with a defect in the leptin signalling pathway. A mutation in the leptin receptor desensitizes the animal to the actions of leptin in the hypothalamus, where its normal function is to signal satiety. As a result, *fa/fa* Zucker rats develop a phenotype of hyperphagia and reduced energy expenditure despite high levels of circulating leptin, and exhibit adipocyte hyperplasia and hypertrophy, leading to morbid obesity [167,168]. They are also hyperlipidemic and have impaired glucose tolerance (insulin resistance). The control animal for this model, the lean Zucker rat, has a leptin receptor which is fully functional. Obese (*fa/fa*) Zucker rats are valuable for the study of the development, etiology, associated pathologies and possible mechanisms of early-onset obesity, as they exhibit many of the traits common to human obesity, but do not develop overt diabetes [169].

The Diet-Induced Obese (DIO) Rat

Outbred strains of the Sprague-Dawley, Wistar, Long Evans and Lewis rats are used as polygenic models of obesity. When exposed to a high-energy diet (often high-fat

or high-sucrose), many rats become DIO, while others maintain a body weight trajectory similar to that of control rats on a low-energy diet. The former are known as obese prone (OP), while the latter are called obese resistant, although the genetic variation resulting in the phenotypic differences between the strains is not well defined. Numerous studies demonstrate that high-energy diets not only promote obesity in OP rats, but also cause characteristics of obesity that are typically seen in humans, such as whole body insulin resistance, hyperlipidemia, elevated insulin levels and decreased adiponectin levels [170]. High-fat feeding in these animals also alters cell and organ function, causing changes in adipocyte morphology and metabolism, impairing insulin-stimulated glucose uptake in muscle, promoting hepatic steatosis, and elevating inflammatory gene expression [170]. For these reasons, DIO models may be more representative of human obesity than monogenic models, since diet and energy excess are major contributors to the development of obesity in an undefined polygenic background. Limitations of the use of DIO models include the rate at which the animals develop obesity (typically longer than genetic models) and the lesser severity of the related outcomes.

In summary, the metabolic disturbances caused by obesity have a broad impact on health, and contribute in a major way to the risk of developing further complications. As will become evident in the following section, the common themes of immune dysregulation and stress due to excess energy form the basis not only for imbalances in metabolism, but also for the development of cardiovascular disease.

2.3 Cardiovascular Disease

Cardiovascular disease is a broad class of disorders involving the heart, the blood vessels (arteries, veins and capillaries), or any part of the cardiovascular system. Many types of cardiovascular disease exist, including hypertension (high blood pressure), heart failure (failure of the heart to maintain blood flow to meet the body's needs), atherosclerosis (stiffening of the arteries), and subtypes of coronary heart disease and cerebrovascular disease (stiffening of arteries leading to the cardiac muscle and the brain, respectively). Cardiovascular disease is the leading cause of death worldwide [171]. Although it mainly affects older adults, many risk factors are established in early life and impact the rate at which the disease develops [172].

2.3.1 Overview of Atherosclerosis

The focus of this dissertation will be on a subtype of cardiovascular disease called atherosclerosis. Atherosclerosis manifests as lesions on the arterial wall, causing the blood vessel to stiffen and narrow [139]. In this way, atherosclerosis causes dysregulation of blood vessel tone (vasoconstriction and vasodilation), affects barrier function of the endothelial lining (permeability), and promotes inflammatory and thrombotic/fibrinolytic processes. In the early stages, atherosclerosis is characterized by endothelial dysfunction as the result of an inflammatory response to an insult to the vascular endothelium due to shear stress, hypertension, infection, free radicals, elevated or modified serum lipoprotein levels, toxins from smoking, or a combination of these and other risk factors [139]. The endothelium becomes more adhesive and

permeable to inflammatory cells such as leukocytes and platelets, and begins to produce a different profile of vasoactive molecules, cytokines and growth factors. These signalling molecules steadily recruit monocytes to the site of injury, which infiltrate the sub-endothelial space and differentiate into macrophages [139]. Due to the elevated permeability of the endothelium, lipoproteins (particularly the cholesterol- and triglyceride-rich LDL) become trapped in the vascular wall, where they undergo oxidative modification by free radicals released from damaged endothelial cells [173]. Oxidation of LDL can occur through enzymatic oxidation or through interactions with reactive oxygen species, and may involve modifications to both protein and lipid components [174]. These modifications increase the affinity of the LDL molecule for scavenger receptors on macrophages [174]. Macrophages in the sub-endothelial space internalize oxidized LDL and rapidly become engorged. These macrophages are then classified as foam cells, due to their lipid-laden “foamy” appearance. Foam cells form a fatty streak in the arterial wall, the earliest visible form of atherosclerosis. As the disease progresses, cholesterol and foam cells continue to accumulate, and prolonged oxidative stress and inflammation contribute to endothelial dysfunction.

Normal physiological endothelial function depends on a delicate balance between pro-oxidative and anti-oxidant mechanisms in the vascular wall. In the healthy state, these mechanisms favour the production of nitric oxide, a critically important signalling molecule in many metabolic pathways. An imbalance in production or bioavailability of nitric oxide or its downstream signalling molecules is implicated in the pathogenesis of endothelial and vascular smooth muscle dysfunction [175].

Haptoglobin, an acute phase protein produced primarily by hepatocytes, is a biomarker for oxidative stress and a risk factor for cardiovascular disease. Haptoglobin irreversibly binds free hemoglobin in the blood, inhibiting heme iron release and protecting the tissues from oxidative damage. Unbound hemoglobin is also a potent scavenger of nitric oxide. Low levels of haptoglobin are thus indicative of hemolysis and a pro-oxidative environment [176].

In progressively later stages of atherosclerosis, the cytokine-rich environment in the sub-endothelial space stimulates vascular smooth muscle cell migration to a position over the lipid-rich core of the lesion and the muscle cells divide continuously, causing the lesion to bulge into the lumen of the vessel and partly obstruct blood flow [177]. The enlarging lesion interferes with nutrient exchange in the cells that make up the vessel wall around the plaque, leading to degeneration of the vessel wall. The damaged area is infiltrated by fibroblasts, which form a cap of collagen-rich connective tissue over the plaque, thereby stabilizing it. Calcium deposition occurs throughout lesion formation, contributing to stenosis of the vessel. These end-stage lesions are influenced by inflammatory cytokines and are constantly remodelled. Atherosclerotic lesion rupture is associated with a combination of biomechanical forces that are dependent on the thickness of the fibrous cap, the thickness of the lipid core region and positive remodelling of the plaque [178,179].

The clinical manifestations of atherosclerosis can vary, depending on the site where the plaques develop. Myocardial infarction can occur if plaques form in the coronary arteries and reduce or partly occlude the blood flow to the heart muscle.

Alternatively, a stroke may occur if a plaque ruptures and a blood clot blocks a narrowed, hardened vessel supplying the brain. Other manifestations of atherosclerosis include kidney failure (renal arteries), aortic aneurisms (aorta), and PAD (arteries of the lower extremities) [180]. The remainder of this section will focus on PAD as a representative atherosclerotic disease.

2.3.2 Peripheral Arterial Disease

Introduction to PAD: PAD, also known as peripheral vascular disease, is an atherosclerotic disease that causes stenosis (narrowing) of non-coronary and non-cerebral arteries. It affects approximately 800,000 Canadians and as many as 29% of the elderly [181-183]. The incidence of PAD increases with age and exposure to atherosclerotic risk factors, but the disease tends to be underdiagnosed and undertreated because the symptoms are often silent (up to one-half of all individuals with PAD are asymptomatic) [184-186]. Patients who develop symptoms usually present with intermittent claudication (aching pain, numbness, weakness or fatigue in the muscles of the legs) [187]. Critical limb ischemia is its most severe manifestation, thus, PAD can lead to loss of limbs or death if not treated promptly.

Diagnosing PAD: The primary symptom of PAD is intermittent claudication, brought on by exercise and relieved by rest; this prevents patients from walking more than short distances and negatively impacts quality of life [187]. The current gold standard technique for diagnosing PAD is the ankle-brachial index (ABI). The ABI is

obtained by measuring systolic blood pressure at the ankle and at the brachial artery (arm) while the individual is at rest, and then dividing ankle pressure by brachial pressure. Normal resting ABI is 1.0 to 1.4; an ABI of <0.9 indicates the presence of PAD [188]. Other methods of diagnosing PAD include imaging techniques, such as Doppler ultrasound and magnetic resonance angiograms, to detect the location and severity of an obstructed blood vessel. Treadmill testing offers information about the severity of the symptoms in response to a particular level of exercise, and provides functional measures of pain-free walking distance (the distance travelled until onset of claudication) and maximum walking distance (the total distance the individual can walk). Although claudication distances can be inaccurate as an absolute measure of disease severity, a change in perceived walking ability over time may be a useful indicator of clinical improvement or deterioration [187].

Determinants of PAD: The risk factors for PAD parallel the classical determinants of atherosclerotic disease and involve both non-modifiable and modifiable risk factors. Non-modifiable risk factors include sex, age and family history of cardiac or vascular disease. PAD occurs mainly in older adults, and its incidence increases with advancing age. It is commonly reported as being twice as prevalent in men as in women [181]. The most significant modifiable risk factor for PAD is smoking [189]; smoking increases the risk of lower extremity PAD by two- to six-fold, and intermittent claudication by three- to ten-fold. There is a threefold increase in amputation and early death in people with claudication [190,191]. Obesity, dyslipidemia, hypertension, T2D, and impaired glucose intolerance are also considered important modifiable risk factors [192]. T2D in particular

increases the risk of PAD and intermittent claudication [190,193,194], drives disease progression, and increases the risk of complications, such as major amputation [195,196].

Consequences of PAD: PAD is associated with an exceptionally high risk of coronary heart disease due to the underlying presence of atherosclerotic disease, and adverse cardiac events are the most common cause of death in PAD patients [197]. PAD is also linked to a decline in cognitive function [198]. PAD has a relatively positive prognosis, with more than 70% of diagnosed individuals remaining stable or improving with risk factor management [199], but the symptoms of PAD can cause considerable impairment in quality of life, due to the associated limited mobility and decline in social activities [200,201].

2.3.3 Management Strategies for PAD

PAD is a chronic condition with a substantial impact on mobility, morbidity and mortality if risk factors and symptoms are not managed from the outset. Treatment addresses the relief of symptoms (intermittent claudication), improves quality of life, and reduces the risk of cardiovascular complications.

Risk Factor Modification: Risk factor modification is the primary means for halting or slowing the progression of PAD and reducing the risk of cardiovascular events [197]. This includes smoking cessation, weight reduction in overweight and obese patients, and exercise. Exercise has been shown to maximize walking distance and

positively impact quality of life [202]. Management of hypertension and hyperlipidemia via lipid modifying therapy is also a priority [203].

Pharmacological Interventions: Pharmacological agents to control risk factors are commonly prescribed: antiplatelet therapy (aspirin or clopidogrel) to improve vasodilation and inhibit platelet aggregation; smoking cessation therapy; insulin or insulin sensitizing drugs to improve glycemic control and glycated hemoglobin levels; anti-hypertensives (angiotensin-converting enzyme inhibitors) to lower blood pressure; and cholesterol-lowering therapies (statins) to reduce the risk of cardiovascular events [204]. A number of clinical trials have assessed pharmacological interventions for intermittent claudication, aimed at improving the hemorrheologic properties of the blood and increasing vasodilation. These drugs, which include pentoxifylline, cilostazol, naftidrofuryl, and buflomedil, and their respective efficacies in improving intermittent claudication, are described in detail in an excellent review [204]. However, among pharmacological agents, only cilostazol has a class IA recommendation according to current guidelines, having demonstrated benefit in both walking distance and quality of life [204].

Vascular Surgery: A vascular surgical intervention tends to be the final treatment option for individuals with PAD, but may be necessary for those with severe disease, lifestyle-limiting claudication, and/or tissue loss [204]. Angioplasty and stenting are minimally invasive procedures to improve blood flow through a partially blocked artery. Bypass surgery may be necessary for more severe blockages.

Dietary Supplements: The use of dietary supplements has also been studied in relation to improving intermittent claudication and walking distance. For example, carnitine functions as a metabolite in pathways of energy production and balance in skeletal muscles, and L-arginine is a mediator of nitric oxide production reported to improve vasoreactivity. Both of these supplements are recommended by the American College of Cardiology-American Heart Association for use in patients with intermittent claudication [189]. The benefit of anti-oxidative substances, such as vitamin E, has been difficult to establish due to inconsistencies in trial design, methods and outcomes [205].

N3 PUFAs in Cardiovascular Disease: Over the last three decades, a large body of evidence has accumulated to support a potential protective role for n3 PUFAs in coronary heart disease and other cardiovascular complications [206]. However, clinical trials of n3 PUFA supplementation in individuals who are healthy, at-risk or have established cardiovascular disease have yielded conflicting results, making it difficult to determine whether these fatty acids are truly beneficial in improving cardiovascular health. The majority of prior studies have focused on the marine n3 PUFAs, EPA and DHA. Some systematic reviews and meta-analyses have not found a significant benefit of n3 PUFA supplementation in reducing the occurrence of major cardiovascular endpoints, including all-cause mortality, cardiac death, myocardial infarction, stroke or atrial fibrillation [207,208], while others report moderate protective effects [209] or reduction in risk factors, such as inflammatory markers [210], hypertension [211] or hypertriglyceridemia [212]. It should be noted that most of the trials measuring patient-centred outcomes (i.e. cardiac events, death) have not found significant benefits with n3

PUFA supplementation, while other trials reporting surrogate markers or risk factors have found improvements. The findings on risk factor modification form the basis for experimental studies to investigate the mechanisms by which n3 PUFAs act in the body; however, research on the efficacy of n3 PUFAs in cardiovascular disease should ultimately focus on clinically relevant endpoints.

In addition to the effects of marine PUFAs, the plant-based n3 PUFA ALA, which is found in products such as canola oil, flaxseed and walnuts, may also confer protection from adverse cardiac events. ALA is the metabolic precursor to EPA and DHA [213-216]. A recent systematic review and meta-analysis of observational studies examining the effects of ALA reported a moderately lower cardiovascular disease risk with higher ALA exposure [217]. However, the reviewers emphasized the need for large high-quality RCTs to establish stronger evidence for the role of ALA in cardiovascular protection.

Studies examining the potential benefits of n3 PUFAs in the high-risk population of PAD patients are scarce. However, a meta-analysis of 10 trials showed that marine n3 PUFA supplementation significantly reduced measures of arterial stiffness (arterial compliance, cardio-ankle pulse wave velocity and ankle-brachial pulse wave velocity) in several cohorts, including healthy individuals, individuals with cardiovascular risk factors, T2D, hypertension and individuals who were overweight [218]. A Cochrane systematic review and meta-analysis of six trials of marine and plant n3 PUFA use in patients with intermittent claudication reported no significant improvement in symptoms [219]. Taking into consideration the findings summarized here and above,

further investigation into the potential of n3 PUFAs as therapeutic agents for PAD is warranted.

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CHAPTER 3: Rationale and Hypotheses

3.1 Rationale

In today's modern world, nutrition research is focused on promoting health, and preventing and managing chronic disease through diet [1]. Improper diet is considered one of the major factors contributing to the rapid increase in the incidence of metabolic disorders such as obesity and cardiovascular disease [2]. In Canada, more than one in four adults are obese and the prevalence of obesity has roughly doubled over the past two decades [3]. The burden of vascular disease, including atherosclerotic manifestations such as PAD, is likewise high, with over 10 million people affected in North America [4]. The consequences of these conditions are severe: increased morbidity and mortality, complications from related diseases, reduced quality of life, and a huge cost to the healthcare system [5,6].

Much of the research in this field examines approaches to prevent and slow the progression of metabolic and vascular disease, and many studies focus on dietary fat. The type of fat in the diet is an important factor in maintaining metabolic balance, since what is consumed determines which fatty acids are available as metabolites, signalling entities and structural components [7]. Thus, dietary fat choices can affect the metabolic health of the whole body. The term “fat quality” has been used to describe the fatty acid composition of the diet [8,9]. A diet containing *trans* fat and higher SFA has low fat quality, because of strong associations between consumption of these fats and cardiovascular disease. On the other hand, foods rich in MUFAs and PUFAs (including many plant-based oils) are of high fat quality, due to demonstrated health

benefits such as reducing cardiovascular disease risk [10], and in the case of n3 fatty acids, exerting anti-inflammatory effects [11].

The fatty acid profiles of flaxseed oil and canola oil (low in SFAs, high in MUFAs and PUFAs and a good source of the n3 fatty acid ALA) are recognized to have many nutritional advantages [12]. However, current dietary recommendations for type and amounts of fatty acids are largely based on cardiovascular disease [13]. Further research is required to demonstrate whether plant-based oil consumption confers benefits in metabolic disease or other types of vascular disease, specifically in regards to blood vessel function. Moreover, although n3 fatty acids sourced from fish oil have been well-studied, very few studies have used dietary interventions with plant-based n3 fatty acid-rich foods such as canola oil or flaxseed oil to assess their effects on vascular function and obesity-related parameters. Thus, the goal of this thesis is to investigate whether dietary plant-based oils containing ALA can improve outcomes in obesity and PAD.

3.2 Theoretical Framework

The theoretical framework for the research is depicted in Figure 3.1. In order to investigate whether the n3 fatty acid ALA from plant-based oils has protective effects in metabolic disease and vascular disease, we designed dietary interventions with different sources of ALA in animal models and in humans. Pre-clinical studies used animal models of metabolic disease. We then turned to clinical research to examine whether our promising findings in animal models would translate to humans.

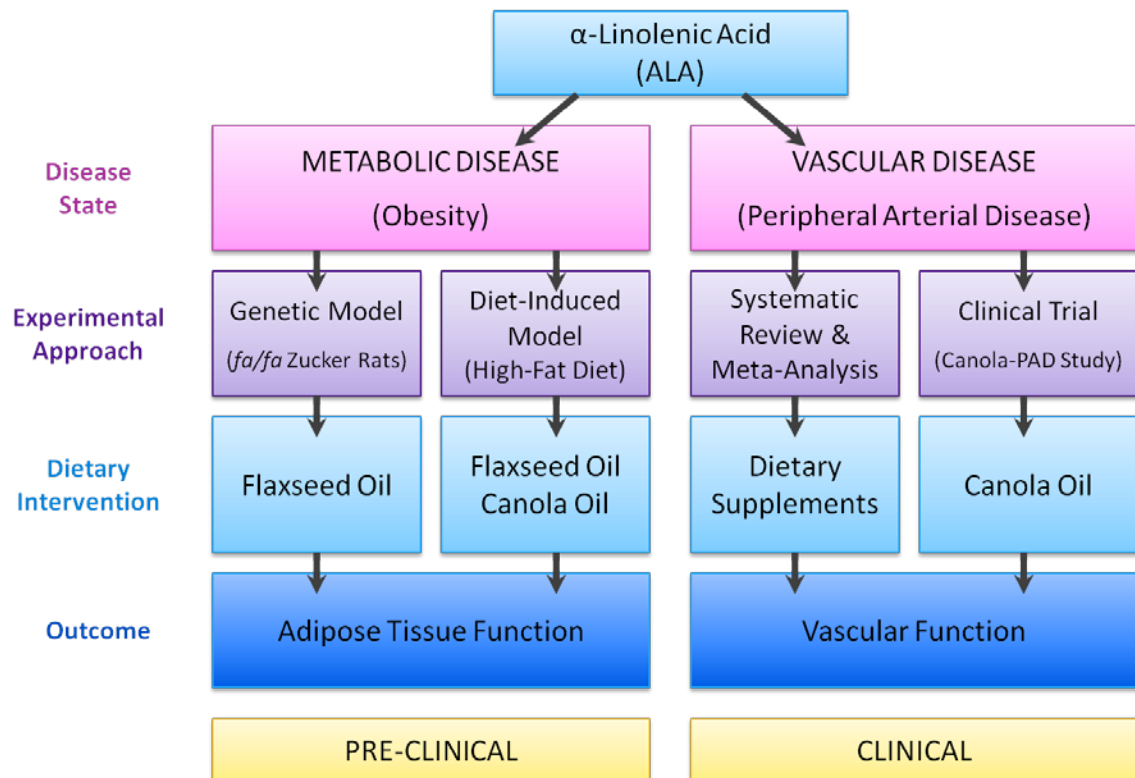


Figure 3.1 The theoretical framework for the research.

3.3 Hypotheses and Objectives

The overarching hypothesis of this body of research is:

The n3 fatty acid alpha-linolenic acid from dietary plant-based oils confers protection against the pathophysiological consequences of metabolic and vascular disease.

This hypothesis was tested using the following detailed approaches:

- 1) Plant-based oils rich in n3 fatty acids (flaxseed oil and canola oil) improve adipose tissue function in obesity (Chapter 4 and Chapter 5).

Objectives:

- To determine whether flaxseed oil containing the n3 fatty acid ALA is an effective modulator of adipose tissue function in obese *fa/fa* Zucker rats by measuring adipocyte size, circulating and adipose tissue adipokines and cytokines, and immune cell infiltration.
- To investigate the effects of plant-based oils with different fatty acid compositions on adipose tissue function in DIO rats by measuring circulating and adipose tissue adipokines and cytokines, expression of proteins involved in fatty acid uptake and metabolism, and fatty acid composition.

- 2) Supplementation with n3 fatty acids improves cardiovascular outcomes in individuals with PAD (Chapter 6).

Objectives:

- To conduct a systematic review and meta-analysis of the literature on the effect of n3 fatty acid supplementation in individuals with PAD.
- To inform the design of a clinical trial that tests the effects of canola oil containing the n3 fatty acid ALA on vascular parameters and serum biochemistry in individuals with PAD.

- 3) A dietary intervention using canola oil improves vascular parameters in individuals with PAD (Chapter 7 and Chapter 8).

Objectives:

- To describe the rationale and design of a RCT evaluating the effect of canola oil on blood vessel function in individuals with PAD.
- To determine the effects of a canola oil intervention on vascular function in a population with PAD by measuring arterial stiffness and endothelial function, anthropometrics, blood pressure, plasma lipid profile, glycemic indicators, serum markers of oxidative stress and inflammation, and fatty acid composition.

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CHAPTER 4

Dietary Flaxseed Oil Reduces Adipocyte Size, Adipose Monocyte Chemoattractant Protein-1 Levels and T-Cell Infiltration in Obese, Insulin-Resistant Rats

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4.1 Contribution Statement

I, Jennifer Enns, made the following contributions to this study:

- conducted the Western blotting analyses in adipose tissue for Figure 4.1 (C-F), Figure 4.2 (A-C), Figure 4.3 and Figure 4.4
- cryosectioned the adipose tissue and conducted immunohistochemical analyses
- performed statistical analyses for above-mentioned data
- interpreted results together with those from other contributors
- contributed approximately 50% to writing the manuscript together with Maria Baranowski, such that we were listed as co-first authors

Other contributors:

- Maria Baranowski
- Heather Blewett
- Uma Yakandawala
- Peter Zahradka
- Carla G. Taylor

4.2 Abstract

Adipocyte dysfunction is characterized by an increase in adipocyte size and changes to their adipokine profiles. Immune cell infiltration into adipose tissue is thought to contribute to the metabolic complications of obesity, with local and systemic consequences for the inflammatory status of the obese individual. Dietary interventions with n3 fatty acids from marine sources have been successful at reducing inflammation. The aim of this study was to determine whether flaxseed oil containing the plant-based n3 fatty acid ALA is an effective modulator of inflammation and adipocyte dysfunction.

Seventeen-week old male *fa/fa* and lean Zucker rats were fed a control diet (faCTL, InCTL) and *fa/fa* rats were fed an ALA-rich flaxseed oil supplemented diet (faFLAX) for 8 weeks. Adipose tissue and serum were collected and analyzed for cytokine (IL-6, IL-10, IL-18, IL-2, interferon [IFN]- γ , TNF- α), haptoglobin, MCP-1 and adipokine (leptin, adiponectin) levels. Splenocytes were isolated and *ex vivo* mitogen-stimulated cytokine production was measured. Digital images of adipose tissue sections were used to quantify adipocyte area. Macrophage and T-cell infiltration were assessed in adipose tissue by immunohistochemistry.

FaFLAX rats had 17% smaller adipocytes and 5-fold lower MCP-1 levels in adipose tissue than faCTL rats. Adipose tissue levels of IL-10 were 72% lower in the faFLAX group compared to baseline, and TNF- α levels decreased 80% (equal to InCTL levels) in the faFLAX group compared to faCTL. There were no changes in *ex vivo* cytokine production by splenocytes between faFLAX and faCTL. Macrophage infiltration was not different among groups; however, faFLAX rats had less T-cell infiltration than faCTL rats.

Dietary intervention with ALA-rich flaxseed oil in obese Zucker rats reduced adipocyte hypertrophy, protein levels of inflammatory markers MCP-1 and TNF- α , and T-cell infiltration in adipose tissue. Modest improvements to other parameters of obesity were also observed. The results suggest that, due to its ability to improve adipocyte function, ALA-rich flaxseed oil confers health benefits in obesity.

4.3 Introduction

Obesity is associated with low-grade inflammation resulting from chronic activation of the innate immune system [1]. Inflammation has been linked to the pathogenesis of several other chronic diseases, including metabolic syndrome, cardiovascular disease, T2D and cancer [2,3]. Adipose tissue actively participates in inflammation and immunity through the production and release of proinflammatory molecules (e.g., leptin, TNF- α , IFN- γ , IL-6, IL-18) and anti-inflammatory molecules (e.g., adiponectin, IL-10) from both the adipocytes and infiltrating immune cells [4]. Obesity results in increased adipocyte size (hypertrophy) and adipose tissue dysfunction, creating an imbalance of proinflammatory and anti-inflammatory factors which can alter the immune response and inflammatory status both locally and systemically. For example, increased fat mass and larger adipocytes lead to elevated circulating leptin concentrations and leptin, in turn, exerts proinflammatory actions by activating various immune cells [5].

The macrophages that infiltrate adipose tissue are a major source of proinflammatory cytokines, including TNF- α and IL-6 [6,7]. Recent research indicates that T-cells also play a prominent role in adipose tissue inflammation and the development of obesity [8]. In fact, it has been suggested that T-cells are among the first immune cells to enter the adipose tissue and may stimulate pre-adipocytes to recruit macrophages via release of factors such as MCP-1 [9].

N3 fatty acids are considered to be anti-inflammatory and thus may counteract some of the damaging effects of the obese condition. Higher intakes of dietary n3 fatty

acids are associated with reduced lymphocyte proliferation [10,11] and Th1 cell development [12], lower circulating levels of leptin [13-15], C-reactive protein [16-20], and other proinflammatory cytokines [12,16,17,20], as well as a lower risk of infection [21]. The majority of studies investigating n3 fatty acids in relation to immune function and inflammation have used dietary fish oil as the source of n3 fatty acids (i.e. EPA and DHA) [22]. Alternatively, plant-based sources of n3 fatty acids contain ALA which can be elongated and desaturated to EPA and DHA [22]. Whether intervention with dietary ALA is an effective modulator of inflammation or adipose dysfunction associated with obesity is unknown.

The *fa/fa* Zucker rat is a model of obesity with a defect in the leptin receptor, and as such, exhibits leptin resistance, adipose tissue dysfunction and impaired immune function [23]. We hypothesized that dietary intervention with n3 fatty acids in the form of ALA-rich flaxseed oil would promote a decrease in proinflammatory markers and an increase in anti-inflammatory markers in *fa/fa* Zucker rats, thereby leading to improved immune function and a healthier adipocyte phenotype in an obese state. Thus, the objective of this study was to investigate the effects of dietary ALA-rich flaxseed oil on the adipose tissue by assessing pro- and anti-inflammatory markers (splenocyte *ex vivo* cytokine production, circulating and adipose tissue levels), adipocyte size, and macrophage/T-cell infiltration in adipose tissue of obese *fa/fa* rats.

4.4 Materials and Methods

Animals and diets

After a 5-8 day acclimatization period, 17-week old male *fa/fa* Zucker rats (Harlan, Indianapolis, IN) were randomly assigned to a baseline group (faBASE; n=7), from which tissues were collected and stored immediately, or groups fed a control diet (faCTL; n=7) or an ALA-rich flaxseed oil diet (faFLAX; n=7) for 8 weeks; lean Zucker rats were assigned to the control diet (lnCTL; n=7) for 8 weeks. The diet formulations were based on the AIN-93G diet [24] (Table 4.1). Although the fatty acid composition of the diets differed, the proportions of PUFAs, MUFAs and SFAs were similar (Table 4.1). Fresh batches of the diet were prepared weekly and stored at -20°C; rats were given fresh feed 3 times per week. Feed intake (corrected for spillage) and weekly body weights were recorded. The University of Manitoba Protocol Management and Review Committee approved the protocol for the animal care procedures and the experimental procedures were in accordance with Canadian Council on Animal Care guidelines [25].

Table 4.1 Diet Formulation and Fatty Acid Profile

Diet Ingredients^a (g/kg)	FLAX diet	CTL diet
Cornstarch ^b	363	363
Maltodextrin	132	132
Sucrose	100	100
Egg white	212.5	212.5
Cellulose	50	50
AIN-93G-MX Mineral mix	35	35
AIN-93-VX Vitamin mix	10	10
Choline	2.5	2.5
Biotin mix ^c	10	10
Tert-butylhydroquinone ^d	0.014	0.014
Soy oil	42.5	85
Flaxseed oil ^e	42.5	0
Fatty Acid Composition^f		
Saturated fatty acids	12.2	15.8
C16:0	7.5	10.5
C18:0	3.9	4.2
Monounsaturated fatty acids	19.1	22.5
C18:1	18.8	22.1
Polyunsaturated fatty acids	67.3	59.9
C18:2 (n6)	33.4	51.6
C18:3 (n3)	33.7	8.0
PUFA:SFA	5.5	3.8
n6:n3	1.0	6.4

^aIngredients from Harlan Teklad (Madison, WI) unless otherwise indicated

^bCornstarch from Castco Inc. (Etobicoke, ON)

^c200 mg biotin/kg cornstarch because egg white was the protein source

^dTert-butylhydroquinone from Sigma-Aldrich (St. Louis, MO)

^eFlaxseed oil from Omega Nutrition Canada Inc. (Vancouver, BC)

^fg/100 g total fatty acids as analyzed by gas chromatography. Only fatty acids present in amounts >1% are included.

Serum and tissue collection

After 8 weeks, rats were fasted overnight and euthanized by CO₂ asphyxiation and decapitation. Trunk blood was collected, placed on ice and centrifuged to separate serum which was stored at -80°C. Dissected peri-renal and epididymal adipose tissue was weighed; a small portion was placed in Cryo-Gel embedding medium (Instrumedics Inc., St. Louis, MO) and frozen in a dry ice/ethanol bath, while the other portion was frozen in liquid nitrogen; both were stored at -80°C. Dissected spleens were weighed and processed immediately.

Splenocyte isolation and stimulation

Single-cell suspensions of spleen were prepared by pressing tissues through nylon screens into sterile Krebs Ringer Bicarbonate Buffer (130 mM NaCl, 10 mM HEPES [pH 7.4], 5.2 mM KCl, 1.4 mM CaCl₂, 1.0 mM NaH₂PO₄, 1.4 mM MgSO₄ and 10 mL antibiotic/antimycotic) containing 0.5% bovine serum albumin (Sigma-Aldrich). Red blood cells in the spleen cell suspensions were lysed by resuspending the splenocytes in 1-2 mL ammonium-chloride-potassium buffer (155 mM NH₄Cl, 10 mM KHCO₃, 0.1 mM ethylenediaminetetraacetate, pH 7.4). Cell concentration was determined using a haemocytometer. Splenocytes (1.25×10^6 cells/mL) were suspended in cell culture medium (RPMI 1640 [Sigma-Aldrich, St. Louis, MO] pH 7.2 supplemented with 5% heat-inactivated fetal calf serum [Invitrogen Corporation, Burlington, ON], 25 mL HEPES buffer, 10 mL antibiotic/antimycotic [Invitrogen] and 4 µL 2-mercaptoethanol) and stimulated (Stim) with 2.5 mg/L concanavalin A (ConA; Catalogue #L2630, Sigma-Aldrich) by incubating cells at 37°C in a 5% CO₂ and 95% humidified air incubator for 48 hours. A

set of splenocytes were incubated without mitogen as controls (un-stimulated; Unstim). After incubation, samples were centrifuged and supernatants were stored at -80°C until analyzed for cytokine production.

Splenocyte cytokine analysis

IL-6 and IL-10 levels in cell culture supernatant fractions were determined simultaneously using a fluorescently labeled microsphere bead array kit (Rat Cytokine 6-Lincoplex Kit; Millipore Corporation, Billerica, MA) on a Lincoplex 200 (Luminex Corporation, Austin, TX).

Serum biochemistry and fatty acid analyses

Fasting serum leptin and adiponectin were measured by ELISA (Alpco, Salem, NH) and serum haptoglobin was measured with a spectrophotometric kit (Tri-Delta Diagnosis, Wicklow, Ireland). Fatty acid composition of diet samples and splenocyte phospholipids (PL) was conducted as previously described [26] using methanolic HCl as the methylating agent and a Shimadzu GC-17A Gas Chromatograph (Shimadzu, Guelph, ON) with a flame ionization detector and a DB-225MS capillary column (30 m × 0.25 mm I.D. with 0.25 µm film thickness).

Western blot analysis

The bicinchoninic acid assay (Thermo Scientific, Rockford, IL) was used to determine total protein in samples isolated from frozen peri-renal adipose tissue using a mortar and pestle and 3× sodium dodecyl sulfate sample buffer. Adipose samples (15 µg protein) were analyzed by Western blotting as described previously [27] with antibodies

diluted 1:1000. Antibodies used included leptin, IL-18 (Santa Cruz Biotechnology Inc., Santa Cruz, CA), adiponectin (Calbiochem, Gibbstown, NJ), IL-6, IL-10 (Invitrogen), p42-44 mitogen-activated protein kinase (MAPK), TNF- α (Cell Signaling Technology Inc., Danvers, MA), F4/80, CD3, eEF2, MAPK and glyceraldehyde 3-phosphate dehydrogenase (Abcam, Cambridge, MA). The marker p42-44 MAPK was used as a loading control in adipose tissue, as levels have been reported to be stable during adipogenesis and in mature adipocytes [28]. The antibody for p42-44 MAPK from Cell Signaling recognizes and binds both p42 and p44 MAPK, resulting in two distinct bands on the blot. Autoradiography and scanning densitometry with Quantity One image analysis software (BioRad, Mississauga, ON) were used to capture and quantify band intensities. Western blot data were expressed as arbitrary units by calculating the ratio of the protein of interest to a house-keeping gene on the same blot. When multiple gels were used, the calculation incorporated an internal control included on all gels.

Adipocyte size

Ten μm thick sections of epididymal and peri-renal adipose tissue were prepared using a cryotome (Thermo Shandon Limited, Astmoor, UK). Digital images were captured with a light microscope fitted with a camera (Olympus BH-2, Carsen Group Inc., Markham, ON) at 200 \times magnification using QCapture Pro software (version 5.1, QImaging Corporation, Surrey, BC). Cell area (μm^2) of 125 adipocytes per treatment group, ~25 adipocytes per rat, was measured with the open-source image analysis program ImageJ as previously described [29].

Immunohistochemistry

Ten μm thick cryostat sections were prepared from snap frozen epididymal fat and peri-renal fat embedded in Cryo-Gel. Sections were mounted on SuperFrost Plus slide (Fisher Scientific, Ottawa, ON), fixed in acetone for 10 min, washed in Tris-buffered saline (TBS; 50 mM Tris-HCl, 150 mM NaCl, pH 7.4) and air dried. Sections were stained following the standard avidin-biotin peroxidase complex (ABC) procedure using a Vectastain ABC kit (Vector Laboratories Inc., Burlingame, CA). Briefly, non-specific staining was blocked using diluted normal rabbit serum for 20 min at room temperature and macrophages and T-cells were identified by incubation with primary antibody overnight at 4°C. Primary antibodies used were F4/80 and CD3 (Abcam). One section per slide was used as a negative control where incubation with the primary antibody was omitted. The following day endogenous peroxidase activity was blocked by incubation in 0.3% H_2O_2 in TBS for 30 min. Sections were incubated with diluted biotinylated secondary antibody followed by Vectastain ABC reagent (containing avidin and biotinylated horseradish peroxidase) at room temperature for 30 min. Antibody was visualized using diaminobenzidine (DAB; Vector Laboratories Inc.). Hematoxylin was used to counterstain. Slides were then dehydrated and cleared with ethanol and xylene washes, and mounted. Five fields were randomly selected from 4 different stained sections for analysis with a Zeiss Axioskop 2 microscope and a Zeiss AxioCam camera using a 20 \times objective and AxioVision V4.6.1.0 software. The total number of macrophages or T-cells in the five fields per animal were counted and reported as the number of cells per 100,000 μm^2 .

Statistical analysis

Data were analyzed by one-way analysis of variance followed by Duncan's multiple range test for means testing (SAS Version 9.2, SAS Institute, Cary, NC). If assumptions for normality and homogeneity were not met, the data were log transformed for analyses. A p-value ≤ 0.05 was accepted as statistically significant.

4.5 Results

Feed intake, body and organ weights, and serum haptoglobin

At the end of the 8-week study period, the *fa/fa* rats had greater total feed intake, body weight, relative peri-renal, epididymal and visceral adipose weights (Table 4.2). Dietary ALA-rich flaxseed oil did not affect the total feed intake, final body weights or relative adipose weights of *fa/fa* rats. Circulating levels of acute phase protein haptoglobin were higher in *fa/fa* rats fed the ALA-rich flaxseed oil diet compared to the InCTL rats but not the faCTL rats (Table 4.2).

Spleen weight and splenocyte PL fatty acids

The *fa/fa* rats had a lower relative spleen weight than lean rats, but there were no differences due to dietary treatment (Table 4.2). Total n6 fatty acids and AA were unchanged; however, LA was lower in the faCTL compared to InCTL. Total n3, EPA and docosapentaenoic acid (DPA), were elevated 1.7-, 18.5-, and 2.0-fold, respectively, in splenocyte PLs from the faFLAX group compared to the faCTL group (Table 4.3), but ALA and DHA were not altered. In addition, the faFLAX group had a lower n6:n3 fatty acid ratio compared to the InCTL and faCTL groups but the PUFA:SFA ratio was unchanged.

Table 4.2 Anthropometric Parameters and Serum Biochemistry

	lnCTL	faCTL	faFLAX
Feed intake (g/8 weeks)	1059 ± 58 ^a	1480 ± 76 ^b	1513 ± 95 ^b
Relative feed intake (g/g BW)	0.24 ± 0.01	0.21 ± 0.01	0.23 ± 0.01
Final body weight (g)	473 ± 13 ^a	790 ± 14 ^b	787 ± 23 ^b
Peri-renal adipose weight (g/100 g BW)	2.12 ± 0.15 ^a	8.01 ± 0.43 ^b	7.82 ± 0.19 ^b
Epididymal adipose weight (g/100 g BW)	1.98 ± 0.11 ^a	2.73 ± 0.09 ^b	3.09 ± 0.12 ^b
Visceral adipose weight (g/100 g BW)	4.1 ± 0.3 ^a	10.7 ± 0.5 ^b	10.9 ± 0.3 ^b
Spleen weight (mg/g BW)	1.3 ± 0.1 ^a	1.1 ± 0.1 ^b	1.1 ± 0.1 ^b
Serum haptoglobin (mg/mL)	0.67 ± 0.12 ^a	1.09 ± 0.14 ^{ab}	1.36 ± 0.20 ^b
Serum adiponectin (μg/mL)	0.633 ± 0.031 ^a	0.779 ± 0.016 ^b	0.779 ± 0.014 ^b
Serum leptin (ng/mL)	1.53 ± 0.18 ^a	47.28 ± 2.86 ^b	45.31 ± 2.37 ^b

Data are presented as mean ± SEM; n=7 for all groups. Statistical differences among means ($p \leq 0.05$) are indicated by different lowercase letters. BW: body weight; lnCTL: lean rats fed control diet; faCTL: *fa/fa* rats fed control diet; faFLAX: *fa/fa* rats fed ALA-rich flaxseed oil diet.

Table 4.3 Splenocyte Phospholipid Fatty Acid Composition

	lnCTL	faCTL	faFLAX
ΣSFA	58.21 ± 2.56	57.99 ± 2.82	56.55 ± 2.12
ΣMUFA	8.91 ± 0.62	10.41 ± 0.77	10.99 ± 0.58
ΣPUFA	29.32 ± 1.69	28.82 ± 2.12	29.68 ± 1.92
C18:2n6 LA	7.16 ± 0.47 ^a	5.34 ± 0.49 ^b	5.49 ± 0.31 ^{ab}
C18:3n3 ALA	0.22 ± 0.09	0.16 ± 0.08	0.26 ± 0.08
C20:4n6 AA	12.87 ± 0.96	13.58 ± 1.04	12.48 ± 1.01
C20:5n3 EPA	0.05 ± 0.04 ^b	0.02 ± 0.02 ^b	0.37 ± 0.12 ^a
C22:5n3 DPA	0.69 ± 0.23 ^b	0.93 ± 0.09 ^b	1.87 ± 0.17 ^a
C22:6n3 DHA	0.90 ± 0.28	0.92 ± 0.28	1.43 ± 0.19
Σn9	5.07 ± 0.44	5.81 ± 0.33	6.47 ± 0.48
Σn6	23.31 ± 1.55	22.36 ± 1.61	21.09 ± 1.56
Σn3	3.13 ± 0.10^a	2.74 ± 0.39^a	4.65 ± 0.46^b
n6:n3	7.83 ± 0.61^a	8.67 ± 0.97^a	4.59 ± 0.15^b
PUFA:SFA	0.51 ± 0.05	0.51 ± 0.06	0.53 ± 0.05

Data are presented as mean ± SEM; n=4. lnCTL: lean rats fed control diet; faCTL: *fa/fa* rats fed control diet; faFLAX: *fa/fa* rats fed ALA diet. Statistical differences among means ($p \leq 0.05$) are indicated by different lowercase letters. AA: arachidonic acid; ALA: alpha-linolenic acid; DHA: docosahexaenoic acid; DPA: docosapentaenoic acid; EPA: eicosapentaenoic acid; LA: linoleic acid; MUFA: monounsaturated fatty acid; PUFA: polyunsaturated fatty acid; SFA: saturated fatty acid.

Splenocyte cytokine production

Pro- and anti-inflammatory *ex vivo* cytokine production by splenocytes was assessed. IL-10 secretion from unstimulated (Unstim) and ConA-stimulated (Stim) splenocytes was 2.1-fold and 1.6-fold lower, respectively, in the faFLAX group compared to the InCTL but was not different from the faCTL group (Figure 4.1A). There were no changes in production of IL-6 (Figure 4.1B) by Unstim or Stim splenocytes due to obesity or n3 supplementation.

Adipose tissue inflammation and adipokine secretion

Adipose tissue inflammatory status was evaluated by assessing pro- and anti-inflammatory adipokines in serum and adipose tissue, and adipocyte size was determined as a measure of adipocyte function. The *fa/fa* rats had 6.4-fold higher levels of anti-inflammatory IL-10 (Figure 4.1C) and 3.3-fold lower levels of proinflammatory IL-6 (Figure 4.1D) in peri-renal adipose tissue compared to their lean counterparts. Dietary intervention with ALA-rich flaxseed oil reduced adipose levels of IL-10 by 34% compared to baseline *fa/fa* rats, but did not change IL-6 compared to the other groups. IL-18 is a proinflammatory cytokine; in peri-renal adipose tissue the faCTL group levels of IL-18 precursor were more than twice those of the faFLAX group, while faBASE and InCTL groups were intermediate (Figure 4.1E). However, the level of mature (active) IL-18 was not different among groups. Mature (active) TNF- α protein levels were up to 10-fold higher in the faCTL group compared to all other groups (Figure 4.1F). Dietary intervention with ALA-rich flaxseed maintained mature TNF- α protein levels similar to the faBASE and InCTL groups. TNF- α precursor levels were also highest in the faCTL

group and the increase from baseline was prevented by consumption of ALA-rich flaxseed oil.

Adiponectin and leptin are anti-inflammatory and proinflammatory adipokines, respectively. Adiponectin protein levels in peri-renal adipose tissue were 2.7-fold lower (Figure 4.2A) while serum adiponectin concentrations were 1.2-fold higher (Table 4.2) in the faCTL group compared to the InCTL group, but there was no effect of dietary ALA-rich flaxseed oil in obese rats. Serum leptin concentrations were 31-fold higher in the faCTL group compared to the InCTL group (Table 4.2); however, leptin protein levels in peri-renal adipose tissue were not influenced by age or obesity (Figure 4.2B). There was a trend ($p=0.07$) towards lower leptin levels in the faFLAX compared to faCTL and faBASE groups, but this change was not significant. The levels of proinflammatory MCP-1 in adipose tissue of faCTL rats were elevated 5-fold compared to InCTL rats, and 3-fold compared to the *fa/fa* baseline group (Figure 4.2C). Dietary intervention with ALA-rich flaxseed oil in *fa/fa* rats reduced MCP-1 levels to those of the InCTL group.

Adipocytes in the faCTL group were nearly double in size compared to the InCTL in sections of epididymal adipose tissue (Figure 4.2D). The baseline *fa/fa* group had a ~15% larger adipocyte size compared to older faCTL rats. Interestingly, adipocyte size in obese rats fed ALA-rich flaxseed oil was reduced by 17% compared to the faCTL group. The range of adipocyte sizes was more than doubled in the obese animals compared to the lean rats (Figure 4.2E and 4.2F). The distribution curve for obese rats fed the ALA-rich flaxseed oil diet revealed a greater proportion of smaller adipocytes compared to the other *fa/fa* groups yet still a wider range of values compared to the InCTL.

Macrophage infiltration

Macrophages infiltrating adipose tissue contribute to low grade chronic inflammation in obesity. Thus, macrophage infiltration was determined by counting F4/80 positive cells in epididymal and peri-renal adipose tissue. No differences among groups were observed in either fat depot (Figure 4.3A). A similar pattern was observed with F4/80 protein levels by Western blotting, except faBASE levels were elevated compared to the other groups (Figure 4.3B). Representative images of immunohistochemical staining with F4/80 are shown (Figure 4.3C-G).

T-cell infiltration

T-cells are among the first immune cells to enter adipose tissue and they may stimulate macrophage infiltration through release of MCP-1. Rats fed a diet with ALA-rich flaxseed oil had 46% lower CD3 protein levels compared to faCTL rats (Figure 4.4A). T-cell infiltration in adipose tissue is illustrated by representative images of immunohistochemistry staining for CD3 in Figure 4.4B-F.

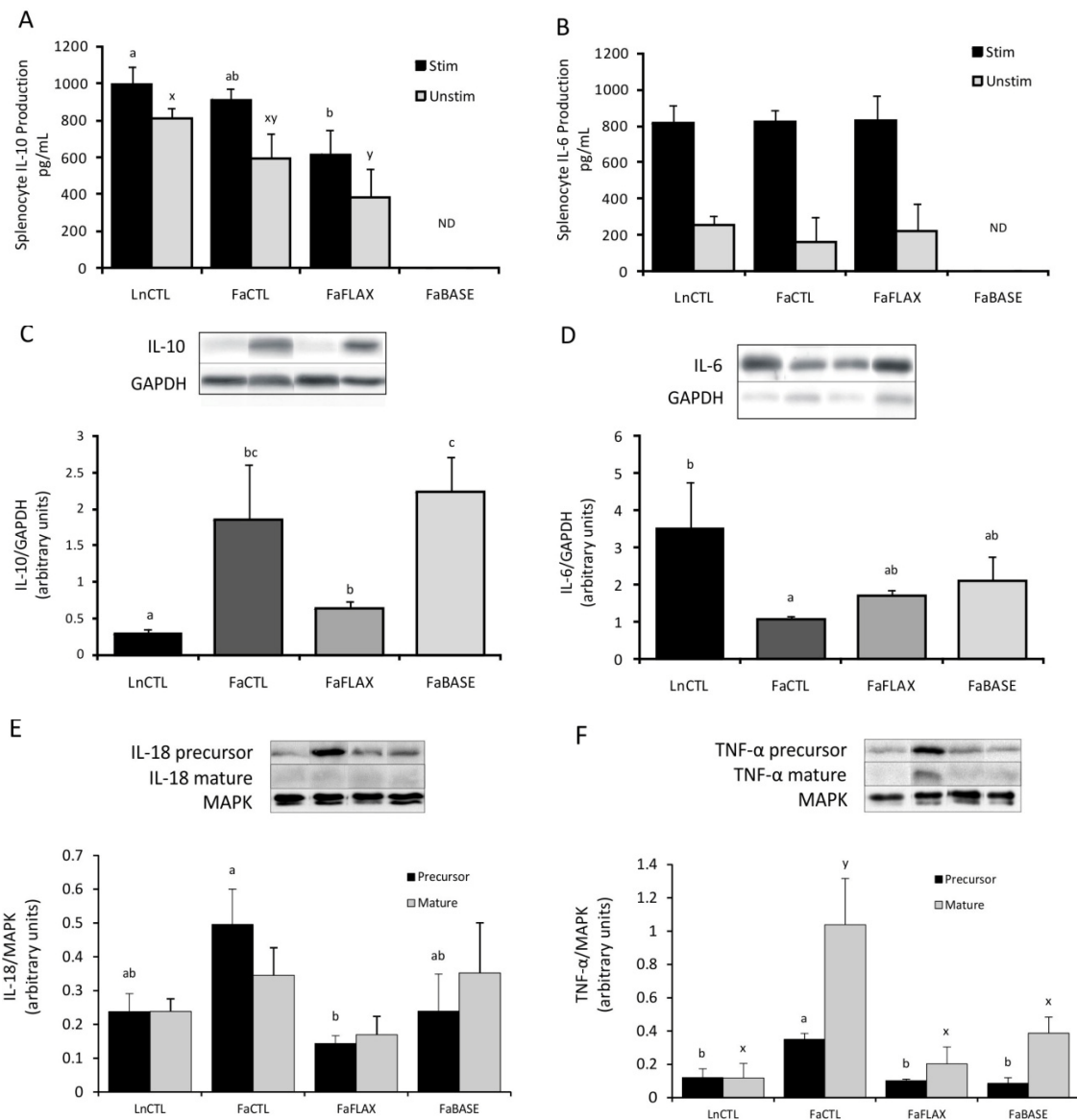


Figure 4.1 Splenocyte and Adipose Tissue Cytokines

Production of IL-10 (A) and IL-6 (B) by unstimulated (Unstim) and ConA-stimulated (Stim) splenocytes was measured in duplicate using a multiplex assay and Luminex instrument. Protein levels of IL-10 (C), IL-6 (D), IL-18 (E) and TNF- α (F) were assessed in peri-renal adipose tissue by Western blot analysis. Relative intensities of the bands were quantified by densitometry and the ratio of cytokine to GAPDH was calculated. Data are expressed as means \pm SEM (n=6/group). Statistical differences among means ($p \leq 0.05$) are indicated by different lowercase letters (either 'abc' or 'xy'). LnCTL: lean rats fed control diet; FaCTL: *fa/fa* rats fed control diet; FaFLAX: *fa/fa* rats fed flax diet; FaBASE: *fa/fa* rats at baseline; ND: not determined.

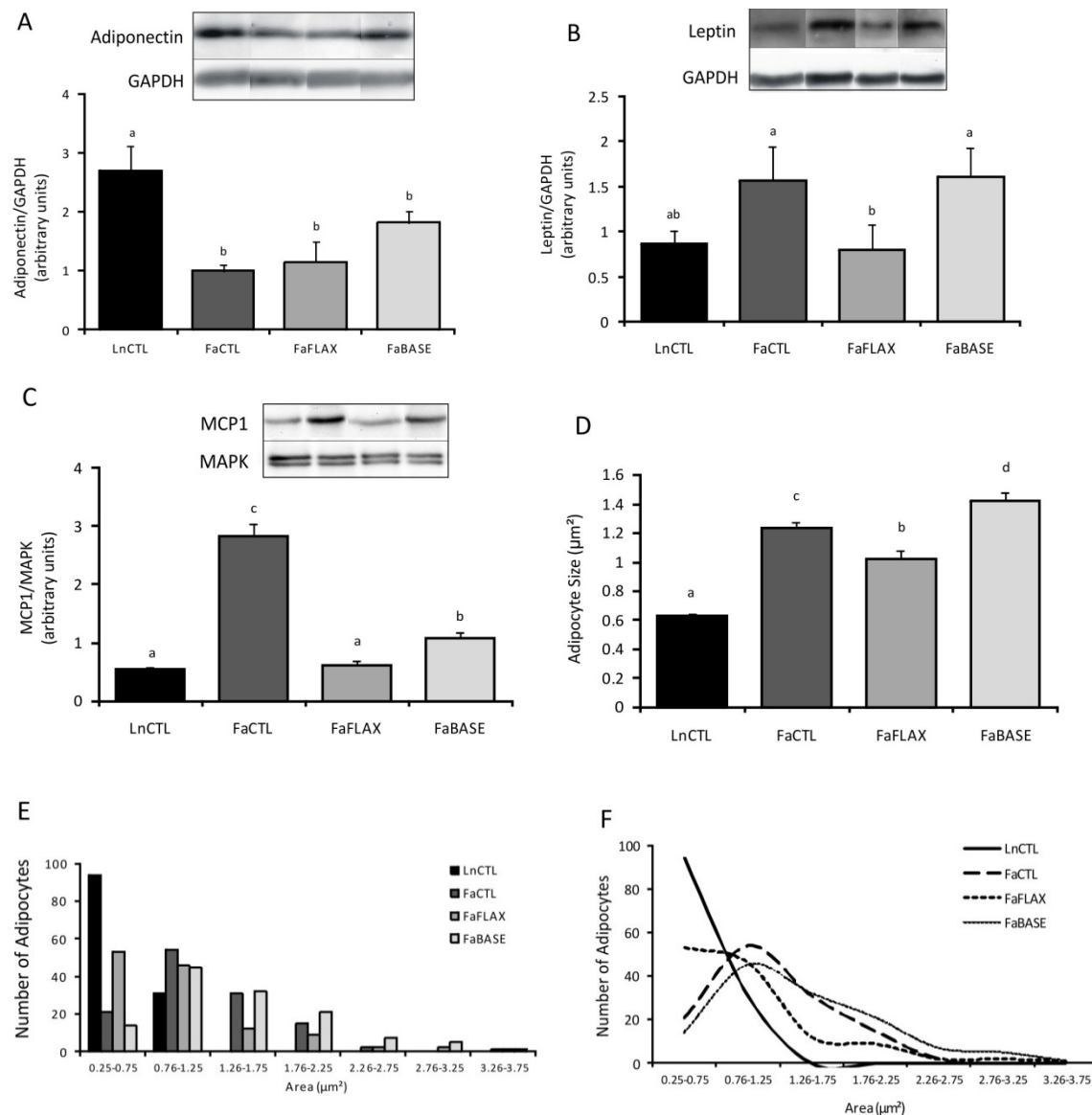


Figure 4.2 Changes in Adipocyte Phenotype

Protein levels of adiponectin (A), leptin (B) and MCP-1 (C) in peri-renal adipose tissue were measured by Western blot analysis. Relative intensities of the bands were quantified by densitometry and the ratio of cytokine to GAPDH (leptin, adiponectin) or MAPK (MCP-1) was calculated. Data are expressed as means \pm SEM ($n=6/\text{group}$ for adiponectin and leptin, $n=5/\text{group}$ for MCP-1). Statistical differences among means ($p \leq 0.05$) are indicated by different lowercase letters; there was a trend ($p < 0.07$) for leptin (B). Mean adipocyte size (D) was determined from digitally captured images of sectioned epididymal adipose tissue as described in Materials and Methods. The size distribution is presented in (E) and (F). Data are presented as means \pm SEM ($n=4-5/\text{group}$). Statistical differences among means ($p \leq 0.05$) are indicated by different lowercase letters. LnCTL: lean rats fed control diet; FaCTL: *fa/fa* rats fed control diet; FaFLAX: *fa/fa* rats fed flax diet; FaBASE: *fa/fa* rats at baseline.

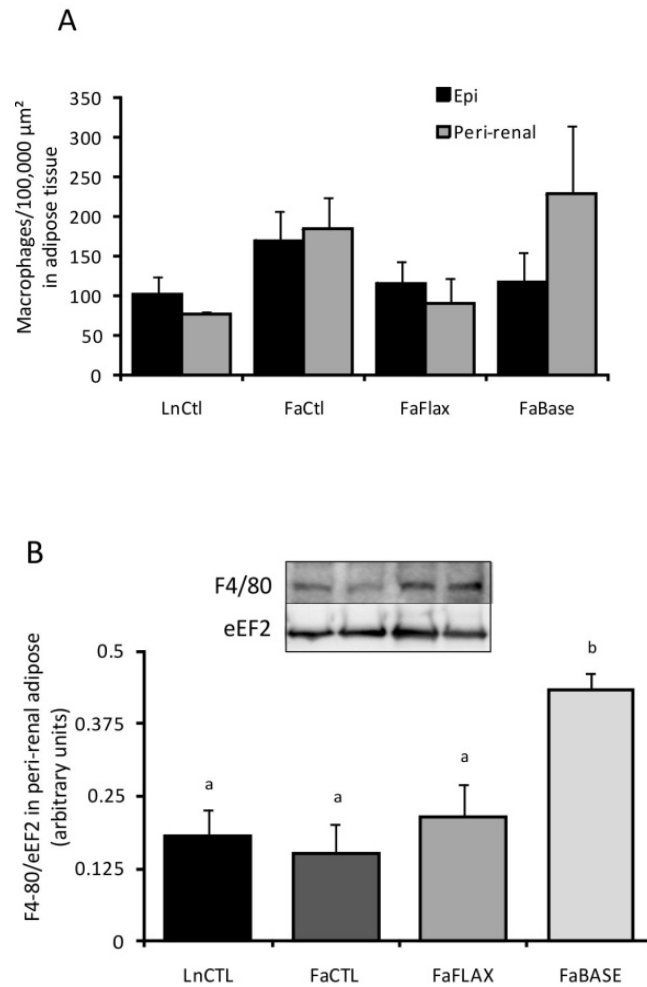


Figure 4.3 Macrophage Infiltration in Adipose Tissue

Macrophage infiltration in adipose tissue was determined by immunohistochemical staining with an HRP-linked F4/80 antibody (macrophages = brown) on frozen sections of tissue counterstained with hematoxylin (nuclei = purple). Digital images were captured using a light microscope and digital camera. The number of macrophages per 100,000 μm^2 (A) was determined in epididymal (epi) and peri-renal adipose tissue. The macrophage content of peri-renal adipose tissue was measured by Western blotting with antibody to F4/80 (B). A representative blot is shown; eEF2 was used as the loading control. Relative intensities of the bands were quantified by densitometry and the ratio of F4/80 to eEF2 loading control was calculated. Data are expressed as means \pm SEM ($n=5/\text{group}$). Statistical differences among means ($p \leq 0.05$) are indicated by different lowercase letters. LnCTL=lean rats fed control diet, FaCTL=*fa/fa* rats fed control diet, FaFLAX=*fa/fa* rats fed flax diet, FaBASE=*fa/fa* rats at baseline.

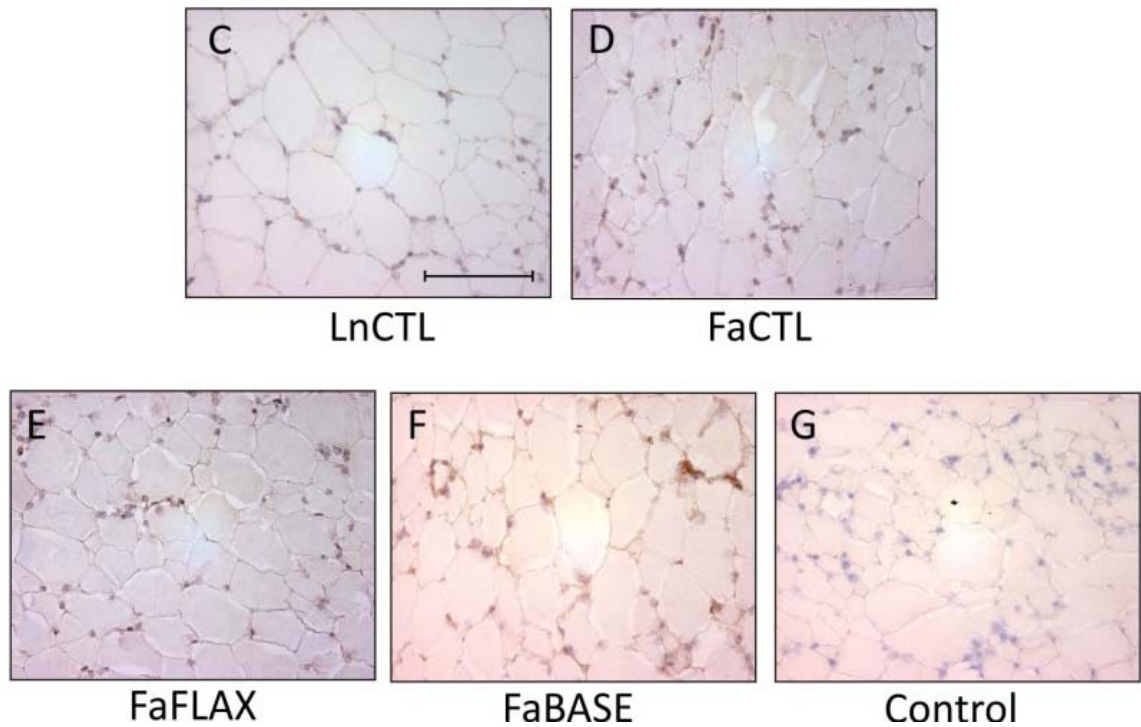


Figure 4.3 Macrophage Infiltration in Adipose Tissue (*Cont'd*)

Representative images of the immunohistochemical staining for each diet group are shown (C-G). The control image (G) had no primary antibody. Scale bar = 200 μ m; all images are to the same scale. LnCTL=lean rats fed control diet, FaCTL=*fa/fa* rats fed control diet, FaFLAX=*fa/fa* rats fed flax diet, FaBASE=*fa/fa* rats at baseline.

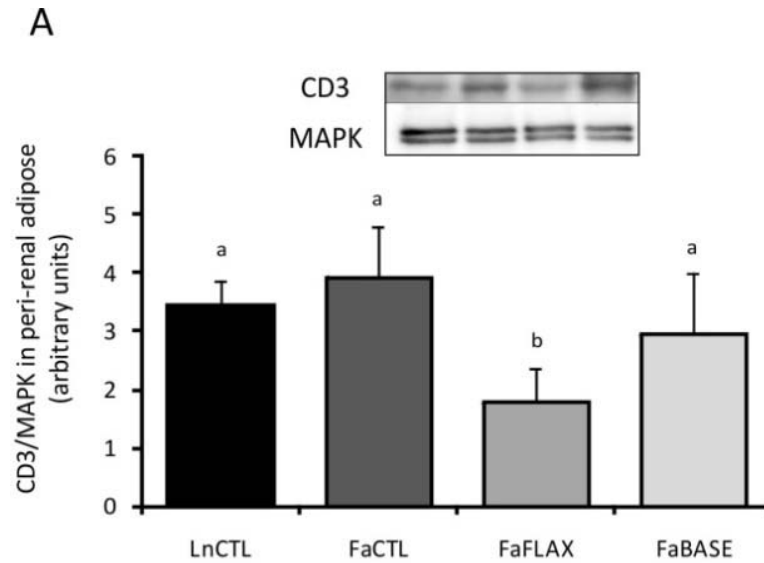


Figure 4.4 T-cell Infiltration in Adipose Tissue

Levels of the T-cell marker CD3 (A) were measured in peri-renal adipose tissue by Western blot analysis; a representative blot is shown with MAPK as the loading control. The relative band intensities were quantified by densitometry and the ratio of CD3 to MAPK was calculated. Data are expressed as means \pm SEM (n=5/group). Statistical differences among means ($p \leq 0.05$) are indicated by different lowercase letters. Immunohistochemical staining for CD3 was performed on frozen sections of peri-renal adipose tissue and images were captured using a light microscope and digital camera. LnCTL=lean rats fed control diet, FaCTL=*fa/fa* rats fed control diet, FaFLAX=*fa/fa* rats fed flax diet, FaBASE=*fa/fa* rats at baseline.

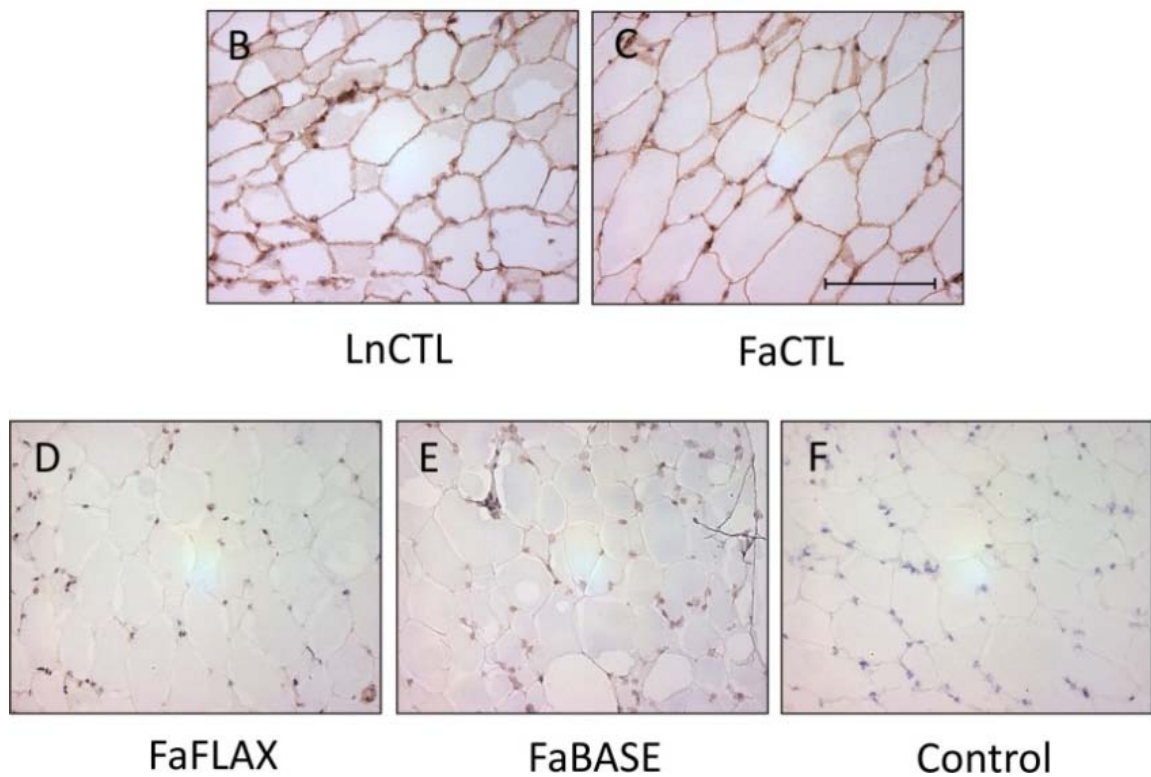


Figure 4.4 T-cell Infiltration in Adipose Tissue (*Cont'd*)

Representative images are shown (B-F). The control image (F) had no primary antibody. Scale bar represents 200 μ m; all images are to the same scale. LnCTL=lean rats fed control diet, FaCTL=*fa/fa* rats fed control diet, FaFLAX=*fa/fa* rats fed flax diet, FaBASE=*fa/fa* rats at baseline.

4.6 Discussion

The major findings of the study were that dietary ALA-rich flaxseed oil reduced adipocyte size (hypertrophy) by 17% in obese Zucker rats, decreased adipose MCP-1 levels to those of lean rats, and prevented T-cell infiltration into adipose tissue, despite no changes in adipose mass or body weight compared to obese Zucker rats fed the control diet. Although dietary flaxseed oil elevated total n3, EPA and DPA in splenocyte PLs of *fa/fa* rats, there were no changes in splenocyte *ex vivo* production of cytokines except less IL-10 production compared to the lean control. IL-10 protein levels in adipose tissue were also reduced in ALA-fed *fa/fa* rats compared to baseline, and TNF- α protein levels were elevated in faCTL rats compared to low levels in all other groups. However, other anti- and proinflammatory molecules in adipose or serum remained unchanged. To our knowledge, this is the first study to investigate the effects of dietary flaxseed oil in obese Zucker rats.

An important novel finding of our study was the reduction in adipocyte size, as well as MCP-1 levels and T-cell infiltration in the adipose tissue of obese rats fed a diet containing supplemental plant-based n3 fatty acid. Considerable obesity research is conducted with growing rats and the present study demonstrates these changes in older rats with established obesity. The faCTL rats had adipocytes 2.0 \times the size of the InCTL group, whereas the faFLAX rats' adipocytes were only 1.6 \times the size of the InCTL adipocytes. The dietary ALA-rich flaxseed oil decreased adipocyte size in epididymal and peri-renal adipose tissue, despite no changes in relative weights of these fat depots. Others have previously reported reduced adipocyte size with dietary n3 fatty acid

supplementation of EPA and DHA in non-obese male Wistar rats [30,31] and *db/db* mice [32], but none have used animals that were already obese when treatment began. Our adipocyte size measurements clearly show that adipocyte hypertrophy was present at baseline, and thus the decline in size observed in animals on the flaxseed oil diet signifies that this treatment is capable of reversing established adipocyte hypertrophy. These results would explain other improvements in obesity parameters (i.e. lowered adipose tissue MCP-1, T-cell infiltration and leptin) since smaller adipocytes would be associated with less dysfunction. The present study also indicates that dietary ALA is effective; however, the present study cannot distinguish if the effects are due directly to ALA or through its elongation and desaturation to EPA and DHA. Although there may be controversy about the efficiency of conversion, dietary ALA is effective at elevating EPA and/or DHA concentrations in various tissues [33], including immune cells (Table 4.3).

Interestingly, we observed less T-cell infiltration in adipose tissue of obese rats fed a diet supplemented with ALA-rich flaxseed oil than those fed a control diet. Several different types of T-lymphocytes are present in adipose tissue under normal and pathological conditions, as described by a recent review [34]. In mice, adipose depots contain immune cells from the innate and adaptive immune systems [35]. In our study, we did not observe a difference in T-cell infiltration between the FaCTL and LnCTL groups. This is surprising, since recent studies have described the accumulation of CD3⁺ T-cells in the adipose tissue of obese mice [36-39] and human patients [9]. The reason for this discrepancy is unclear, but it should be noted that there are currently no published studies investigating T-cell infiltration in adipose tissue of the Zucker rat

model. Thus, factors such as the age of the rats during the study or the model itself may explain our findings. In addition, the use of a T-cell marker that distinguishes between T-cell subtypes may have aided in determining the actions of T-cells in our model. The literature suggests that T-cell infiltration precedes macrophage entry in adipose tissue [9], but despite the changes in T-cell infiltration in our study, no differences in the degree of macrophage infiltration were observed in either epididymal or peri-renal adipose tissue. This finding is in contrast to several other reports of increased macrophage infiltration in obesity (reviewed in [34]), and is surprising, since in our study 5-fold higher levels of MCP-1 were detected in adipose tissue of faCTL rats compared to InCTL rats. MCP-1 is a potent chemotactic factor associated with macrophage infiltration [40]. Two previous studies have reported 5- to 10-fold higher MCP-1 mRNA levels in epididymal adipose tissue of *fa/fa* Zucker rats compared to lean Zucker rats [41,42] and this was accompanied by increased macrophage infiltration into adipose tissue. The reason for the lack of macrophage infiltration observed in our study is unclear; however, it is important to note that our results are based on an objective assessment of macrophage and T-cell counts during which the authors were blinded to the sample being analyzed, whereas other studies that observed changes in macrophage number utilized a qualitative method [41,42]. The present study also demonstrates that dietary n3 supplementation to obese rats can attenuate MCP-1 protein levels in adipose tissue to the level of lean rats and lower than the faBASE level. This extends previous observations that EPA treatment of 3T3-L1 adipocytes reduces adipocyte size and MCP-1 mRNA levels [43].

Metabolic differences exist between smaller and larger adipocytes [44] specifically in relation to the adipokine profile expressed by these cells. The larger adipocytes of *fa/fa* Zucker rats would be expected to shift the balance to more proinflammatory and less anti-inflammatory adipokines/cytokines; however, this was not always the case. The changes in the profile of pro- and anti-inflammatory adipokines in adipose tissue of the obese *fa/fa* fed control diet were both expected (\uparrow proinflammatory: mature and precursor TNF- α ; \downarrow anti-inflammatory: adiponectin) and unexpected (\downarrow or unchanged proinflammatory: IL-18, IL-6, leptin; \uparrow anti-inflammatory: IL-10) compared to lean rats. Despite no differences among groups in the levels of mature IL-18 in adipose tissue, the faCTL group exhibited elevated precursor IL-18 levels compared to the faFLAX group. It appears that IL-18 synthesis is sensitive to obesity and dietary flaxseed oil, with a possible gene mechanism suppressing transcription in the flax-fed animals. Both precursor and mature TNF- α protein levels were elevated in the faCTL group compared to all other groups. This finding exemplifies the proinflammatory tendency of adipose tissue in obesity, and demonstrates the ability of n3-rich flaxseed oil to decrease levels of this inflammatory marker to InCTL levels. Higher IL-10 protein levels have also been reported in subcutaneous adipose tissue of obese individuals compared to lean subjects, and elevated IL-10 was interpreted as a possible defensive response of fat tissue to inflammation [45]. In the present study, dietary flaxseed oil reduced adipose tissue IL-10 levels in *fa/fa* rats by 4-fold compared to baseline suggesting a reduction in the chronic inflammatory state associated with obesity. Likewise, there was a trend ($p=0.07$) for a reduction in adipose tissue levels of

proinflammatory leptin in obese rats fed flaxseed oil. Both serum leptin and adiponectin concentrations were positively associated with obesity and neither was altered by dietary ALA-rich flaxseed oil. Although there is evidence that diets rich in EPA and DHA can decrease circulating leptin and increase circulating adiponectin in animals and humans [13,14,46], dietary ALA intervention in humans has not increased serum adiponectin [47-49]. Serum adiponectin is typically reduced in obese states [50]; however, our group [26,28] and others [51,52] have reported elevated serum adiponectin in *fa/fa* Zucker rats compared to their lean counterparts. Lean rats would be expected to produce more adiponectin based on their smaller adipocytes and 2.7-fold higher protein levels of adiponectin in adipose tissue. However, the ~2.6-fold greater adipose mass of *fa/fa* rats may overall produce more adiponectin despite lower adipose levels of adiponectin and less adiponectin production by large adipocytes [28]. In a similar vein, adipose tissue IL-6 is typically elevated in obese individuals and animals, but in our study, there was no change in adipose tissue IL-6 between the lean and *fa/fa* Zucker rats, similar to other reports using this model [53,54]. The fact that macrophage infiltration between these groups did not differ would lead us to expect no change in IL-6 levels, since macrophages are a source of proinflammatory IL-6 in adipose tissue [6]. Likewise, serum haptoglobin was not different between lean and obese Zucker rats; however, it was elevated in the flaxseed oil-fed obese rats compared to lean control rats, and may represent a state of systemic inflammation which was not attenuated by the supplementation of plant-based n3. An alternative interpretation of these results regarding modest changes in adipokines may relate to the new concept of

“metabolically healthy obesity”, a term used to describe individuals who are obese, yet do not exhibit any metabolic disorder [55,56]. With respect to this study, Farb et al [57] recently described an obese population that does not exhibit inflammation. In addition, these persons do not show changes in circulating adiponectin levels. As such, these characteristics resemble those we have reported for the *fa/fa* Zucker rats used in the current study in regards to lack of macrophage infiltration and slight increase in adiponectin levels, both of which are a departure from the findings reported in other obesity models. These animals may thus be a model of intermediate cardiometabolic obesity phenotype. If this is the case, then increased macrophage infiltration would not be expected.

Dietary interventions with n3 fatty acids are known to influence the membrane PL composition of immune cells, typically with increased appearance of the fatty acid in which the diet has been enriched [58]. In a study by Ruth et al [59], diets supplemented with fish oil containing pre-formed EPA and DHA resulted in a reduced n6:n3 ratio and elevated total n3, EPA and DPA, but not DHA, in splenocyte PL of JCR:LA-*cp* rats, another model of obesity and impaired leptin signalling. In our study, we observed similar changes in splenocyte PL n3 fatty acids with supplementation of ALA-rich flaxseed oil in *fa/fa* rats, indicating that plant-based ALA is also effective for enhancing n3 fatty acid composition. Interestingly, neither fish oil nor ALA supplementation was effective at altering the DHA levels of splenocyte PL in either model. The immune function mediators generated from EPA and DHA (eg. eicosanoids, resolvins and docosanoids) are less biologically active than those produced from AA [60-62], and in some cases have

been shown to be anti-inflammatory, inflammation-resolving and immunomodulatory [63-65]. Thus, a diet rich in n3 fatty acids has the potential to reduce adipose tissue inflammation by modifying the PL composition of immune cell membranes and subsequent production of signalling molecules. There is also evidence that n3 fatty acids play a role in modulating T-cell function and cytokine production [66]. In the present study, dietary ALA-rich flaxseed oil did not alter *ex vivo* splenocyte production of proinflammatory IL-6, possibly due to the leptin receptor deficiency model affecting the response to the dietary treatment. However, there was a reduction in anti-inflammatory IL-10 produced by unstimulated and mitogen-stimulated splenocytes of obese compared to lean rats. If IL-10 is produced as part of a defensive response to inflammation, then the reductions in IL-10 in both adipose tissue and splenocytes support the view that intervention with ALA-rich flaxseed oil produced a less proinflammatory environment in obese rats.

We acknowledge that this study has several limitations. One of these is the relatively small sample size (n=5), which was nevertheless large enough to yield significant differences in many of our analyses. Secondly, the animals in our study were fed relatively high amounts of ALA-rich flaxseed oil that would not be attainable in humans. However, the flaxseed oil diet was designed specifically to achieve a lower n6:n3 ratio than the control diet while maintaining ~10% fat by weight. In the context of our study, changing the composition of PUFAs provided to obese insulin-resistant rats had modest effects on inflammatory and metabolic parameters.

4.7 Conclusion

We conclude that plant-based n3 fatty acids, administered as ALA-rich flaxseed oil for 8 weeks, are effective for reducing adipocyte size, adipose MCP-1 levels and T-cell infiltration in adipose tissue of a rodent model of obesity. Dietary flaxseed oil had a striking effect on MCP-1 and TNF- α in adipose tissue, lowering levels of these proinflammatory cytokines 5-fold in each case compared to *fa/fa* controls. Changes in other cytokines and adipokines in the flaxseed oil-treated *fa/fa* group were limited to a decrease in splenocyte production of IL-10 compared to the lean control, a reduction in adipose IL-10 compared to baseline, and a trend for lower leptin levels in adipose tissue. Our data indicate that there are no harmful or detrimental effects associated with dietary ALA-rich flaxseed oil intake in an obese state. The reduction in adipocyte size to smaller, more functional cells and the decrease in T-cell infiltration in obese rats fed ALA-rich flaxseed oil provide encouraging evidence for the health benefits of plant-based n3 fatty acids on adipose tissue and the inflammatory process in obesity. Furthermore, in light of our findings that *fa/fa* Zucker rats appear to be resistant to major changes in circulating inflammatory factors and do not exhibit increased adipose macrophage infiltration, these animals may serve as a model of intermediary cardiometabolic disease, analogous to the “healthy obese” phenotype in humans.

4.8 Literature Cited

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CHAPTER 5

Diets High in Mono- and Polyunsaturated Fatty Acids Decrease Fatty Acid Synthase Protein Levels in Adipose Tissue But Do Not Alter Other Markers of Adipose Function and Inflammation in Diet-Induced Obese Rats

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5.1 Contribution Statement

I, Jennifer Enns, made the following contributions to this study:

- conducted the Western blotting analyses
- performed the serum biochemistry analyses
- designed the primers for the real-time qPCR experiments, developed and tested protocols to optimize experimental conditions, and trained an undergraduate student to carry out these experiments
- performed statistical analyses for above-mentioned data
- interpreted results together with those from other contributors and wrote the manuscript

Other contributors:

- Danielle Hanke
- Angela Park
- Peter Zahradka
- Carla G. Taylor

5.2 Abstract

This study investigates the effects of MUFAs and PUFAs from different fat sources (High-Oleic Canola, Canola, Canola-Flaxseed (3:1 blend), Safflower, or Soybean Oil, or a Lard-based diet) on adipose tissue function and markers of inflammation in Obese Prone rats fed high-fat (55%E) diets for 12 weeks. Adipose tissue fatty acid composition reflected the dietary fatty acids profiles. Protein levels of fatty acid synthase, but not mRNA levels, were lower in adipose tissue of all groups compared to the Lard group. Adiponectin and fatty acid receptors GPR41 and GPR43 protein levels were also altered, but other metabolic and inflammatory mediators in adipose tissue and serum were unchanged among groups. Overall, rats fed vegetable oil- or lard-based high-fat diets appear to be largely resistant to major phenotypic changes when the dietary fat composition is altered, providing little support for the importance of specific fatty acid profiles in the context of a high-fat diet.

5.3 Introduction

Obesity has become a worldwide epidemic, affecting millions of adults and children in developed and developing nations alike. In Canada, more than one in four adults are obese and the prevalence of obesity has roughly doubled over the past two decades [1]. In large epidemiological studies, body mass index and waist circumference (the most widely used measures of obesity) show strong associations with mortality and cardiovascular disease [2,3].

Adipose tissue is central to the development of obesity. Once thought to serve merely as an energy storage depot, adipose tissue is now known to be a dynamic endocrine organ, secreting bioactive molecules termed “adipokines” that contribute to the regulation of metabolic homeostasis. In obesity, excessive adiposity and the dysregulated production of adipokines, such as adiponectin, leptin, and several classes of proinflammatory molecules, contribute to the chronic low-grade inflammation that drives the pathogenesis of related conditions, including insulin resistance and cardiovascular disease [4,5].

Although the etiology of obesity is complex, diet is one of the most important determinants [1]. While high fat intake has been shown to play a role in promoting adipose tissue inflammation [6-8], the findings from carefully controlled weight-gain and weight-loss trials that compare low-, moderate- and high-fat diets have suggested that the amount of fat in the diet is less important than the type of fat consumed [9-11]. In this context, researchers have begun to assess whether diets containing certain proportions of MUFAs and PUFAs might favourably affect obesity parameters. Many of

these studies have focused on the very long-chain n3 PUFAs EPA (C20:5n3) and DHA (C22:6n3), which are obtained primarily from fatty fish or fish oil supplements. However, less is known about the essential n3 fatty acid ALA (C18:3n3), found in plant-based products such as canola and flaxseed oils. ALA can be elongated and desaturated in the body to form EPA and DHA, but its conversion occurs with very low efficiency [12]. At the same time, the effects of ALA in obesity have not been well studied, despite reports that ALA blood levels and cardiovascular endpoints are inversely associated [13-16]. Other promising findings include the inflammation-reducing effects of ALA supplementation in obese subjects [17] and in the Δ^6 -desaturase null mouse [18], as well as our previous study in the obese *fa/fa* Zucker rat that indicated a role for ALA-rich flaxseed oil in improving adipose tissue function [19].

While n3 PUFAs are widely believed to be beneficial to health, a recent meta-analysis has indicated that high intakes of n6 PUFAs without a concurrent increase in n3 PUFAs has deleterious effects on cardiovascular events and death [20,21]. Although some n6 PUFAs are precursors to eicosanoids, which modulate pro-thrombotic, pro-constrictive and proinflammatory processes [22], emerging data on fatty acid derivatives involved in the resolution of inflammation indicate that those derived from n6 PUFAs can have both pro- and anti-inflammatory properties [23].

Diets rich in MUFAs, which have been studied mainly in relation to the oleic acid (C18:1n9)-rich Mediterranean Diet, may also be beneficial in reducing the risk of co-morbidities associated with obesity [24]. The National Institute of Medicine does not propose a specific dietary requirement for MUFAs; instead, MUFA intake makes up the

balance of dietary fats after the requirements for SFAs and PUFAs are met, and MUFA intake may therefore vary widely [25]. Although there is some evidence that MUFAs favourably alter serum lipids [26] and improve insulin sensitivity [27], their contribution to adiposity is uncertain [25] and they have no confirmed independent role in the prevention of chronic disease [28].

Given the high prevalence of obesity and obesity-related conditions as well as dietary recommendations to reduce SFA intake, it is important to determine the contributions of MUFAs and PUFAs to the development of these metabolic imbalances. Our study compared the effects of MUFA- and PUFA-rich diets containing different amounts and ratios of n6 and n3 fatty acids in a DIO rodent model, which closely mimics the phenotype and pathogenesis of human obesity. The fatty acid profiles of the vegetable oils making up the fat content of the diets allowed us to evaluate the metabolic consequences of two high-n6 PUFA diets containing very low or moderate levels of ALA, and three high-MUFA diets with relatively constant amounts of LA and low, moderate and high levels of ALA. Thus, the objective of our study was to investigate the effects of the different fatty acid profiles of vegetable oils on adipose tissue function and inflammation and relationships with adipose tissue fatty acid composition in DIO rats.

5.4 Materials and Methods

Animals and diets

The animal and diet protocol has been described previously [29]. Briefly, six week-old obese prone-CD (Charles River Sprague-Dawley) male rats (Charles River, St-Constant, PQ) were acclimatized for two weeks and randomized (n=10/group) to one of six dietary treatments for 12 weeks. The experimental diets were formulated with different oils (high-oleic canola oil, conventional canola oil, a 3:1 blend of conventional canola and flaxseed oil, safflower oil or soybean oil), which allowed comparisons of MUFAs and PUFAs with varying proportions of n6 and n3 fatty acids among diet groups (Table 5.1). A lard-based diet high in SFA that is commonly used to develop DIO in obese prone rats served as the control [30,31]. All diets contained 55% of total energy as fat, 30% as carbohydrate and 15% as protein; this formulation was based on similar interventions that induced changes in body weight, glucose, insulin and other metabolic parameters in Wistar rats [30,31]. The diets were prepared in 10 kg batches as needed and stored at 4°C. Feed intake (corrected for spillage) and weekly body weights were recorded. The experimental protocol was approved by the University of Manitoba Protocol Management and Review Committee and conducted according to the Canadian Council on Animal Care Guidelines. Other indices for these rats related to hepatic steatosis, glucose homeostasis and inflammatory markers have been reported elsewhere [29].

Table 5.1 Diet Formulations

	Lard	High-Oleic Canola Oil	Canola Oil	Canola- Flaxseed Oil	Safflower Oil	Soybean Oil
Ingredients (g/kg):						
Cornstarch ¹	209	209	209	209	209	209
Maltodextrin ¹	69.4	69.4	69.4	69.4	69.4	69.4
Sucrose ¹	100	100	100	100	100	100
Cellulose ¹	63.8	63.8	63.8	63.8	63.8	63.8
Casein ¹	186.2	186.2	186.2	186.2	186.2	186.2
Lard ²	279.8	0	0	0	0	0
High-Oleic Canola Oil ²	0	308.3	0	0	0	0
Canola Oil ²	0	0	308.3	231.2	0	0
Flaxseed Oil ³	0	0	0	77.1	0	0
High-Linoleic Safflower Oil ⁴	0	0	0	0	308.3	0
Soybean Oil ²	28.5	0	0	0	0	308.3
Mineral Mix AIN-93G ¹	44.6	44.6	44.6	44.6	44.6	44.6
Vitamin Mix AIN-93G ¹	12.7	12.7	12.7	12.7	12.7	12.7
L-cysteine ¹	3	3	3	3	3	3
Choline Bitartrate ¹	3.2	3.2	3.2	3.2	3.2	3.2
t-Butylhydroquinone ⁵	0.006	0.006	0.006	0.006	0.006	0.006
Fatty Acid Composition (g/100 g fatty acids)⁶:						
SFA	49	7	7	8	10	15
MUFA	42	78	66	54	17	21
PUFA	9	16	27	38	73	63
C18:2n6 (LA)	8	14	19	18	73	54
C18:3n3 (ALA)	1	2	8	20	0.2	9
LA:ALA	8:1	7:1	2:1	1:1	365:1	6:1

¹Dyets Inc., Bethlehem, Pennsylvania; ²Bunge Canada, Oakville, Ontario; ³Omega Nutrition, Vancouver, British Columbia; ⁴Alnoroil Company, Inc., Valley Stream, New York; ⁵Sigma-Aldrich, St. Louis, Missouri; ⁶Analyzed by gas chromatography as described in the Methods; SFA: saturated fatty acid; MUFA: monounsaturated fatty acid; PUFA: polyunsaturated fatty acid; LA: linoleic acid; ALA: alpha-linolenic acid.

Blood and tissue collection

Fasting blood samples were obtained via the jugular vein at weeks 0, 4, and 8, and trunk blood was collected at 12 weeks (study end) after rats were euthanized with carbon dioxide. Blood samples were centrifuged at 1500 *g* for 15 min at 4°C. The serum layer was collected, aliquoted and stored at -80°C for biochemical analyses. Various organs, including adipose tissue (epididymal fat pads), were dissected, weighed, flash-frozen in liquid N₂, and stored at -80°C for analysis.

Serum biochemistry

Serum concentrations of leptin, MCP-1 and active plasminogen activator inhibitor (PAI)-1 at weeks 0, 4, 8 and 12 were determined using a Rat Serum Adipokine Milliplex® MAP multiarray kit (EMD Millipore, St. Charles, MO). Serum adiponectin at study end was measured using an ELISA kit (Alpco Diagnostics, Salem, NH).

Fatty acid composition

The fatty acid composition of epididymal adipose tissue was analysed as previously described [32] using methanolic hydrochloric acid as the methylating agent. The methylated samples were analysed by gas chromatography (GC) using a Varian 450-GC Gas Chromatograph with FID detector (Varian, Lake Forest, CA) and a GC capillary column (length 100 m, diameter 0.25 mm and film thickness 0.25 µm; Varian, Lake Forest, CA). Fatty acid composition of diet samples was also verified by GC.

Western immunoblotting

The protein fraction was extracted from frozen epididymal adipose tissue and quantified as previously described [33]. Western immunoblotting was performed by separating proteins (15 µg) by SDS-PAGE, transferring to a polyvinylidene fluoride membrane and probing with primary (1:1000 dilution) and HRP-conjugated secondary antibodies (1:10 000 dilution). Quantification of band intensities was carried out using a FluorChem®Q gel scanning system with a charge-coupled device camera (Proteinsimple, Santa Clara, CA) and AlphaView® Software (Version 1.3.0.6; Alpha Innotech Corporation, Proteinsimple). Data are expressed as arbitrary units relative to the loading control. Western immunoblot analysis was conducted using the following antibodies: Fatty acid synthase, p42/44 MAPK, eEF2 and Perilipin (Cell Signaling Technology, Inc., Danvers, MA); IL-18, GPR41 and GPR43 (Santa Cruz Biotechnology, Inc., Dallas, TX); MCP-1, Pigment Epithelial-Derived Factor, Fatty Acid Binding Protein 4 and CD36 (Abcam, Cambridge, MA); and Adiponectin (EMD Millipore).

Quantitative PCR analysis

Total RNA was extracted from epididymal adipose tissue using an EZNA®Total RNA Kit II (Omega Bio-Tek, Norcross, GA) according to the manufacturer's instructions. RNA concentration and integrity were measured with the Experion™ RNA StdSens Analysis Kit and Automated Electrophoresis System (Bio-Rad, Mississauga, ON). Conversion to cDNA was conducted using qScript™ cDNA SuperMix (Quanta BioSciences Inc., Gaithersburg, MD). Each reaction tube contained 1 µg of total RNA as template. A PTC-100™ Programmable Thermal Controller (MJ Research Inc., Waltham, MA) was used

to carry out reverse transcription at optimal temperatures. Real-time PCR was conducted using PerfeCTa® SYBR® Green FastMix® (Quanta BioSciences) in a Mastercycler® RealPlex System (Eppendorf, Hauppauge, NY). Primer sequences for fatty acid synthase and GAPDH (internal control) were as follows: Fatty acid synthase Forward 5'-CAGACAACAGCAACCTCACG-3' and Reverse 5'-ACTCTCAGACAGGCACTCAGG-3'; GAPDH Forward 5'-GTTCAACGGCACAGTCAAGG-3' and Reverse 5'-ATCTCGCTCCTGGAAGATGG-3'.

Statistical analysis

Experimental data were analysed using SAS statistical software (SAS Institute, Inc.). Endpoint data were analysed using one way analysis of variance (ANOVA) followed by Duncan's Multiple Range test for means testing. Time course data were analysed by repeated measures ANOVA. Data that were not normal and/or homogeneous were analyzed by Kruskal-Wallis, a non-parametric test, followed by post-hoc testing by least significant differences. All results are reported as mean values with standard error of the mean (SEM). Differences were considered statistically significant at $p \leq 0.05$.

5.5 Results

Physical parameters

At baseline, body weight among diet groups was not different (Figure 5.1). Throughout the rest of the study, the Soybean Oil group had higher body weight than the High-Oleic Canola Oil, Canola Oil, and Canola-Flaxseed Oil groups. At week 4, the body weight of the Soybean Oil group was also higher than the Safflower Oil group, and at study end, both the Soybean Oil and the Lard group were higher than the three groups fed diets containing canola oil. There were no differences in total feed intake, which ranged from 1992 ± 66 g/12 weeks to 2119 ± 58 g/12 weeks [29]. Epididymal fat mass (expressed as percent of body weight) ranged from 2.77 ± 0.16 to 3.34 ± 0.10 and was not different among groups.

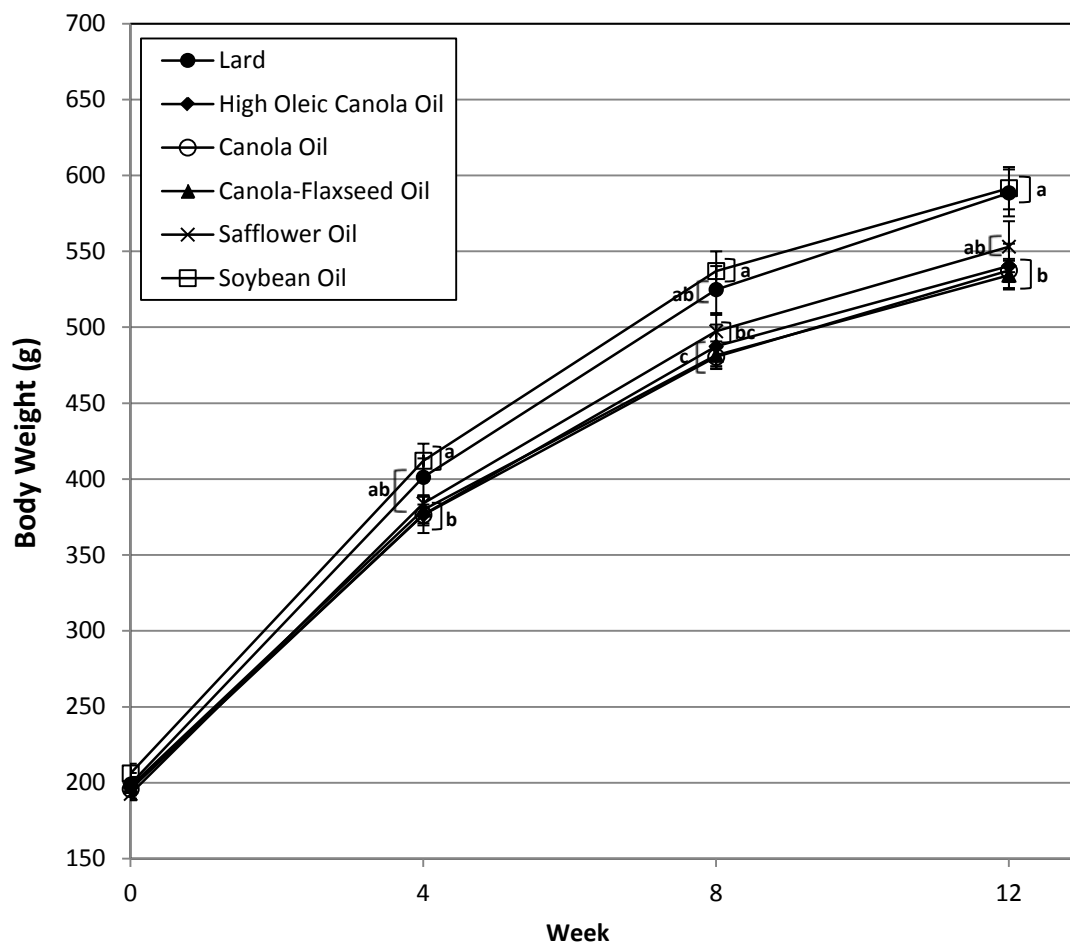


Figure 5.1 Weekly Body Weights

Data are means \pm SEM, $n=10/\text{group}$. At each time point, brackets with different lowercase letters are significantly different from each other ($p \leq 0.05$).

Serum biochemistry

Leptin, MCP-1, PAI-1 and adiponectin concentrations in fasting serum are presented in Table 5.2. PAI-1 was higher in the Lard and Canola-Flaxseed Oil groups than the High-Oleic Canola Oil and Canola Oil groups at 4 weeks. At 8 weeks, the Canola Oil group had elevated leptin and lower MCP-1 compared to the Canola Flaxseed Oil group. However, at the end of the study, there were no differences in any of these adipokines/cytokines due to dietary treatment. Serum adiponectin was higher in the Lard group than the High-Oleic Canola Oil group at the end of the study. Over the course of the study, leptin concentrations were increased and PAI-1 concentrations were decreased in all groups at week 12 compared to baseline, while MCP-1 levels were elevated in the Canola Oil and Canola-Flaxseed Oil groups at study end compared to baseline.

Table 5.2 Serum Biochemistry¹

		Lard	High-Oleic Canola Oil	Canola Oil	Canola- Flaxseed Oil	Safflower Oil	Soybean Oil
Week 0	Leptin	2658 ± 283	2456 ± 247	2398 ± 252	2324 ± 196		
	MCP-1	190 ± 28	222 ± 31	187 ± 44	145 ± 21	ND ²	ND
	PAI-1	3586 ± 287	3073 ± 256	3679 ± 329	3324 ± 214		
Week 4	Leptin	5290 ± 545	6014 ± 1033	7261 ± 859	5412 ± 215		
	MCP-1	197 ± 26	139 ± 29	121 ± 19	169 ± 40	ND	ND
	PAI-1	2840 ± 386 ^a	1633 ± 227 ^b	1850 ± 244 ^b	2872 ± 311 ^a		
Week 8	Leptin	5913 ± 469 ^{ab}	6528 ± 505 ^{ab}	6715 ± 398 ^a	5008 ± 649 ^b		
	MCP-1	113 ± 16 ^{ab}	142 ± 31 ^{ab}	92 ± 17 ^b	177 ± 30 ^a	ND	ND
	PAI-1	2357 ± 319	1791 ± 153	2560 ± 321	2197 ± 370		
Week 12	Leptin	8075 ± 598 ^{†‡§}	9132 ± 1096 ^{†‡§}	9820 ± 1763 [†]	7901 ± 1183 ^{†‡§}	8243 ± 627	8888 ± 1130
	MCP-1	209 ± 39	307 ± 30 ^{‡§}	324 ± 51 ^{†‡§}	202 ± 35 [†]	266 ± 39	284 ± 32
	PAI-1	335 ± 39 ^{†‡§}	191 ± 59 ^{†‡§}	417 ± 100 ^{†‡§}	357 ± 74 ^{†‡§}	384 ± 71	398 ± 64
	Adn	14.1 ± 0.9	11.0 ± 0.7	12.0 ± 0.8	12.3 ± 0.5	13.5 ± 0.9	11.8 ± 0.6

¹Leptin, MCP-1 and PAI-1 values are pg/mL; Adn (Adiponectin) values are ng/mL;

All values are presented as mean ± SEM, n=6-8/group. Statistical differences ($p \leq 0.05$) among dietary groups at the same time point are indicated across horizontal rows by different lowercase letters; statistical differences ($p \leq 0.05$) between week 12 and other time point means within a dietary group are denoted in vertical columns by the following symbols: † different from week 0; ‡ different from week 4; § different from week 8.

²ND: not determined.

Fatty acid composition

The effects of the dietary interventions on the adipose tissue fatty acid composition of phospholipids (pool of membrane signalling molecules) and triglycerides (intracellular storage) are summarized in Table 5.3 and Table 5.4, respectively.

Phospholipids: SFAs were unchanged, however, the High-Oleic Canola Oil and Canola Oil groups had elevated MUFAs compared to Safflower Oil and Soybean Oil groups, largely due to oleic acid. The Safflower Oil and Soybean Oil groups had greater total PUFAs, n6 PUFAs and LA compared to Lard, High-Oleic Canola Oil and Canola Oil groups. The Canola Oil, Canola-Flaxseed Oil and Soybean Oil groups had higher total n3 than the Safflower Oil group. DHA, the primary n3 fatty acid in adipose phospholipids, was unchanged by the experimental diets and there were only minor changes in EPA. The n6:n3 ratio was highest in the Safflower Oil group compared to all other groups, and the PUFA:SFA ratio was greater in the Safflower Oil group compared to Lard and High-Oleic Canola Oil groups.

Triglycerides: The diet fatty acid composition was also reflected in the triglyceride fatty acid composition, with changes in total SFAs, MUFAs, PUFAs, n6 PUFAs, and n3 PUFAs corresponding to dietary intake. Of interest was the 2-3 fold increase in n3 PUFAs due to ALA in the groups fed Canola Oil, Canola-Flaxseed Oil and Soybean Oil compared to the other groups. Notably, the other major n3 fatty acid in adipose triglycerides was docosapentaenoic acid (DPA, C22:5n3) and not DHA. DPA was elevated in High-Oleic Canola Oil and Canola Oil groups compared to the Safflower Oil group. The

Safflower Oil group had the highest n6:n3 ratio and PUFA:SFA ratio due to the large increase in n6 PUFAs and specifically LA.

Table 5.3 Adipose Tissue Phospholipid Fatty Acid Composition (g/100 g fatty acids)

	Lard	High-Oleic Canola Oil	Canola Oil	Canola-Flaxseed Oil	Safflower Oil	Soybean Oil
ΣSFA	51.2 ± 1.8	47.1 ± 3.4	46.7 ± 2.0	47.1 ± 1.2	49.5 ± 2.2	50.4 ± 2.2
C16:0 (PA)	17.5 ± 1.6	15.3 ± 0.9	16.0 ± 0.8	15.6 ± 0.7	15.6 ± 0.5	16.9 ± 0.8
C18:0 (SA)	26.0 ± 1.2	21.7 ± 1.0	24.1 ± 2.2	25.5 ± 2.3	24.2 ± 2.2	24.3 ± 2.3
ΣMUFA	16.5 ± 2.8^{ab}	20.0 ± 2.2^a	18.7 ± 1.4^a	16.6 ± 1.0^{ab}	9.5 ± 0.95^c	11.2 ± 1.8^{bc}
C18:1n7 (VA)	1.23 ± 0.15^b	1.82 ± 0.10^a	1.84 ± 0.15^a	1.84 ± 0.17^a	0.96 ± 0.02^b	1.19 ± 0.20^b
C18:1n9 (OA)	12.6 ± 2.7^{ab}	14.8 ± 1.6^a	13.9 ± 1.1^a	11.4 ± 0.7^{abc}	6.2 ± 0.78^c	7.5 ± 1.29^{bc}
ΣPUFA	31.5 ± 1.8^c	30.4 ± 1.34^c	33.6 ± 0.8^{bc}	36.1 ± 0.7^{ab}	39.4 ± 1.1^a	38.6 ± 0.5^a
Σn6	19.9 ± 1.7^b	19.9 ± 0.8^b	19.7 ± 1.1^b	22.2 ± 1.8^b	29.9 ± 1.5^a	28.1 ± 1.5^a
C18:2n6 (LA)	10.4 ± 1.2^{bc}	8.8 ± 0.87^c	11.1 ± 0.8^{bc}	12.6 ± 1.2^b	15.9 ± 0.7^a	15.6 ± 0.7^a
C20:4n6 (AA)	6.95 ± 1.1	8.36 ± 1.07	6.25 ± 0.59	6.07 ± 1.70	9.89 ± 0.91	7.29 ± 1.08
Σn3	11.5 ± 0.6^{ab}	10.4 ± 0.7^{ab}	12.5 ± 1.4^a	13.8 ± 1.1^a	8.6 ± 0.85^b	12.6 ± 1.5^a
C18:3n3 (ALA)	0.629 ± 0.312	0.318 ± 0.067	0.912 ± 0.354	1.04 ± 0.47	0.314 ± 0.272	0.864 ± 0.508
C20:5n3 (EPA)	0.329 ± 0.086^{ab}	0.0499 ± 0.0343^{bc}	0.0228 ± 0.0218^c	0.130 ± 0.055^{abc}	0.0338 ± 0.0328^c	0.0651 ± 0.0419^{bc}
C22:5n3 (DPA)	1.01 ± 0.22	0.530 ± 0.149	1.15 ± 0.13	0.899 ± 0.255	1.03 ± 0.22	0.862 ± 0.360
C22:6n3 (DHA)	9.07 ± 0.43	9.15 ± 0.86	9.95 ± 1.99	11.39 ± 1.02	6.89 ± 1.21	8.70 ± 0.79
n6:n3	1.75 ± 0.17^b	1.94 ± 0.11^b	1.57 ± 0.30^b	1.70 ± 0.29^b	4.15 ± 0.81^a	2.36 ± 0.35^b
PUFA:SFA	0.616 ± 0.032^c	0.663 ± 0.061^{bc}	0.725 ± 0.040^{abc}	0.768 ± 0.025^{abc}	0.956 ± 0.157^a	0.903 ± 0.135^{ab}

Data are presented as mean ± SEM; n=5/group. SFA: saturated fatty acid; MUFA: monounsaturated fatty acid; PUFA: polyunsaturated fatty acid. Within a row, statistical differences ($p \leq 0.05$) among means are indicated by different lowercase letters, and the whole row is bolded.

Table 5.4 Adipose Tissue Triglyceride Fatty Acid Composition (g/100 g fatty acids)

	Lard	High-Oleic Canola Oil	Canola Oil	Canola- Flaxseed Oil	Safflower Oil	Soybean Oil
ΣSFA	33.49 ± 1.28^a	12.26 ± 0.63^d	12.23 ± 0.28^d	13.23 ± 0.29^d	14.53 ± 0.21^c	17.11 ± 0.28^b
C16:0 (PA)	22.00 ± 0.67 ^a	8.48 ± 0.24 ^b	8.81 ± 0.19 ^b	9.48 ± 0.24 ^b	10.52 ± 0.22 ^b	8.63 ± 2.55 ^b
C18:0 (SA)	8.35 ± 0.52 ^a	1.76 ± 0.05 ^e	1.82 ± 0.05 ^e	2.16 ± 0.11 ^d	2.49 ± 0.07 ^c	3.00 ± 0.16 ^b
ΣMUFA	51.96 ± 0.86^c	68.09 ± 0.26^a	59.84 ± 0.66^b	52.96 ± 1.74^c	17.94 ± 0.63^e	22.74 ± 0.60^d
C18:1n7 (VA)	1.50 ± 0.43	2.57 ± 0.03	1.46 ± 0.82	1.95 ± 0.65	0.75 ± 0.31	1.27 ± 0.30
C18:1n9 (OA)	46.79 ± 0.96 ^d	65.08 ± 0.52 ^a	57.44 ± 0.81 ^b	50.51 ± 1.98 ^c	16.55 ± 0.39 ^f	21.16 ± 0.42 ^e
ΣPUFA	13.95 ± 0.88^f	19.12 ± 0.59^e	27.31 ± 0.49^d	33.00 ± 1.56^c	67.10 ± 0.68^a	59.55 ± 0.67^b
Σn6	9.56 ± 0.36^e	13.25 ± 0.35^d	17.03 ± 0.23^c	17.35 ± 0.20^c	62.69 ± 0.78^a	50.11 ± 1.70^b
C18:2n6 (LA)	8.78 ± 0.20 ^e	12.40 ± 0.23 ^d	16.50 ± 0.21 ^c	16.88 ± 0.16 ^c	60.67 ± 0.85 ^a	48.12 ± 0.92 ^b
C20:4n6 (AA)	0.11 ± 0.05	0.10 ± 0.05	0.15 ± 0.04	0.11 ± 0.03	0.39 ± 0.22	0.19 ± 0.05
Σn3	4.31 ± 0.76^c	5.84 ± 0.88^c	10.07 ± 0.47^b	15.61 ± 1.45^a	4.22 ± 0.99^c	9.19 ± 1.75^b
C18:3n3 (ALA)	0.41 ± 0.13 ^c	0.66 ± 0.21 ^c	4.28 ± 0.07 ^b	10.20 ± 1.23 ^a	0.14 ± 0.04 ^c	4.53 ± 0.91 ^b
C20:5n3 (EPA)	0.245 ± 0.078 ^a	0.040 ± 0.009 ^b	0.050 ± 0.005 ^b	0.0788 ± 0.017 ^b	0.101 ± 0.027 ^b	0.091 ± 0.020 ^b
C22:5n3 (DPA)	3.56 ± 0.63 ^{ab}	4.82 ± 0.68 ^a	5.03 ± 0.48 ^a	4.45 ± 0.47 ^{ab}	2.48 ± 0.63 ^b	3.31 ± 0.91 ^{ab}
C22:6n3 (DHA)	0.19 ± 0.01	0.51 ± 0.13	0.66 ± 0.18	0.80 ± 0.19	1.78 ± 0.87	1.22 ± 0.59
n6:n3	2.52 ± 0.40^b	2.66 ± 0.54^b	1.71 ± 0.10^b	1.17 ± 0.14^b	20.68 ± 5.13^a	4.59 ± 0.38^b
PUFA:SFA	0.42 ± 0.04^f	1.59 ± 0.12^e	2.24 ± 0.07^d	2.50 ± 0.12^c	4.62 ± 0.10^a	3.49 ± 0.08^b

Data are presented as mean ± SEM; n=6/group. SFA: saturated fatty acid; MUFA: monounsaturated fatty acid; PUFA: polyunsaturated fatty acid. Within a row, statistical differences ($p \leq 0.05$) among means are indicated by different lowercase letters, and the whole row is bolded.

Epididymal adipose tissue protein analysis

Levels of fatty acid synthase protein, a multimeric enzyme complex that catalyzes the synthesis of SFAs, were significantly lower in all dietary treatment groups compared to the Lard group (Figure 5.2). Adiponectin protein was significantly higher in the High-Oleic Canola Oil group compared to the Lard and Soybean Oil groups (Figure 5.3). Levels of short-chain fatty acid receptors GPR41 and GPR43 were significantly lower in the Soybean Oil group compared to the Lard group (Figure 5.4).

Fatty acid synthase expression in epididymal adipose tissue

To further explore the effect of the dietary treatments on fatty acid synthase in adipose tissue, we conducted real-time qPCR to measure mRNA levels. No differences in fatty acid synthase mRNA were observed among the groups measured: Lard, High-Oleic Canola Oil, Canola Oil and Canola-Flaxseed Oil (data not shown).

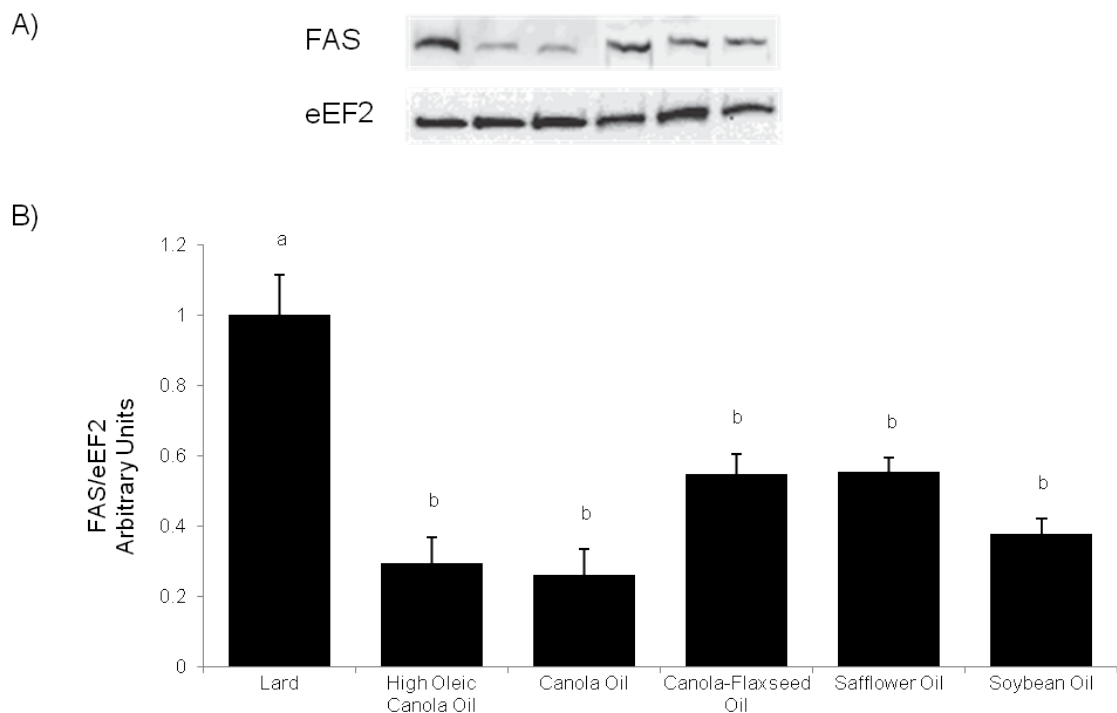


Figure 5.2 Fatty Acid Synthase Protein Levels in Epididymal Adipose Tissue

A) Representative Western blots of fatty acid synthase (FAS) and eEF2, the loading control. B) Relative intensities of the bands were quantified by densitometry and the ratio of fatty acid synthase to eEF2 was calculated in arbitrary units. Data are expressed as mean \pm SEM (n=6/group). Columns with different lowercase letters are significantly different ($p \leq 0.05$) from each other.

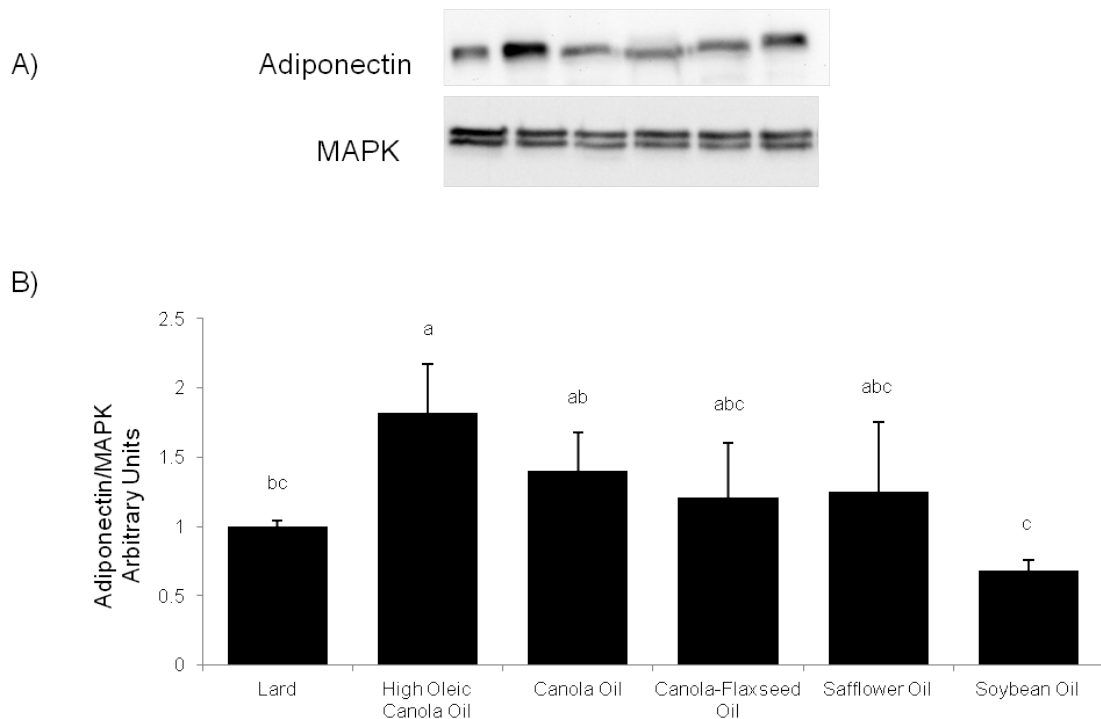


Figure 5.3 Adiponectin Protein Levels in Epididymal Adipose Tissue

A) Representative Western blots of adiponectin and p42/44 MAPK, the loading control.

B) Relative intensities of the bands were quantified by densitometry and the ratio of adiponectin to MAPK was calculated in arbitrary units. Data are expressed as mean \pm SEM (n=6/group). Columns with different lowercase letter are significantly different ($p \leq 0.05$) from each other.

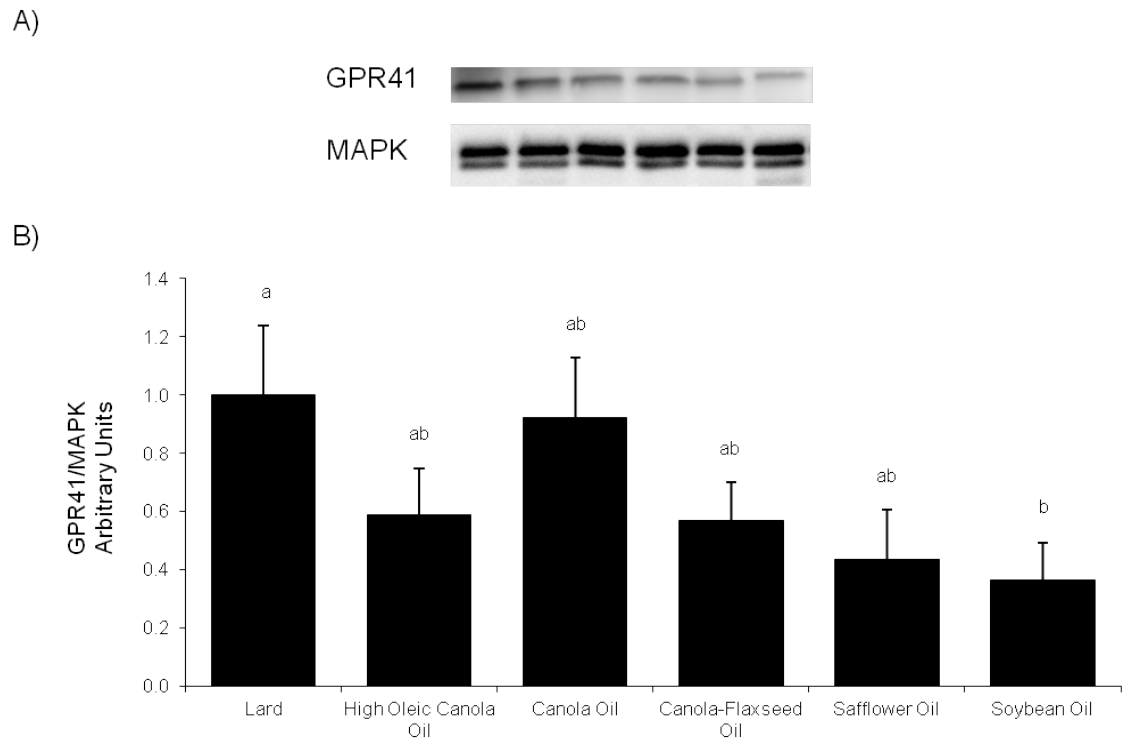


Figure 5.4 GPR41 Protein Levels in Epididymal Adipose Tissue

A) Representative Western blots of GPR41 and MAPK, the loading control. B) Relative intensities of the bands were quantified by densitometry and the ratio of GPR41 to p42/44 MAPK was calculated in arbitrary units. Data are expressed as mean \pm SEM (n=6/group). Columns with different lowercase letters are significantly different ($p \leq 0.05$) from each other.

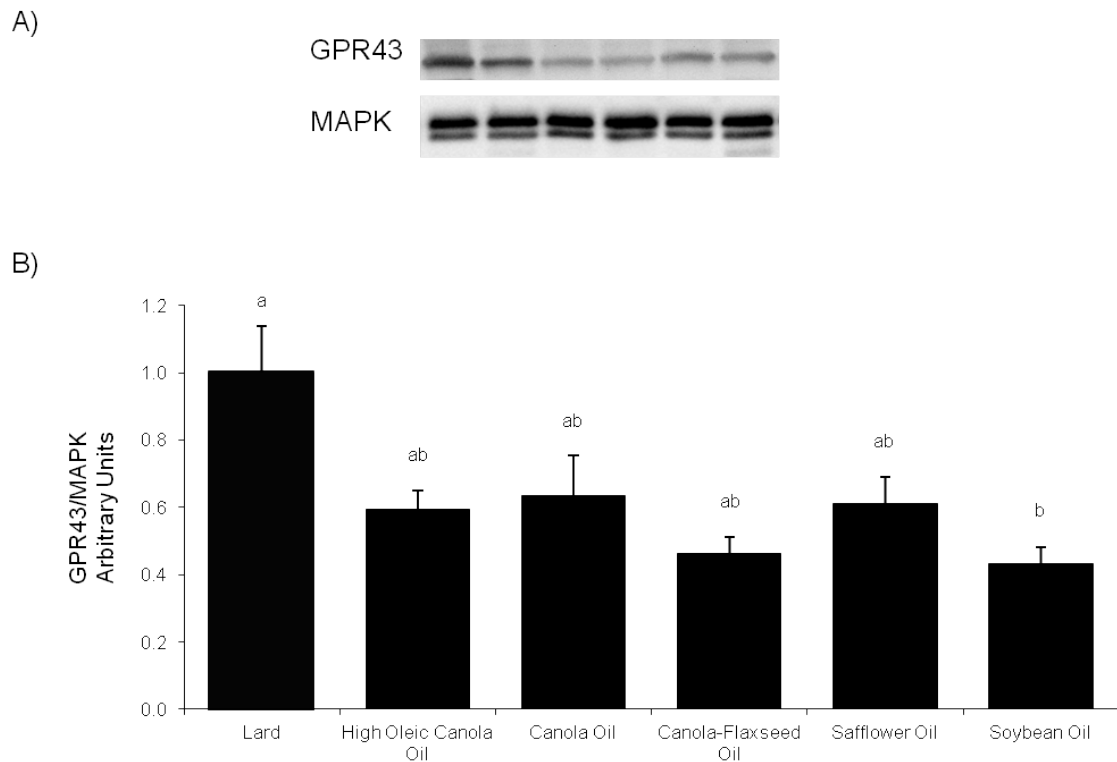


Figure 5.5 GPR43 Protein Levels in Epididymal Adipose Tissue

A) Representative Western blots of GPR43 and MAPK, the loading control. B) Relative intensities of the bands were quantified by densitometry and the ratio of GPR43 to p42/44 MAPK was calculated in arbitrary units. Data are expressed as mean \pm SEM (n=6/group). Columns with different lowercase letters are significantly different ($p \leq 0.05$) from each other.

5.6 Discussion

The present study was designed to determine the effects of high-fat diets containing different proportions of MUFAs and PUFAs on adipose tissue function and inflammation in the context of obesity. Although the groups fed the MUFA-rich diets had 10% lower body weight at the end of the study, there was no difference in adiposity based on relative fat pad mass. One of the main findings of the study is the decrease in fatty acid synthase protein observed in all dietary treatment groups compared to the Lard group. The adipose tissue is one of two major sites of fatty acid synthesis, second only to the liver [34]. The fatty acid synthase multimeric enzyme is transcriptionally regulated by several factors responsive to insulin/glucose, including sterol regulatory element binding protein and upstream stimulatory factors -1 and -2 [35]. PUFAs also play a role in suppressing the expression of lipogenic genes such as fatty acid synthase, but this effect is hepatocyte-specific [36]. The mechanism for the observed decrease in fatty acid synthase protein in our dietary treatment groups consuming PUFA- or MUFA-rich diets remains uncertain, since we did not observe any change in fatty acid synthase mRNA levels in the adipose tissue. Thus, the change in fatty acid synthase protein may instead be the result of regulation at either the post-transcriptional or the translational level.

Another interesting finding of the study is the increase in adiponectin protein in the adipose tissue of the High-Oleic Canola Oil group compared to the Lard and Soybean Oil groups. Expression of this anti-inflammatory adipokine is positively associated with adipocyte function [37]. Our previous research has demonstrated that even when

adipose tissue mass remains the same following dietary treatments, adipocyte size may decrease, resulting in an improved adipocyte phenotype associated with this more functional state [19]. However, since the increased adiponectin in the adipose tissue of our animals is not reflected in the serum values, and other cytokine and adipokine protein levels do not correspond with an improved adipocyte phenotype, it is unlikely that the adipose tissue in the High-Oleic Canola Oil group was truly more functional than in other groups. The elevated adipose tissue adiponectin may instead be linked to the high MUFA content of the High-Oleic Canola Oil diet, as other studies have demonstrated an association between MUFAs and adiponectin. For instance, in rats fed a high-fat diet rich in MUFAs, adiponectin mRNA levels were increased in subcutaneous and visceral adipose tissue [38]. As well, plasma adiponectin was negatively associated with MUFA intake in Filipino-American women [39], but positively associated with a MUFA-rich diet (compared to diets high in carbohydrates or protein) in hypertensive adults [40]. In addition, in vitro treatment of 3T3-L1 adipocytes with oleic acid increased adiponectin mRNA levels [41]. Although the variation in the study design of the available reports makes it difficult to determine whether dietary MUFAs regulate adiponectin production, there is some evidence for the anti-inflammatory and cardioprotective effects of MUFA consumption in obesity [42,43]. The findings of our study, however, do not fully support an anti-inflammatory role for MUFAs, as levels of inflammatory mediators MCP-1 and IL-18 in adipose tissue did not change among groups. The association between circulating adiponectin and a high-MUFA diet requires further investigation.

In our study, the short-chain fatty acid receptors GPR41 and GPR43 were decreased in the Soybean Oil group compared to the Lard group. These receptors play a role in bacteria-derived short-chain fatty acid sensing in the colon [44]. However, they are also expressed in adipose tissue, where both receptors are reported to stimulate leptin secretion upon binding of short-chain carboxylic acids, including propionate and butyrate [45-47]. Leptin is important in regulating appetite and satiety by binding to the leptin receptor in the hypothalamus, but also serves as an adiposity signal. Leptin circulates at levels directly proportional to body fat, and overproduction of this proinflammatory adipokine is characteristic of hypertrophic, dysfunctional adipocytes [5]. We speculate that the short-chain fatty acids present in the high-saturated fat lard diet in our study could play a role in activating GPR41 and/or GPR43, contributing to leptin-driven inflammation. It should be noted, however, that the availability of receptor ligands does not necessarily result in a corresponding increase in receptor protein; furthermore, in our study, serum leptin was not different among groups. Given that little information is available on the mechanism of GPR-stimulated leptin production in adipocytes, the precise role of dietary fatty acid signalling via this pathway remains unclear.

Changes to the fatty acid composition in the phospholipids and triglycerides of adipose tissue occurred as expected, corresponding to the content of SFAs, MUFAs and PUFAs in the diets. Membrane phospholipid fatty acids can modulate cellular signalling pathways by serving as precursors to fatty acid-derived messengers. For example, some n6 PUFAs are substrates for eicosanoid biosynthesis, which may drive local tissue

inflammation [21]. Despite the large differences in LA and ALA intake, there were no changes in AA and only small differences in EPA in phospholipids, suggesting that the pool of substrates for eicosanoid production was relatively unchanged. Furthermore, enrichment of n6 PUFAs in the Safflower and Soybean Oil diets and a high level of n3 PUFAs in the Canola-Flaxseed Oil diet did not produce changes in protein levels of pro- or anti-inflammatory adipokines in the adipose tissue of our animals. Although the diets varied in the amounts and ratios of n6 and n3 fatty acids, in adipose tissue, there were no differences among groups in the n6:n3 ratio in either phospholipid or triglyceride fatty acids. The sole exception was the Safflower Oil group, in which the n6:n3 ratio in adipose tissue was elevated due to the high content of LA in the oil. The same is true of the PUFA:SFA ratio in adipose tissue phospholipid fatty acids, while the triglyceride fatty acid composition closely reflects the dietary composition of PUFAs and SFAs.

Even though the n6:n3 ratios varied across the dietary treatments, a dietary n6:n3 ratio of 8:1 or less did not produce differences in adipose tissue n6:n3 ratios. However, DHA, the main n3 PUFA in adipose tissue phospholipids was similar across all groups, indicating that the range of dietary ALA intakes was able to maintain tissue DHA by elongation and desaturation of ALA. Interestingly, DPA was the major n3 PUFA in adipose tissue triglycerides, where it was elevated in the Canola Oil and High-Oleic Canola Oil groups compared to the Safflower Oil group. The findings of Gregory et al [48] and Pawlosky et al [49] suggest that increased ALA intake elevates tissue DPA due to competition between EPA and DPA for Elongase2 (Elovl2), the enzyme that acts on both these substrates in the final steps for conversion to DHA. The prevalence of DPA in

the adipose tissue triglycerides of our animals fed diets containing ALA may similarly be the result of competition between EPA and DPA. Overall, the changes in adipose fatty acid composition were insufficient to alter inflammatory adipokines. Furthermore, our study indicates that very few obesity-related parameters were altered by 12 weeks of feeding MUFA- or PUFA-rich diets containing different proportions of n3 and n6 PUFAs in the context of a high-fat diet. The findings of this study parallel those of Hanke et al [29], who investigated the efficacy of the same diet regimen for modulating hepatic steatosis. Despite a 20% decrease in liver lipid concentration and similar changes to the liver fatty acid composition as observed in the present study, markers of glucose homeostasis, hepatic inflammation, and fatty acid metabolism exhibited only minor differences among groups.

Influence of the study design

Several features of the study design may help to explain the results. Although changes in some of the markers of adipocyte function and inflammation we measured were detected among dietary groups during the course of the study (for example, serum PAI-1 was different at week 4, and serum leptin and MCP-1 were different at week 8), most groups were not different from each other by the end of the 12-week study. This finding suggests that the animals were able to adapt to the dietary treatments over time, and demonstrates that long-term high-fat feeding may yield fewer phenotypic physiological changes than previously thought. In addition, although the PUFA:SFA ratios and the n6:n3 ratios in the adipose tissue phospholipids and triglycerides closely

reflected the differences in fatty acid composition among diets, they appeared to have little effect on adipokine production and adipocyte function in this model.

5.7 Conclusion

We observed changes in fatty acid synthase, adiponectin and short-chain fatty acid receptors GPR41 and GPR43 in animals fed high-fat diets containing different proportions of MUFAs and PUFAs. These findings indicate that different types of dietary fatty acids regulate adipokine production and proteins involved in fatty acid metabolism in adipose tissue. However, although the fatty acid compositions of the diets were variable (particularly in PUFA, MUFA and ALA content) and there were changes in the fatty acid profile of adipose tissue, the diets had little impact on adipose tissue function. Additionally, although there were some differences in body weight among groups at the end of the study, these differences did not translate into changes in adiposity (fat mass) or inflammation, suggesting that the dietary treatments had little impact on the broader physiological function of these obese animals. Overall, the findings of this study indicate that using vegetable oils to replace PUFAs with MUFAs and to increase intake of ALA has minimal effects on altering adipose tissue function and inflammation in the context of a long-term high-fat diet.

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CHAPTER 6

The Impact of n3 Polyunsaturated Fatty Acid Supplementation on the Incidence of Cardiovascular Events and Complications in Peripheral Arterial Disease: A Systematic Review and Meta-Analysis

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6.1 Contribution Statement

I, Jennifer Enns, made the following contributions to this study:

- conceived of and designed the review with PZ, CGT, RZ, and AAS
- developed the search strategy with CF and conducted the literature search
- conducted the literature screening and data extraction with AY as a second reviewer
- performed statistical analyses with guidance from RZ and AAS
- interpreted results and wrote the manuscript

Other contributors:

- Azadeh Yeganeh
- Ryan Zarychanski
- Ahmed M. Abou-Setta
- Carol Friesen
- Peter Zahradka
- Carla G. Taylor

6.2 Abstract

Individuals with PAD are at higher risk for cardiovascular events than the general population. While supplementation with n3 PUFAs has been shown to improve vascular function, it remains unclear if supplementation decreases serious clinical outcomes. We conducted a systematic review and meta-analysis to determine whether n3 PUFA supplementation reduces the incidence of cardiovascular events and complications in adults with PAD.

We searched five electronic databases (MEDLINE, EMBASE, CENTRAL, Scopus and the International Clinical Trials Registry Platform) from inception to 6 December 2013 to identify randomized trials of n3 PUFA supplementation (from fish or plant oils) that lasted ≥ 12 weeks in adults with PAD. No language filters were applied. Data on trial design, population characteristics, and health outcomes were extracted. The primary outcome was major adverse cardiac events (MACE); secondary outcomes included the components of MACE (myocardial infarction, cardiovascular death, stroke and angina), amputation, revascularization procedures, maximum and pain-free walking distance, adverse effects of the intervention, and quality of life. Trial quality was assessed using the Cochrane Risk of Bias tool.

Of 741 citations reviewed, we included five trials enrolling 396 individuals. All included trials were of unclear or high risk of bias. There was no evidence of a protective association of n3 PUFA supplementation against major adverse cardiac events (pooled risk ratio 0.73, 95% CI 0.22 to 2.41, I^2 75%, 2 trials, 288 individuals) or other serious clinical outcomes. Adverse events and compliance were poorly reported. Our results

showed that insufficient evidence exists to suggest a beneficial effect of n3 PUFA supplementation in adults with PAD with regard to cardiovascular events and other serious clinical outcomes.

6.3 Introduction

PAD is an atherosclerosis-induced blockage of non-coronary and non-cerebral arteries. Individuals diagnosed with PAD most often present with ischemic pain in the lower leg following exercise, and as the disease progresses, pain may also occur at rest [1]. The management of PAD includes therapies that reduce atherosclerotic disease progression and cardiovascular events, such as vasoactive drugs, statins, smoking cessation therapy, exercise, and in severe cases, angioplasty or bypass surgery [2]. Risk factor modification unfortunately has little effect on the primary symptom of PAD, intermittent claudication [2]. PAD is estimated to occur in 5.9% of people older than 40 years, corresponding to 7.2 million affected individuals in the USA alone [3]. While nearly 60% of all individuals with PAD are asymptomatic [4], they are still at high risk for coronary heart disease due to the underlying presence of atherosclerosis. Cardiac events are the most common cause of death in persons with PAD [5].

N3 PUFAs are a group of dietary fats obtained from fish and plant oils that are reported to have a protective role in coronary heart disease and other cardiovascular complications [6]. N3 PUFAs may exert beneficial effects in cardiovascular disease by lowering hepatic triglyceride production and increasing clearance from the circulation [7], and by incorporating into phospholipids in cell membranes, thereby reducing the availability of substrates for the production of proinflammatory molecules [8]. Systematic reviews of n3 PUFA supplementation cohort studies and RCTs have focused primarily on the marine-sourced n3 PUFAs EPA and DHA. Many of these reviews have demonstrated reductions in cardiovascular mortality in populations with and without

established cardiovascular disease [6,9-14], while others have shown mixed results [15-18] or no benefits [19-21] following marine n3 PUFA supplementation. However, little is known about the role of the plant-based n3 PUFA ALA in cardiovascular disease, particularly in the high-risk population of individuals with PAD.

Several clinical trials of n3 PUFA supplementation have been conducted in the PAD population, given the high incidence of cardiovascular events in individuals with PAD [22] and the demonstrated benefits of n3 PUFAs in individuals with cardiovascular disease [6]. While some of these trials have shown improvements in vascular function [23-25] or inflammatory status [26], it is unclear if supplementation decreases the incidence of major adverse cardiovascular events and other relevant clinical outcomes. The objective of this systematic review was to determine whether dietary supplementation with fish and/or plant-based n3 PUFAs reduces the incidence of cardiac events and complications in the high-risk population of PAD patients.

6.4 Methods

Using an *a priori* published protocol [27], we conducted a systematic review using methodological approaches outlined in the *Cochrane Handbook for Systematic Reviewers* [28] and reported according to the Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) criteria [29].

Data Sources and Searches

We searched the following bibliographic databases from inception to Dec 6, 2013: PubMed/MEDLINE (National Library of Science), EMBASE (Ovid), CENTRAL (Cochrane library - Wiley), followed by forward searching for key articles in Scopus. To identify ongoing and unpublished trials, we searched the World Health Organization's International Clinical Trials Registry Platform. We supplemented electronic searches by hand-searching the bibliographies of included trials, and relevant narrative and systematic reviews. Our search strategy used both controlled vocabulary and free text, and searches in MEDLINE and EMBASE were combined with a high-sensitivity filter for RCTs [28]. The detailed search strategy for MEDLINE is included in Table 6.1.

Table 6.1 Search Strategy

1.	(omega 3 fatty acid OR n3 fatty acid OR n 3 fatty acid OR n-3 fatty acid OR n-3 polyunsaturated fatty acid)
2.	(dietary supplement OR dietary supplementation OR dietary fat)
3.	(flax OR flaxseed OR flaxseed oil OR linseed oil)
4.	(fish OR fish oil OR fatty fish OR marine)
5.	(canola OR canola oil OR rapeseed oil)
6.	(dietary supplements[MeSH Terms]) OR (dietary fats[MeSH Terms]) OR (flax[MeSH Terms]) OR (linseed oil[MeSH Terms]) OR (fishes[MeSH Terms]) OR (fish oils[MeSH Terms])
7.	(eicosapentaenoic acid OR EPA)
8.	(docosahexaenoic acids OR DHA)
9.	(alpha-linolenic acid OR ALA)
10.	(fatty acids, n3[MeSH Terms]) OR (eicosapentaenoic acid[MeSH Terms]) OR (docosahexaenoic acids[MeSH Terms]) OR (alpha-linolenic acid[MeSH Terms])
11.	#1 OR #2 OR #3 OR #4 OR #5 OR #6 OR #7 OR #8 OR #9 OR #10
12.	(peripheral arterial disease OR peripheral artery disease OR peripheral arterial diseases OR peripheral artery diseases)
13.	(peripheral vascular disease OR peripheral vascular diseases OR peripheral angiopathy OR peripheral angiopathies)
14.	(ankle brachial index OR ankle-brachial index OR ankle brachial indices OR ankle-brachial indices OR intermittent claudication)
15.	(peripheral arterial disease[MeSH Terms]) OR (peripheral vascular diseases[MeSH Terms]) OR (ankle-brachial index[MeSH Terms]) OR (intermittent claudication[MeSH Terms])
16.	#12 OR #13 OR #14 OR #15
17.	(randomized controlled trial[pt] OR controlled clinical trial[pt] OR randomized[tiab] OR placebo[tiab] OR drug therapy[sh] OR randomly[tiab] OR trial[tiab] OR groups[tiab]) NOT (animals[mh] NOT humans[mh])
18.	#11 AND #16 AND #17

Study Selection

To identify a population at high risk for cardiovascular events, we included only randomized trials of adults aged 40 years or older with established PAD, which was diagnosed by the presence of stable intermittent claudication and/or $ABI \leq 0.9$. The intervention period was required to be at least 12 weeks in duration to allow the intervention to impact cardiovascular function. The minimum intervention duration of 12 weeks was chosen based on studies of the appearance of dietary fatty acids in the plasma and blood cell plasma membranes (which are considered robust markers of n3 PUFA consumption), demonstrating that plasma membrane fatty acid composition changes are apparent within two weeks of a dietary fatty acid intervention [30]. Thus, it is feasible that downstream physiological effects of the diet could be demonstrated within 12 weeks. We included interventions with any n3 PUFAs in diet or supplement form where the dose per day was reported and with appropriate comparators (i.e. placebo, n3 PUFA deficient diet or usual diet). We used a two-stage process for trial screening and selection employing standardized and piloted screening forms. Two reviewers independently screened the titles and abstracts of the search results to determine whether each citation met the inclusion criteria. Trials published in languages other than English were translated, and the full text of citations classified as *include* or *unclear* were independently reviewed with reference to the predetermined inclusion and exclusion criteria. Discrepancies between reviewers were resolved through consensus or by discussion with a third reviewer, as required.

Data Extraction and Quality Assessment

From each trial, two reviewers independently abstracted population characteristics (including age, body mass index, and diagnosis of PAD), number of participants, trial duration and follow-up, intervention design (including type and dose of n3 PUFA, comparators, and co-interventions), clinical health outcomes (incidence of major adverse cardiac events, myocardial infarction, cardiovascular death, angina, and stroke, amputation, symptom-driven revascularization procedures (e.g., bypass surgery), maximum and pain-free walking distance, and quality of life), and adverse effects. Any disagreements were resolved through consensus. Where data were incompletely or imprecisely reported, we contacted study authors for clarification. We assessed the internal validity of the included trials using the Cochrane Collaboration Risk of Bias Tool [31]. This tool consists of six domains, each of which is rated “low risk,” “unclear risk,” or “high risk.” If one or more individual domains were assessed as having a high risk of bias, the overall rating was a high risk of bias. We considered the overall risk of bias low only if all components were rated as having a low risk of bias. We rated the risk of bias for all other trials as unclear. Information regarding methodological quality was used to guide subgroup analyses and to explore sources of heterogeneity.

Data Synthesis and Analysis

We conducted meta-analyses of the data from included trials using Review Manager (Version 5.2, The Cochrane Collaboration, Copenhagen, Denmark). Pooled binary data were weighted using the Mantel-Haenszel method, and presented as risk ratios (or Peto odds ratios for rare events) with 95% confidence intervals (CI) [32].

Pooled continuous data were weighted by the inverse of variance and expressed as a weighted mean difference with 95% CI. We explored and quantified statistical heterogeneity of the data using the I-squared test [33]. We assessed publication bias by viewing the overlap of confidence intervals and using funnel plot techniques. For the primary outcome of major adverse cardiac events, we performed the following *a priori* subgroup analyses: short versus extended duration of intervention, high versus low n3 PUFA dose (g/day), type of n3 PUFA (EPA, DHA, ALA, or a combination of these), supplements or capsules vs. dietary sources of n3 PUFA, and the duration of follow-up. Final subgroup analyses were limited by the number of trials included and the availability of reported outcomes and covariates.

6.5 Results

Characteristics of Trial Populations and Interventions

Of 741 citations identified from electronic and hand-searches, we included 5 unique trials enrolling a total of 396 individuals (Figure 6.1; Table 6.2). All were single-centre trials published between 1990 and 2010. All trials were adjudicated to be of high (3/5) [34-36] or unclear (2/5) [37,38] risk of bias (Table 6.3). Due to the small number of trials included, formal assessment of reporting bias (e.g., by using a funnel plot) was not possible.

Four trials were conducted in Europe [34-37] and one in Japan [38]. One trial recruited individuals who were diagnosed with PAD and were also hypercholesterolemic and on statin therapy [38]. One trial included individuals who previously had angina or a myocardial infarction [34], while the rest excluded individuals with a recent history of cardiovascular/cerebrovascular events or revascularization surgery. Three trials [34,37,38] studied capsules containing varying doses of EPA alone or EPA and DHA combined, compared to capsules of sunflower oil [34] or corn oil [37], or no treatment [38]. The intervention in the remaining two trials [35,36] studied a specially developed dairy product enriched with EPA, DHA and ALA, as well as Vitamins A, B₆, D and E, folate and oleic acid, compared to a control group who received a product identical in appearance enriched only with Vitamins A and D. None of the trials had follow-up periods beyond the duration of the intervention.

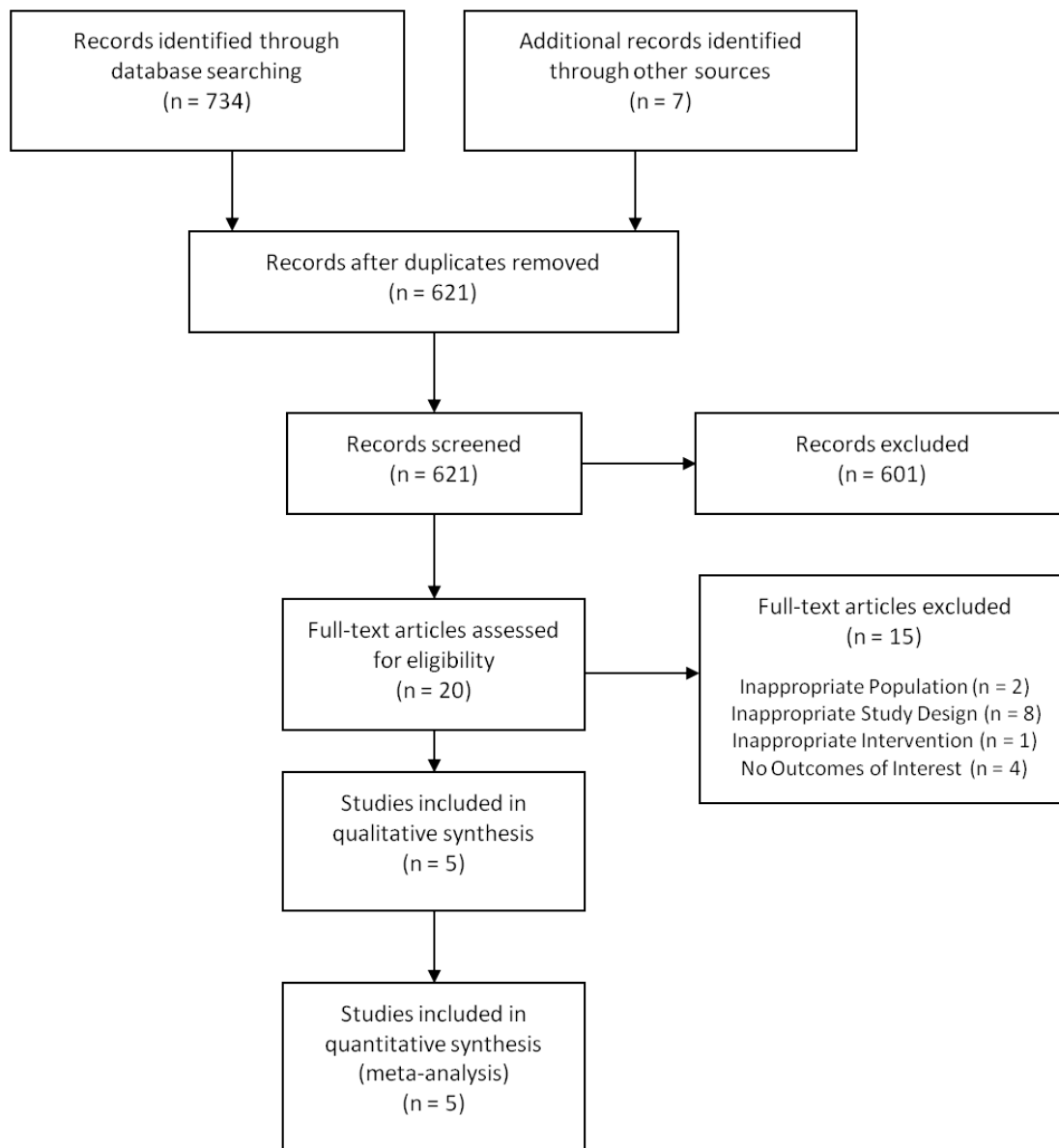


Figure 6.1 PRISMA flow diagram. Summary of the evidence search and selection.

Table 6.2 Patient Populations and Study Characteristics

STUDY	POPULATION					INTERVENTION			
	Age (years)	BMI (kg/m ²)	Diagnosis of PAD	Medication/Therapy	Co-morbidities	n3 PUFA (g/day)	Comparator (g/day)	Co-intervention	Duration
Gans 1990	66.1 ± 8.2	NR	Treadmill testing for IC classification; IC stable for ≥ 1 yr	Excluded patients on lipid-lowering or platelet-active drugs	None reported	EPA (1.8) + DHA (1.2)	Corn oil (3.0)	none	16 wk
Leng 1998	65.7 ± 7.1	26.7 ± 4.2	Edinburgh Claudication Questionnaire, IC stable for ≥ 6 mth; ABI < 0.9	Included patients taking aspirin	Excluded patients with critical ischemia, previous or impending surgery, unstable angina or MI, or severe concurrent illnesses	EPA (0.18-0.27)	Sunflower oil (2.0 – 3.0)	Gamma-linolenic acid	2 yr
Carrero 2005	64.0 ± 9.0	27.9 ± 3.9	Presence of IC, ABI < 0.7	Excluded patients eligible for vascular surgery; excluded patients taking statins	Excluded patients with history of cardiac events or with endocrine or metabolic disturbances. Included patients who were smokers, had T2D or hypertension	EPA (0.2) + DHA (0.13) + ALA (0.06)	Placebo (dairy product)	Vitamins B ₆ and E, folate, oleic acid	1 yr
Carrero 2006	65.5 ± 9.5	27.7 ± 3.4	Presence of IC, ABI < 0.7	Excluded patients eligible for vascular surgery; excluded patients taking statins	Excluded patients with history of cardiac events or with endocrine or metabolic disturbances. Included patients who were smokers, had T2D or hypertension	EPA (0.2) + DHA (0.13) + ALA (0.06)	Placebo (dairy product)	Vitamins B ₆ and E, folate, oleic acid	1 yr
Ishikawa 2010	65.2 ± 7.4	23.3 ± 2.8	Presence of IC, physical findings (e.g., ulcer) and ABI	All patients included were taking statins	Hyperlipidemia (total serum cholesterol)	EPA (1.8)	No treatment	Simvastatin or Pravastatin	up to 5 yr

Data are presented as mean ± SD. ABI, ankle-brachial index; ALA: alpha-linolenic acid; BMI: body mass index; DHA: docosahexaenoic acid; EPA: eicosapentaenoic acid; IC: intermittent claudication; MI: myocardial infarction; T2D: type 2 diabetes.

Table 6.3 Risk of Bias Assessment

STUDY	Selection Bias		Performance Bias	Attrition Bias	Reporting Bias	Other Sources of Bias	Overall Risk of Bias
	Random Sequence Generation	Allocation Concealment	Blinding of Participants and Personnel	Incomplete Outcome Data	Selective Reporting		
Gans 1990	Low risk	Low risk	Unclear risk	Unclear risk	Unclear risk	Low risk	Unclear risk
Leng 1998	Low risk	Low risk	Low risk	High risk [¶]	Unclear risk	Unclear risk*	High risk
Carrero 2005	Low risk	Unclear risk	Low risk	Unclear risk	Unclear risk	High risk [§]	High risk
Carrero 2006	Low risk	Unclear risk	Low risk	Unclear risk	Unclear risk	High risk [§]	High risk
Ishikawa 2010	Low risk	Unclear risk	Unclear risk	Unclear risk	Unclear risk	Low risk	Unclear risk

* Intervention group received gamma-linolenic acid, which was not supplied in control.

[§] Intervention group received Vitamins B₆ and E, folate and oleic acid, which were not supplied in control.

[¶] Drop-out rate was > 30%.

Risk of Bias in Included Trials

While all trials had random sequence generation (5/5), fewer reported adequate allocation concealment (2/5) [34,37] and blinding of outcome assessment (3/5) [34-36], and all trials were subject to unclear or high risk of attrition bias due to incomplete outcome data following substantial loss to follow-up or poor reporting of outcomes (5/5) (Table 6.3). The presence of co-intervention nutrients in 3/5 [34-36] trials did not allow proper controlling for n3 PUFA effect.

Major Adverse Cardiac Events

Two trials reported the incidence of major adverse cardiac events following an intervention with EPA only [34,38]. The risk ratio for major adverse cardiac events was not significantly decreased with supplementation of EPA (risk ratio 0.73; 95% CI 0.22 to 2.41; I^2 75%; 2 trials [34,38]; 288 individuals; Figure 6.2). High statistical heterogeneity observed may relate to differences in the trial populations (Japanese vs. British, hyper- vs. normo-cholesterolemic), the ten-fold difference in daily dose of EPA (1.8 g vs. 0.18 g), the comparator in the control group (no treatment vs. sunflower oil), the co-intervention (statins vs. gamma-linolenic acid), or the length of the intervention (five vs. two years). In one of the trials (213 individuals), the risk ratio for major adverse cardiac events associated with 1.8 g/day EPA compared to no treatment for 5 years was 0.41; 95% CI 0.19 to 0.87 [38]. In the second trial (75 individuals) comparing 0.18 g/day EPA to sunflower oil for 2 years, the risk ratio for major adverse cardiac events was 1.38; 95% CI 0.55 to 3.50 [34].

Secondary Clinical Outcomes

Two trials with a total of 288 individuals reported the incidence of myocardial infarction, cardiovascular death and revascularization procedures (Table 6.4) [34,38]. The pooled Peto odds ratios for myocardial infarction (Peto odds ratio 0.64; 95% CI 0.22 to 1.88), cardiovascular death (Peto odds ratio 0.60, 95% CI 0.19 to 1.90) and the pooled risk ratio for revascularization surgery (risk ratio 0.81, 95% CI, 0.13 to 4.91) demonstrated no significant difference between the EPA intervention group and control (no treatment or sunflower oil).

Four trials [34-37] with a total of 187 individuals reported pain-free walking distance (metres), an indicator of intermittent claudication (Figure 6.3). We observed considerable heterogeneity and no statistically significant differences in pain-free walking distance associated with n3 PUFA supplementation (mean difference 115.40 metres; 95% CI -42.24 to 273.05; I^2 89%). None of the trials reported on quality of life. The remaining secondary outcomes (stroke, angina, amputation and maximum walking distance) were each reported by only one trial and were not found to be statistically different with n3 PUFA intervention (Table 6.4). Gastrointestinal upset, reported in a single trial [34], was more frequent with EPA treatment (risk ratio 1.58; 95% CI 1.01 to 2.48; 60 individuals).

Subgroup Analyses

Limitations of the data (i.e. the number of trials included, and the availability of appropriate outcome data and covariates reported) precluded most subgroup analyses. Comparisons of the effects of n3 PUFA intervention length, dose and type of n3 PUFA on

pain-free walking distance did not reach significance, and statistical heterogeneity remained high (Table 6.5).

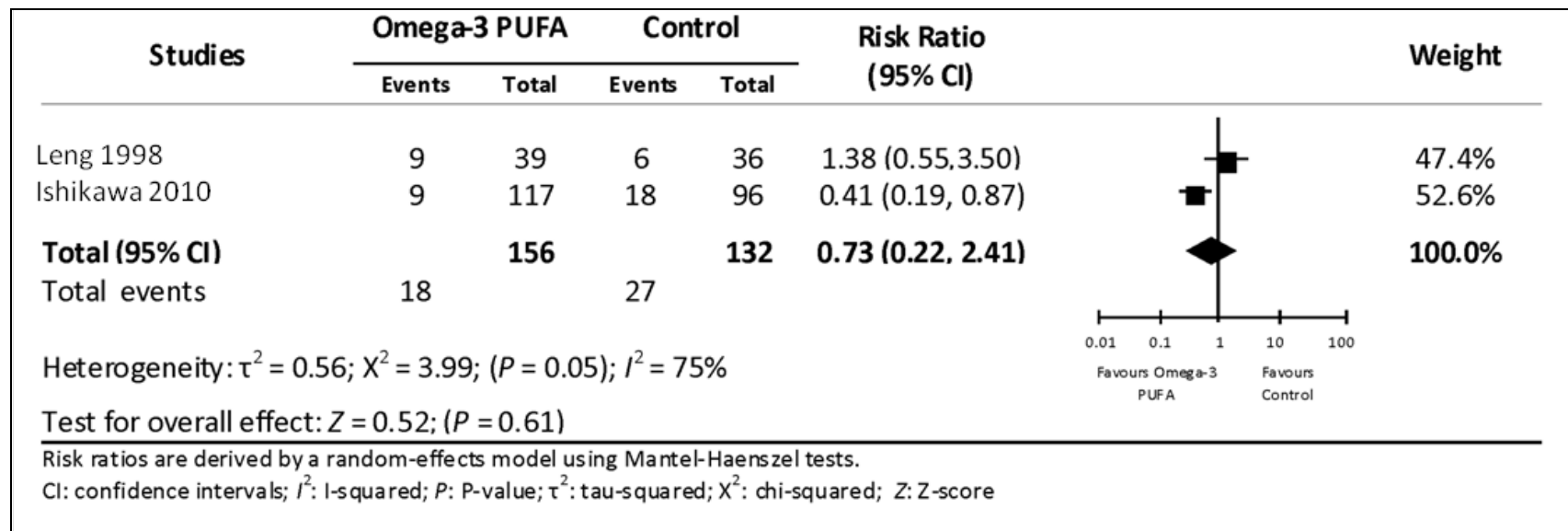


Figure 6.2 Effect of n3 PUFA supplementation on major adverse cardiac events in individuals with PAD.

Table 6.4 Secondary Outcome Measures

Outcome	No. of Trials	No. of Events/ Total Patients in Cohort		Effect Estimate (95% CI)	I^2
		n3 PUFA	Control		
Myocardial Infarction (Leng 1998, Ishikawa 2010)	2	6/156	8/132	Peto OR, 0.64 (0.22, 1.88)	0%
Cardiovascular Death (Leng 1998, Ishikawa 2010)	2	5/156	7/132	Peto OR, 0.60 (0.19, 1.90)	0%
Stroke (Leng 1998)	1	3/60	1/60	Peto OR, 2.79 (0.38, 20.31)	NE
Angina (Ishikawa 2010)	1	5/117	8/106	RR, 0.57 (0.19, 1.68)	NE
Adverse Effects					
All Adverse Events (Gans 1990, Leng 1998)	2	17/76	21/76	RR 0.81 (0.48, 1.38)	NE
Gastrointestinal Upset (Leng 1998)	1	30/60	19/60	RR 1.58 (1.01, 2.48)	NE
Revascularization Surgery (Leng 1998, Ishikawa 2010)	2	9/156	13/132	RR, 0.81 (0.13, 4.91)	59%
Amputation (Leng 1998)	1	0/60	1/60	Peto OR, 0.14 (0.00, 6.82)	NE
Pain-Free Walking Distance (Gans 1990, Leng 1998, Carrero 2005, Carrero 2006)	4	95	88	MD, 115.40 (-42.24, 273.05)	89%
Maximum Walking Distance (Gans 1990)	1	16	16	MD, -26.00 (-71.92, 19.92)	NE

Walking distances (mean differences) are expressed in metres.

CI, confidence intervals; MD, mean difference; NE, not estimable; OR, odds ratio; RR, risk ratio; I^2 , I-squared

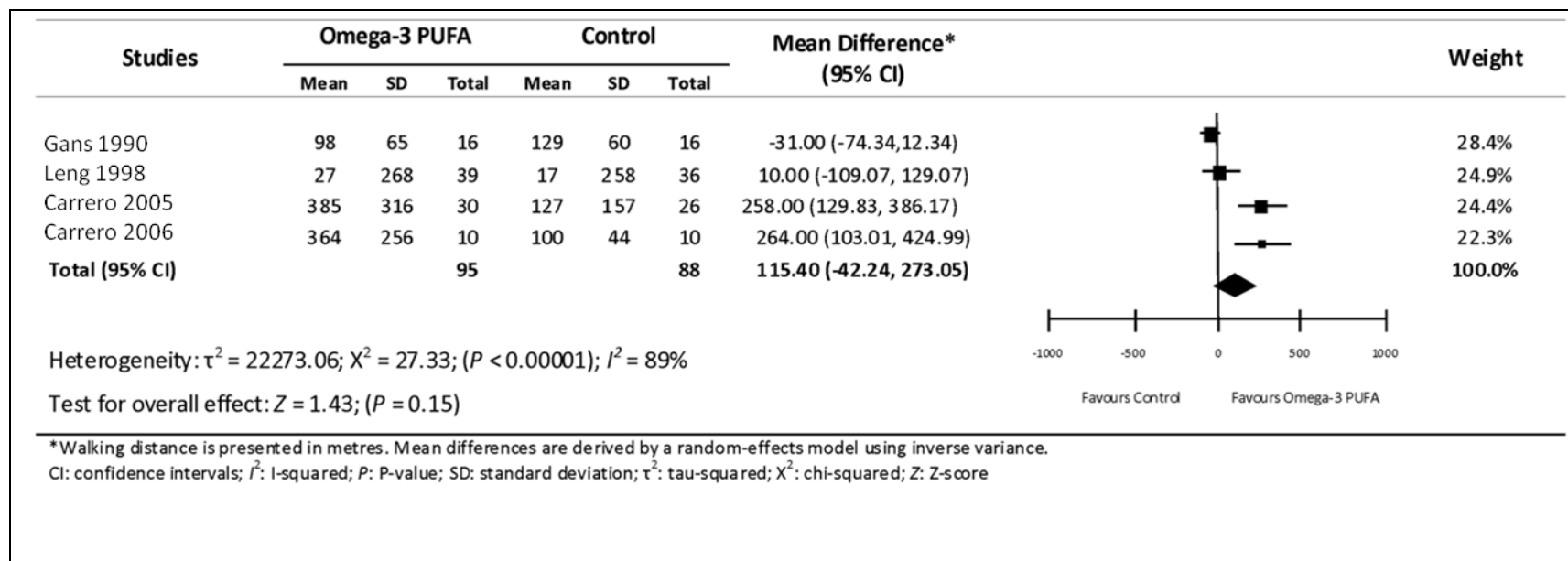


Figure 6.3 Effect of n3 PUFA supplementation on pain-free walking distance in individuals with PAD.

Table 6.5 Subgroup Analysis: n3 PUFA and Pain-Free Walking Distance

Subgroup	No. of Trials	Total Patients in Cohort		Effect Estimate (95% CI)	I ²
		n3 PUFA	Control		
<i>Effect of Intervention Duration on PFWD</i>					
Less than 6 months (Gans 1990)	1	16	16	MD, -31.00 (-74.34, 12.34)	NE
6 months or longer (Leng 1998, Carrero 2005, Carrero 2006)	3	79	72	MD, 172.98 (-0.82, 346.77)	80%
<i>Effect of n3 PUFA Dose on PFWD</i>					
More than 0.3 g/day (Gans 1990)	1	16	16	MD, -31.00 (-74.34, 12.34)	NE
Less than or equal to 0.3 g/day (Leng 1998, Carrero 2005, Carrero 2006)	3	79	72	MD, 172.98 (-0.82, 346.77)	80%
<i>Effect of n3 PUFA Type on PFWD</i>					
EPA + DHA + ALA (Carrero 2005, Carrero 2006)	2	40	36	MD, 260.33 (160.05, 360.60)	0%
EPA + DHA (Gans 1990)	1	16	16	MD, -31.00 (-74.34, 12.34)	NE
EPA (Leng 1998)	1	39	36	MD, 10.00 (-109.07, 129.07)	NE

ALA, alpha-linolenic acid; CI, confidence intervals; DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; MD, mean difference; PFWD, pain-free walking distance; I^2 , I-squared

6.6 Discussion

In this systematic review and meta-analysis of n3 PUFAs in adults with PAD, we found no evidence to suggest a protective association between n3 PUFA supplementation and clinical cardiovascular outcomes, including myocardial infarction, cardiovascular death, angina, stroke, amputation, revascularization, pain-free walking distance or quality of life. One trial indicated that n3 PUFA intake may be associated with increased gastrointestinal side effects.

The effect of n3 PUFA supplementation in cardiovascular disease is controversial. Some reviews and meta-analyses have demonstrated reductions in adverse events with both plant-based ALA and marine-sourced EPA and DHA supplementation in cardiovascular [9-14,39] and cerebrovascular disease [40], and marine n3 PUFAs may be effective in preventing atrial fibrillation after cardiac surgery [41,42], although this finding remains controversial [43]. Other reviews have demonstrated mixed [6,15-18] or no benefits [19-21] following marine and/or plant-based n3 PUFA supplementation. In PAD populations, supplementation with EPA and DHA has been shown to significantly reduce measures of arterial stiffness in several cohorts, including healthy and overweight individuals, and individuals with cardiovascular risk factors, T2D, or hypertension [23-25,44]. Even so, adequate long-term data on serious adverse events in PAD populations is lacking.

In this review, the small number of trials available and the lack of uniformity in population and design of the included trials contributed to the relatively high statistical heterogeneity for some outcomes, and thus caution must be exercised in interpreting

these results. Some of the key differences among the included trials included geographic differences (e.g., the Japanese population's n3 PUFA consumption is up to 15 times greater than in Western countries [45,46]), variable intervention durations (from 16 weeks up to several years), differences in doses of n3 PUFAs (doses of 0.13 g/day DHA and 0.2 g/day EPA are probably too low to have an effect), and concomitant statin and vitamin therapy in some trials (which may have provided beneficial effects on cardiovascular function [47]). Finally, the potential influence of the trial sponsor should also be noted, as trials conducted by Carrero et al [35,36] were sponsored by the manufacturers of n3 PUFAs.

Only two of the included trials [37,38] supplied therapeutic n3 PUFA doses of >1 g/day, according to the recommendations from the American Heart Association and the World Health Organization [48,49]. Subgroup analyses suggest that a longer period of treatment (6 months or longer) even at a low n3 PUFA dose may improve walking distance. This exploratory finding must be confirmed in future studies. It should also be noted that only two trials [34,37] reported on adverse side effects: while the trial by Gans et al [37] reported no significant adverse effects, half of the individuals in the n3 PUFA group in the trial conducted by Leng et al [34] experienced gastrointestinal upset. Growing recognition for nutritional components and supplements as therapeutic agents highlights the need for thorough safety testing during clinical trials.

The internal validity of the trials was often unclear due to underreporting of methods, which potentially could have biased the results. All of the trials rated poorly in the risk of bias assessment, mostly due to a lack of detailed reporting and uncertainty

around the true effect of n3 PUFAs alone (Table 6.3). If meaningful and reliable results are to be obtained from future RCTs, substantial improvements in trial design must be made to reduce the risk of bias, ensure sufficient statistical power, and test for the effect of n3 PUFAs without confounding factors.

Strengths of this review include the broad bibliographic screening of multiple citation databases and trial registries, and rigorous testing for bias. We focused on patient-centred outcomes and evaluated efficacy in the context of relevant side effects. We used an *a priori* published protocol and followed established methodological guidelines in the conduct and reporting of this review. It should be noted that the results of the recent update to the Cochrane review [50] on n3 PUFA supplementation in individuals with intermittent claudication are in agreement with our findings, with very little indication for recommending n3 PUFAs as a therapeutic approach in individuals with PAD. However, the focus of the Cochrane review was primarily on marine n3 PUFAs, and the presence of ALA in some of the interventions was not acknowledged.

Limitations of this review include the restricted amount of clinical outcome data available for pooling among trials, which may have affected the overall findings. We chose to pool data from trials that were variable in intervention design and population.

6.7 Conclusion

In individuals with PAD, insufficient evidence exists to suggest a beneficial effect of n3 PUFA supplementation with regard to major adverse cardiac events, need for revascularization or amputation, pain-free walking distance, or quality of life. Rigorously designed randomized trials powered to detect these clinically relevant outcomes are needed to establish the efficacy and safety of n3 PUFAs in individuals with PAD.

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CHAPTER 7

A Randomized Controlled Trial to Evaluate the Effect of Canola Oil on Blood Vessel Function in Peripheral Arterial Disease: Rationale and Design of the Canola-PAD Study

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October 2014;6:117-125 doi <http://dx.doi.org/10.2147/OAJCT.S70576>.

7.1 Contribution Statement

I, Jennifer Enns, made the following contributions to this study:

- wrote the manuscript

Other contributors:

- Carla G. Taylor
- Peter Zahradka
- Randolph P. Guzman
- Alanna Baldwin
- Brendon Foot

Note: Chapter 7 is a detailed description of the **rationale and design** of the Canola-PAD Study, a clinical trial investigating the effects of conventional canola oil consumption on functional and metabolic parameters of PAD. This paper was published in its current form in the Open Access Journal of Clinical Trials in 2014. The next chapter (Chapter 8) contains the data analyses and discussion of the findings of the Canola-PAD Study.

7.2 Abstract

Individuals with PAD are at high risk for cardiac events due to atherosclerosis. Dietary fatty acid composition has been shown to modulate blood vessel properties, but whether a diet enriched in conventional canola oil can improve clinical endpoints in PAD is not known. The purpose of this paper is to describe the rationale and design of a clinical trial testing the effect of canola oil consumption on vascular function and cardiovascular risk factors in an 8-week dietary intervention in individuals with PAD.

The Canola-PAD Study was a single center, prospective, double-blind RCT in 50 patients over 40 years old with PAD. Participants were randomized into 2 groups and consumed food items containing either conventional canola oil (25 g/day) or an oil mixture representing the Western diet (25 g/day) for 8 weeks as part of their usual diet. The primary outcome included indexes of vascular function (ABI, arterial stiffness, endothelial dysfunction, walking capacity, and cognitive function). Secondary measurements included anthropometrics, serum lipid profile and fatty acid composition, markers of inflammation and glycemic control, and serum metabolite profile. The Canola-PAD Study used an innovative and non-invasive approach to evaluate the effect of canola oil on clinically relevant outcomes in individuals with PAD, including arterial stiffness, walking capacity and cognitive function. The findings will help to inform clinical guidelines and recommendations for dietary fat intake.

7.3 Introduction

PAD, a type of cardiovascular disease affecting over 10 million individuals in North America, is characterized by the formation of atherosclerotic lesions in the blood vessels of the lower limbs. This disease leads to the loss of the vessels' elastic properties (arterial stiffness) and reduces blood flow in the legs [1,2], causing ischemia and painful muscle cramping (intermittent claudication), which limits walking capacity. The gold standard for diagnosis of PAD is an ABI <0.9 [2]. As a result of underlying atherosclerotic disease, individuals with PAD have a significantly greater risk of heart attack and stroke than the general population. It has also been demonstrated that increased arterial stiffness is associated with vascular dementia and decreases in indices of memory and cognition [3,4].

Dietary fat has long been implicated in the etiology of cardiovascular disease. Epidemiological studies have collectively demonstrated that both the amount of fat in the diet and the fatty acid composition play a role in the maintenance of normal vascular tone [5]. Vegetable oil consumption and higher intakes of MUFAs and n3 fatty acids such as ALA are associated with a decreased risk of PAD [6-9]. Conventional canola oil is low in SFAs, high in MUFAs and is the best source of n3 fatty acids among commonly consumed vegetable oils [10]. Diet modeling studies have demonstrated that dietary recommendations for SFAs (<7 -10% energy), MUFAs (10% energy) and ALA (0.6-1.2 % energy) can be achieved with canola oil as the primary dietary fat source [11]. However, these dietary recommendations for amount and type of fatty acids are largely

based on heart disease, whereas evidence for effects of canola oil in PAD, and particularly on vascular function, is lacking.

Noticeable symptoms are often absent in individuals with PAD, making it difficult to use preventative approaches in managing this condition. Although there has been considerable focus on serum cholesterol as a risk factor for cardiovascular disease, there is increasing emphasis on the use of evidence-based clinical endpoints [12], such as vascular function, to identify pre-symptomatic disease and to evaluate the efficacy of therapeutic interventions. Indices of blood vessel function such as arterial compliance (elasticity/stiffness) and flow-mediated dilatation (endothelial dysfunction) can be monitored with instruments that provide rapid results with high sensitivity. It is generally agreed that these and related methods are capable of monitoring changes in vascular properties that become compromised early in the atherogenic process [13-17], and that they are appropriate for examining the changes that occur during short-term dietary interventions [18].

We designed the Canola-PAD Study to determine the effect of conventional canola oil consumption on functional and metabolic parameters of PAD. The primary outcome measure was vascular function. We used ABI and non-invasive assessments of function *in vivo* as well as anthropometrics and serum biochemistry to establish whether canola oil has positive effects on vascular function and cardiovascular risk factors in individuals with PAD.

7.4 Methods and Design

Study Design and Research Aims

The Canola-PAD Study was a single-site, double-blinded, randomized, controlled dietary intervention trial conducted at the I.H. Asper Clinical Research Institute, St. Boniface Hospital in Winnipeg, Canada. The primary aim of the study was to examine the effects of canola oil consumption on vascular function and cardiovascular risk factors in individuals with PAD. Specifically, we wanted to determine whether consumption of food products containing canola oil (25 g/day) is superior to those containing an oil mixture representing the Western diet (25 g/day). Participants in the two study arms (n=25/group) were provided with food items containing the study oils for consumption as part of their usual food intake for 8 weeks. The primary outcome was vascular function (ABI, arterial stiffness, endothelial dysfunction, walking capacity, and cognitive function) and secondary outcomes included anthropometrics, serum lipid profile and fatty acid composition, markers of inflammation and glycemic control, and serum and urinary metabolite profile. Assessments were completed at baseline and 8 weeks. The protocol was approved by the University of Manitoba Research Ethics Board and the St. Boniface Hospital Research Review Committee. The study was registered with ICMJE (NCT01250275, November 2010).

Inclusion and Exclusion Criteria

Patients diagnosed with PAD (ABI <0.9) who exhibited moderate hyperlipidemia (10 year risk 20-30%, as defined by the recommendations for the management and

treatment of dyslipidemia [19]), and absence of chronic illness or genetic causes of hypercholesterolemia were asked to join the study. The inclusion and exclusion criteria are detailed in Table 7.1.

Table 7.1 Canola-PAD Study Inclusion and Exclusion Criteria

Inclusion Criteria

- Male or female, >40 years of age;
- Documented PAD including those with claudication (ABI <0.9) or asymptomatic carotid stenosis >50% or having had a previous intervention for PAD;
- Stable medication profile for the past 3 months with no changes anticipated for the duration of the study;
- Willing to comply with the protocol requirements;
- Willing to provide informed consent;
- At least 3 months have passed since participation in another food-related study.

Exclusion Criteria

- Currently smoking, or smoking within last 6 months;
 - Renal failure requiring dialysis;
 - Having experienced an acute cardiovascular event or medical illness within the last 3 months precluding study participation;
 - Hormone (estrogen) replacement therapy;
 - Amputation of upper or lower extremities on both sides;
 - History of gastrointestinal reaction or allergies to dietary oils, or to one or more ingredients in the study foods that significantly limits the number of study foods that can be consumed;
 - Inability to adhere to a regular diet;
 - Daily consumption of n3 supplements.
-

Recruitment and Screening Visit

Recruitment and enrollment in the study are detailed in Figure 7.1. Participant recruitment began in October 2011 and the final participant completed the intervention in December 2012. Potential participants were identified through the Vascular Surgery Clinic at St. Boniface Hospital, and were provided with a brief description of the study. Interested individuals attended a screening visit where they provided written informed consent before any study-related procedures were performed. The general consent form described the study schedule and dietary intervention, and detailed the biological materials that were to be collected (blood and urine), the procedure for storing the samples, the specific analyses planned, and the potential risks associated with sampling of biological fluids. A separate consent form gave participants the option of allowing non-targeted analysis of plasma and urine samples using a metabolomics approach, and gene expression analysis of saliva samples.

Participants who gave consent underwent a complete medical history including age, sex, alcohol use, exercise habits, current medical problems including documented PAD, medication intake including supplements, and a family history of previous events (e.g., hypertension, heart disease, stroke, and diabetes). A blood sample was obtained by finger tip puncture for measurement of glycated hemoglobin. Participants completed the Mini Mental State Examination (MMSE) to screen for cognitive impairment. Participants who scored ≤ 23 on the MMSE were not included in further cognitive testing; however, they did remain in the study and participated in all of the other non-cognitive assessments. Participants also completed a food frequency questionnaire [20]

to determine usual food consumption, including visible oils/fats and foods containing fat, as well as a questionnaire to record any food allergies and food dislikes/dietary preferences. To assess background dietary intake (including fat intake), a 3-day food record was distributed and completed before the baseline visit.

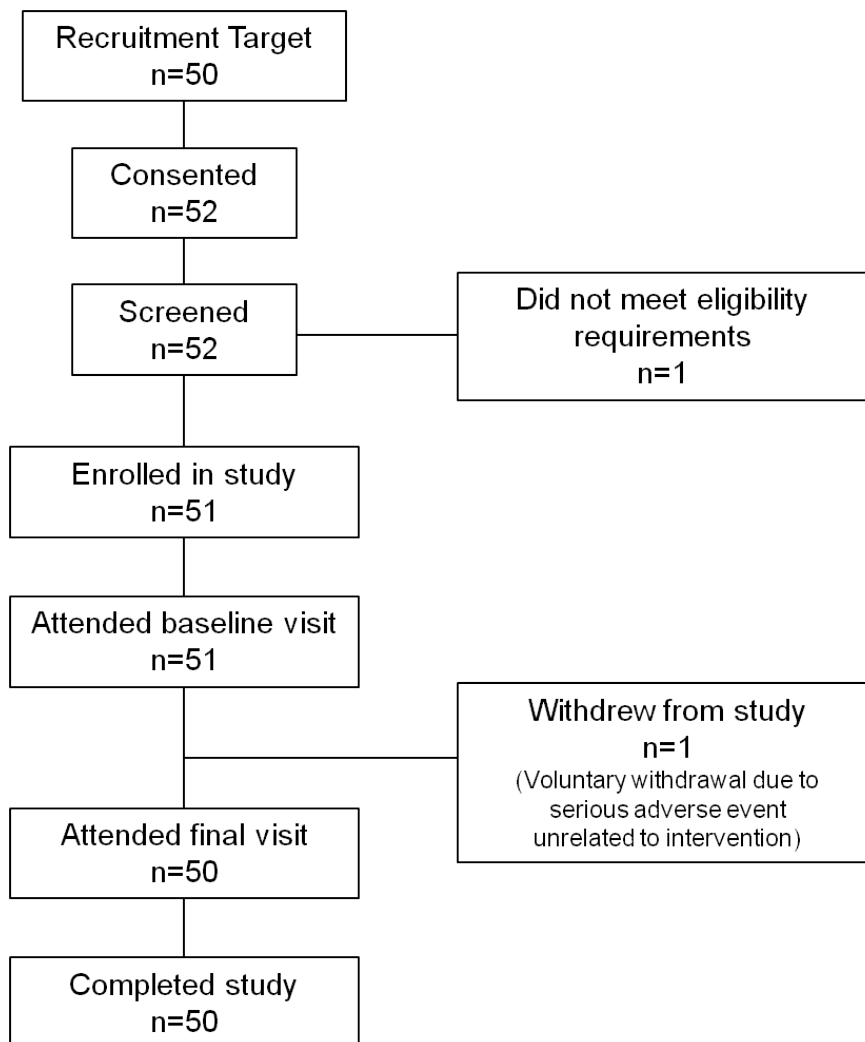


Figure 7.1 Canola-PAD Study Flow Chart

Randomization and Blinding

The randomization sequence was prepared from a random numbers table and individual assignments were stored in opaque envelopes. To ensure that there was equal representation of T2D within both arms of the study, participants were first stratified according to their glycated hemoglobin levels ($\leq 6.5\%$ or $>6.5\%$), and then randomly assigned to one of the 2 dietary treatment groups. To reduce potential bias during data collection and evaluation of study endpoints, both the research team (i.e., individuals who interacted directly with the participants and those involved in analysis of the samples) and the participants were blinded to the dietary intervention from the time of randomization for the duration of the study.

Baseline and Week 8 Assessments

Participants were assessed for changes to their medication profile and current medical conditions throughout the study. Anthropometrics (height, body weight, body mass index, body fat distribution, waist circumference) and blood pressure were obtained. A fasting blood sample was drawn for assessment of fasting plasma lipid profile (total cholesterol, LDL-cholesterol, HDL-cholesterol, triglycerides), glucose, insulin, glycated hemoglobin, markers of vascular function, oxidative stress and inflammation, fatty acid composition, and non-targeted metabolomics analysis (where consent was given). A urine sample was also collected to assess markers of vascular function and metabolism.

Non-invasive assessment of vascular function included measurement of ABI and arterial stiffness (brachial-ankle pulse wave velocity, baPWV) using the Omron VP-1000

Vascular Profiler (Omron Healthcare Inc, Lake Forest, IL). Radial pulse wave analysis was conducted using the SphygmoCor Pulse Wave Monitoring System (AtCor Medical, Sydney, Australia) and SphygmoCor Px Aortic BP Waveform Analysis software (AtCor Medical). Endothelial function was assessed using the Itamar EndoPAT-2000 device (Itamar Medical Inc, Franklin, MA). Advanced glycation end products (AGEs) were assessed by AGE Reader (Diagnoptics Technologies, Groningen, The Netherlands). A selection of cognitive tests was administered to assess various aspects of the participant's level of functioning cognition. Each of the following tests was administered by trained study staff: i) Hopkins Verbal Learning Test, an assessment of verbal learning and memory; ii) Digit Symbol Similarities Test, an assessment of executive functioning, including perceptual speed, motor speed, visual scanning and memory; and iii) Digit Span Test, a measurement of short-term memory and working memory. Cognitive testing data from patients with severe internal carotid artery stenosis ($\geq 70\%$) will be excluded from analysis due to the potential of this condition to influence cognitive function [21]. Participants were asked to undertake a treadmill test [22] to establish pain-free walking distance (distance and time at the onset of claudication) and maximum walking distance (total distance and time).

Intervention

Participants were provided with a 2-3 week supply of frozen food items (including entrées, side dishes, salad dressing, and baked goods; Table 7.2) containing a) conventional canola oil (25 g/day), or b) an oil mixture representing the typical Western diet (25 g/day), to be consumed daily as part of their usual food intake. A dose

of 25 g/day was chosen as it would provide ~40% of the daily fat intake (based on a total fat intake of 67 g/day assuming that this population consumes ~1800 kcal/day and 30% energy from fat) and this amount could be accommodated within 1-2 food items per day. The two study arms provide a comparison of conventional canola oil (low SFAs, high MUFAs and good source of n3 PUFAs) with an oil mixture representing the Western diet. The Western diet is characterized by high intakes of high-fat (high SFAs and n6 PUFAs [23,24]) and high-sugar foods. In our study, the fatty acid composition of the canola oil (100% conventional canola oil) was 8% SFAs, 64% MUFAs, 28% PUFAs (2:1 ratio for n6:n3 PUFAs) whereas the fatty acid composition of the oil mixture representing the Western diet (2:1:1 ratio of butter, coconut oil, high-linoleic safflower oil) was 57% SFAs, 21% MUFAs, and 22% PUFAs (20:1 ratio for n6:n3 PUFAs); these fatty acid compositions will be verified by gas chromatography by sampling food products throughout the study. Furthermore, the analysis of the 3 day food records (before baseline and during week 6) will be used to interpret how the consumption of the study foods changes the overall dietary fatty acid profile in canola oil and control groups.

The food items were based on standard recipes and modified appropriately for the dose of oil and according to sensory testing. Food preparation was done in the Department of Human Nutritional Sciences at the University of Manitoba. The food items were similar in appearance, calories, and macronutrient and micronutrient composition between the two groups. Participants received a 2-week supply of frozen food items at the baseline visit. For convenience, the food items for the rest of the study

were delivered to participants at their homes at the end of Week 2 and Week 5. The food items were also available to immediate family members to assist with compliance.

Table 7.2 Two-Week Cycle of Study Food Products

Schedule	Food Item(s)[†]
Day 1	Chicken Fried Rice
Day 2	Banana Muffin Snack Bar
Day 3	Pizza Pretzel
Day 4	Flatbread Tangy Mustard Salad Dressing
Day 5	Pad Thai Noodle Dish
Day 6	Pumpkin Raisin Muffin Oatmeal Chocolate Chip Cookies
Day 7	Spanish Rice
Day 8	Apple Coconut Muffin Nuts & Pretzel Snack
Day 9	Cheddar Baking Powder Biscuit
Day 10	Greek Pasta Salad Butter Tart Square
Day 11	Pizza
Day 12	Rice Pilaf
Day 13	Herbed Flatbread Caesar Salad Dressing
Day 14	Fresh Tomato & Pepper Pasta Carrot Cake

[†]Each food item contained 25 g study oil/serving, unless two items per day are listed, in which case each item contained 12.5 g study oil/serving.

Dietary Adherence and Safety Measures

During Week 2 and Week 5, participants were contacted by the study staff by telephone to assess their compliance and tolerability of the study foods, report any adverse changes in medical conditions or adverse events, and to identify food preferences. Participants were given the opportunity to modify the selection of food items they were provided for the next 3-week period, and they were reminded to complete a 3-day food record at Week 6 to be submitted at the final study visit. Dietary adherence will also be assessed by reviewing the 3-day food records for inclusion of study food items and by analyzing the plasma phospholipid fatty acid profile before and after the intervention.

Although consumption of the study food products was not considered to pose significant risk to the participants, any adverse changes in medical conditions or adverse events taking place during the study period were recorded, whether or not they required medical attention. The severity, duration, relation to the study food product, clinical action taken, and outcome of each adverse event was noted and reviewed by the clinical co-investigator (RPG). Twenty-one adverse events were reported: two occurrences of nausea, one each of flatulence and indigestion, and all others were not related to the food products. One serious adverse event was reported; this serious adverse event was not related to the study food products, but required the individual to withdraw from the study. The serious adverse event was promptly reported to the University of Manitoba Research Ethics Board. Participants were free to withdraw from the study at any time for any reason, without penalty.

Statistical Considerations and Data Analysis

We calculated sample size based on a previous study we conducted in PAD participants using pulses (dried beans, dried peas, lentils and chickpeas) as the 8 week dietary intervention and ABI as the primary outcome, since there have been no previous studies investigating a canola oil intervention in PAD [25]. This resulted in a total sample size of 44 participants (n=22/group) to give a statistical power of 0.80 to detect a 5% change in ABI from baseline to 8 weeks with a probability of $p=0.05$. We rounded up the sample size to 50 (n=25/group) to allow for the differing interventions, and any dropouts were replaced.

To compare the data on vascular function, anthropometrics, and biochemical assessments between the dietary groups at 8 weeks, we will use ANCOVA models with 8 week values as the outcome, diet as the main effect, and baseline measurements as a covariate. Additional covariates will be considered for inclusion in the model when it is believed they are strongly associated with the outcome being modeled, and when randomization does not lead to sufficiently comparable baseline values between the groups. This approach provides optimal statistical power for detecting a diet effect and adjusts for any baseline differences between groups. Analyses will be performed with PROC GLM using Statistical Analysis Software [26]. The level of significance will be set at $p<0.05$.

7.5 Discussion

PAD is a type of atherosclerotic disease that occurs in the vessels of the limbs, and is associated with a 10-fold increased risk for cardiovascular events, changes in cognitive function, and a reduction in quality of life due to limited walking capacity and mobility [1,2]. PAD is present in as many as 14% of Americans between 50-80 years of age [27]. Lifestyle changes are important in preventing and treating this condition, as smoking and a sedentary lifestyle are major risk factors for PAD [28,29]. Dietary choices can also impact vascular function, as nutrient and dietary interventions have been shown to provide an acute means for improving arterial stiffness by targeting the functional mechanisms that regulate vascular tone [30].

This trial aims to provide valuable data on the effects of canola oil consumption on vascular function and cardiovascular risk factors in individuals with PAD. To the best of our knowledge, this is the first RCT in PAD to use canola oil, which is low in SFAs, high in MUFAs and the best source of n3 fatty acids among commonly consumed vegetable oils, as the primary source (up to one-half) of fat in the diet. A recent review describes the positive effects of consuming canola oil on circulating lipid levels and insulin sensitivity [31]. The combination of MUFAs and the n3 fatty acid ALA in canola oil may also deliver synergistic benefits to PAD individuals with T2D and obesity by modulating gene expression, insulin signalling, fatty acid metabolism, and tissue lipid accumulation [10]. Vascular function has been shown to improve with fish oil consumption in individuals with cardiovascular disease [32,33], but very few trials have investigated the

effects of MUFAs or ALA on blood vessels, and these have used food sources such as flaxseed oil, sunola oil and walnuts [34-37], but not canola oil.

The Canola-PAD Study employed several non-invasive vascular testing methods to assess vascular function. Pulse wave velocity is a measure of the time taken for a pressure wave to travel a specific distance, and is considered the gold standard method for measuring arterial stiffness because it is simple, non-invasive, robust and reproducible [38]. Pulse wave velocity is increased in people with arterial stiffness and age-specific reference values have been established [39]. Pulse wave analysis examines the shape of the pulse wave and provides an indirect measure of arterial stiffness through augmentation of the pulse wave by the reflected waveform. In our study, the peripheral pulse wave was recorded at the radial artery via applanation tonometry. The software uses algorithms to calculate the aortic augmentation index, a measure of how much the central aortic pressure is augmented by the increased pulse wave reflection in people with vascular disease [38], and this affects the workload on the heart. Together, pulse wave velocity and pulse wave analysis provide information on systemic arterial stiffness, central aortic pressure, and other hemodynamic parameters not obtained during measurement of peripheral blood pressure. The utility of these measurements in predicting adverse cardiovascular outcomes has been validated [40,41] and is supported by extensive clinical data [38]. Measuring endothelial function using EndoPAT technology provides data on the responsiveness of the endothelial vasomotor response before and during reactive hyperemia. Peripheral arterial tonometry (PAT) involves recording the finger arterial pulse wave amplitude, which is a measure of pulsatile

volume changes [42]. Abnormalities in the pulse wave amplitude are indicative of atherosclerotic disease [43]. Endothelial dysfunction has been demonstrated to be an adequate surrogate marker for vascular function, and is associated with cardiovascular risk factors [44,45]. The AGE Reader measures tissue accumulation of advanced glycation end-products (AGEs) by means of skin auto-fluorescence. While not strictly a measure of vascular function, this non-invasive technology provides data on cardiovascular risk, since AGEs have been shown to contribute to oxidative stress and are associated with microvascular and macrovascular complications in T2D [46].

Cardiovascular factors have long been recognized as drivers in the vascular pathogenesis of cognitive decline [47,48]. Arterial stiffness is one such pathophysiological process that has been linked to accelerated cognitive aging [3,48-50]. The neuropsychological function of PAD patients has not been well studied, especially in response to intervention. In the Canola-PAD Study, we used three tests to obtain a well-rounded assessment of performance in various cognitive domains. The Hopkins Verbal Learning Test [51] assesses verbal learning and semantic memory. This test has been used in patients with coronary heart disease to demonstrate an improvement in cognitive function following cardiac surgical procedures [52,53]. The Digit Symbol Similarities Test [54] assesses the participants' executive functioning, including perceptual speed, motor speed, visual scanning and memory. Lower performance in this test has been associated with the presence of vascular disease (ABI <0.9 or intermittent claudication) [55-57]. The Digit Span Test [54] measures short-term memory, attention and concentration. Several studies have demonstrated a link between PAD and

neuropsychological deficits using this test score [58,59]. These tests were selected for the Canola-PAD Study because they are well validated and internationally recognized with high re-test reliability and inter-administrator dependability [60-64]. Our study will examine whether data from the cognitive function tests are correlated to changes in arterial stiffness in individuals with PAD consuming the different dietary fats.

Positive Aspects of the Canola-PAD Study

Several aspects of the trial design aided participant recruitment and compliance. All participants were recruited through the St. Boniface Hospital Vascular Surgery Clinic, and remained under the care of their vascular surgeon (RPG), proving continuity of care during the intervention. Participants were provided with their results from the vascular function assessments, and most were interested to learn about their vascular health. The study food items were generally well accepted and tolerated. The ability to tailor the food items participants received to their individual dietary preferences was appreciated, and making the food items available to participants' family members was also thought to improve compliance.

Challenges and Limitations

One of the challenges in conducting this trial was working with participants who were for the most part elderly and not very mobile. Some participants declined to undergo treadmill testing for various reasons, such as needing a walking aide (cane/walker), or they felt that the 12% treadmill incline would be too difficult to manage safely. Obtaining blood samples was sometimes problematic, since the

participants' peripheral blood vessels could be frail and easily collapse. Some participants found the three-hour visit required to conduct all of the vascular assessments and tests to be taxing and too lengthy. However, they were satisfied with the organization of the study where all phlebotomy services and vascular testing occurred in a single clinic at the Asper Clinical Research Institute.

7.6 Conclusion

In summary, we expect that the Canola-PAD Study will provide data on the effectiveness of the canola oil intervention on clinically relevant outcomes for vascular function, including ABI, arterial stiffness, walking capacity and cognitive function, in individuals with PAD. We will be able to evaluate direct effects on blood vessel function via the non-invasive assessments of pulse wave analysis and pulse wave velocity, and to compare these results with changes in cardiovascular disease risk markers. The study will provide important information on the safety and tolerability of a diet enriched in canola oil compared to a typical Western diet oil mixture in a PAD population. The findings may also support current recommendations for canola oil consumption as a healthy source of dietary fat for management of PAD.

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CHAPTER 8

Impact of a Dietary Intervention Containing Canola Oil on Vascular Function in Adults with Peripheral Arterial Disease: The Canola-PAD Study

8.1 Contribution Statement

I, Jennifer Enns, made the following contributions to this study:

- performed serum biochemistry analyses in Table 8.3 (apolipoproteins and markers of inflammation and oxidative stress)
- conducted the statistical analyses
- interpreted the results and wrote the manuscript

Other contributors:

- Carla G. Taylor
- Peter Zahradka
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8.2 Abstract

PAD is a type of atherosclerotic disease that manifests in the arteries of the legs. People with PAD are at high risk for adverse cardiovascular events due to underlying atherosclerotic disease, and intermittent claudication (muscle cramping) limits their walking range. Dietary fatty acid composition has previously been shown to modulate blood vessel properties, and may be effective at improving indices of vascular function (arterial stiffness and flow-mediated dilatation). However, whether a diet enriched in conventional canola oil, which is a good source of n3 fatty acids and has an otherwise favourable fatty acid composition, can improve clinical endpoints in PAD is not known. The objective of this trial was to assess the impact of a dietary intervention containing canola oil on vascular function in people with PAD.

The Canola-PAD Study was a single-site, double-blind, randomized, controlled trial conducted at St. Boniface Hospital in Winnipeg, Canada. A random numbers table was used to assign participants to treatment groups. The participants were 50 adults (mean age 70.8 [SEM, 0.8]; mean body mass index 28.3 [SEM, 0.4]) diagnosed with PAD ($ABI \leq 0.9$) and with moderate hyperlipidemia; 14 of these had T2D ($HbA1c > 6.5$). The participants consumed food products containing 25 g/day of canola oil (Canola Oil group, n=25) or an oil mixture typical of the Western diet (Western group, n=25) as part of their usual food intake. The primary outcome was vascular function (ABI, arterial stiffness, and endothelial function). Secondary outcomes were physiological parameters (body weight, blood pressure), serum biochemistry (lipids and inflammatory biomarkers), and fatty acid composition.

Fifty participants were analyzed. The dietary intervention altered the phospholipid fatty acid profile of participants, with increases in total monounsaturated, n3 and n9 fatty acids in the Canola Oil group, and increases in saturated fatty acids in the Western group. However, these changes did not translate into downstream effects in vascular function, and only minor changes were observed in serum lipids, indices of glycemic control and markers of inflammation. One participant withdrew from the trial, but for reasons unrelated to the intervention.

The intervention had little impact on blood vessel function in people with established PAD. Further research is required to determine whether the favourable fatty acid profile of canola oil may have more robust benefits in less advanced vascular disease.

8.3 Introduction

The underlying cause of most heart disease can be attributed to a reduction in nutrient and oxygen delivery by the blood vessels [1]. Changes to blood vessel function occur as a result of endothelial dysfunction, and the consequences include dysregulation of blood vessel tone, arterial remodeling, and the development of atherosclerotic disease, all of which are associated with a loss of blood vessel elasticity. The reduced blood flow and vessel hardening typical of atherosclerosis increase the risk of many other cardiovascular conditions, including hypertension, heart failure, stroke, ischemia and PAD [2].

PAD is characterized by reduced blood flow to the legs [3,4], and is diagnosed by the ABI, the ratio of the systolic blood pressure at the ankle to that of the arm (brachial artery), where an ABI of <0.9 is indicative of PAD. People with PAD are not only at higher risk than the general population for adverse cardiovascular events, but have a limited walking range due to intermittent claudication (muscle cramps that occur due to obstructed blood flow to the limbs) [5]. Changes in the physical properties of blood vessels (e.g., onset of arterial stiffness) are difficult for patients to detect before the onset of symptoms such as claudication. There is increasing emphasis on the use of evidence-based clinical endpoints, such as blood vessel function, to identify pre-symptomatic disease and to evaluate the efficacy of therapeutic interventions. Pulse wave velocity, the rate of blood flow measured by Doppler ultrasound either from the carotid to the femoral artery, or from the brachial artery to the ankle, is strongly predictive of coronary events [6,7]. Other indices of blood vessel function such as flow-

mediated dilatation (endothelial dysfunction) can be monitored with instruments that provide rapid results non-invasively with high sensitivity. It is generally agreed that these and related methods are capable of monitoring changes in vascular properties that become compromised early in the atherogenic process [8-12].

Although current treatment for PAD is based primarily on pharmaceutical interventions to improve the hemorrheologic properties of the blood and increase vasodilation [13], dietary interventions, and specifically interventions containing n3 fatty acids, may also improve blood vessel function. While there is some evidence for a beneficial effect of fish oil, which contains the long chain n3 fatty acids EPA and DHA, on blood vessel properties [14-17], the effects of MUFAs and ALA, a plant-based n3 fatty acid, on blood vessel function have not been studied. Therefore, we conducted a RCT to assess the impact of a dietary intervention containing canola oil, which is high in MUFAs and a good source of ALA, on blood vessel function in individuals with PAD.

8.4 Methods

Trial Design

The design of the Canola-PAD Study has been previously described [18]. The study protocol was approved by the University of Manitoba Research Ethics Board and the St. Boniface Hospital Research Review Committee, and the trial was registered with clinicaltrials.gov (NCT01250275). Fifty adults with PAD were recruited through the St. Boniface Hospital Vascular Clinic for this double-blind, randomized, controlled trial. Individuals with an ABI of <0.9 and who also exhibited moderate hyperlipidaemia and absence of chronic illness or genetic causes of hypercholesterolaemia were asked to join the study. Interested individuals attended a screening visit where they provided written informed consent before any study-related procedures were performed.

Randomization and Blinding

The randomization sequence was prepared from a random numbers table and individual assignments were stored in opaque envelopes. Twenty-five participants were assigned to each of the two dietary treatment groups. To ensure that there was equal representation of type 2 diabetes in both arms of the study, participants were first stratified according to their HbA1c levels ($\leq 6.5\%$ or $>6.5\%$), and then randomly assigned to one of the two groups. To reduce potential bias during data collection and evaluation of study endpoints, both the research team and the participants were blinded to the dietary intervention from the time of randomization to the end of the study.

Intervention

Participants were randomized to one of two dietary intervention groups and provided with food items (including entrées, side dishes, salad dressing, and baked goods) containing either conventional canola oil (25 g/day; Canola Oil group), or an oil mixture representing the fatty acid profile in a typical Western diet (25 g/day; Western group), to be consumed daily as part of their usual food intake for 8 weeks. The fatty acid composition of the canola oil was 8% SFAs, 64% MUFAs, and 28% PUFAs, resulting in a 2:1 ratio for n6:n3 PUFAs, whereas the fatty acid composition of the Western diet oil mixture (2:1:1 ratio of butter, coconut oil, high-linoleic safflower oil) was 57% SFAs, 21% MUFAs, and 22% PUFAs (20:1 ratio for n6:n3 PUFAs). Thus, the intervention groups received a similar amount of n6 PUFAs, but the composition of the diets differed in the amounts of n3 PUFAs, MUFAs and SFAs, and the ratios of n6:n3 PUFAs and PUFA:SFA (Table 8.1).

Outcomes

The primary outcome of the study was vascular function (ABI, arterial stiffness and endothelial function). Arterial stiffness was measured using the Omron VP-1000 Vascular Profiler (Omron Healthcare Inc, Lake Forest, IL). Radial pulse wave analysis was conducted using the SphygmoCor Pulse Wave Monitoring System (AtCor Medical, Sydney, Australia) and SphygmoCor Px Aortic BP Waveform Analysis software (AtCor Medical). Endothelial function (Itamar EndoPAT-2000, Itamar Medical Inc, Franklin, MA) and advanced glycation end products (AGE Reader, Diagnoptics Technologies,

Groningen, The Netherlands) were also assessed. In addition, anthropometric parameters (height, body weight, BMI and waist circumference) were recorded, and a fasting blood sample was drawn for measurement of the lipid profile and other serum markers. Participants underwent these assessments at baseline and at the end of the 8-week intervention. The plasma phospholipid fatty acid composition was analyzed by gas chromatography as previously described [19]. Any changes in medical conditions or adverse events taking place during the study period were recorded, whether or not they required medical attention. Participants were free to withdraw from the study at any time for any reason, without penalty.

Sample Size

The sample size of 25/group was determined from the best available data from a parallel study, the ABI values in another dietary intervention that measured the effect of pulse varieties (beans, peas, chickpeas and lentils) on vascular function in a PAD population [20].

Statistical Analysis

Continuous variables were expressed as mean \pm SEM. Outliers (mean \pm 2 \times SD) were removed from the data set before analysis. For the initial statistical analysis reported herein, two-tailed Student's T-tests were used to compare the difference between means. Paired T-tests were used to compare means within the Canola Oil group (week 0 vs week 8) and within the Western group (week 0 vs week 8); unpaired T-

tests were used to compare means between groups at the end of the study (Canola Oil week 8 vs Western week 8). If the T-test determined that a parameter was different at baseline between the two groups, ANOVA was used to determine main effects (participant, diet, time, and diet*time interaction) for the delta values (week 8 minus week 0 for each participant). Differences were considered significant when $p < 0.05$, with trends ($p = 0.05-0.1$) noted as such. Pearson product-moment correlation coefficients (r) were calculated to assess linear relationships between selected parameters.

Table 8.1. Fatty Acid Composition of Canola-PAD Study Oils

Fatty Acids (g/100g)	Canola Oil	Western Oil Mixture
Total SFA	7.7	57.3
C4:0 Butyric acid	0.582	0.965
C6:0 Caproic	ND	1.141
C8:0 Caprylic acid	ND	2.733
C10:0 Capric acid	ND	2.915
C12:0 Lauric acid	ND	12.844
C14:0 Myristic acid	0.054	9.565
C15:0 Pentadecylic acid	0.046	0.588
C16:0 Palmitic acid	3.935	19.49
C17:0 Margaric acid	0.149	0.301
C18:0 Stearic acid	1.789	6.396
C20:0 Eicosanoic acid	0.633	0.241
C22:0 Docosanoic acid	0.353	0.141
C24:0 Tetracosanoic acid	0.151	ND
Total MUFA	63.5	21.8
C14:1n5	ND	0.493
C16:1n9 Palmitoleic acid	0.222	0.791
C17:1	0.072	0.171
C18:1n7 Vaccenic acid	3.486	1.436
C18:1n9 Oleic acid	58.054	18.565
C20:1 Eicosenoic acid	1.322	0.208
C22:1 Docosenoic acid	0.079	ND
C24:1 Tetracosenoic acid	0.202	ND
Total PUFA	28.8	20.9
Total n3 PUFA	9.9	1.0
C18:3n3 α -Linolenic acid	9.823	1.018
C20:3n3 Eicosatrienoic acid	ND	ND
C20:5n3 Eicosapentaenoic acid	ND	ND
C22:5n3 Docosapentaenoic acid	0.034	ND
Total n6 PUFA	18.9	19.9
C18:2n6 Linoleic acid	18.897	19.804
C20:3n6 Dihomo- γ -linolenic acid	ND	0.024
C20:2n6 Eicosadienoic acid	0.077	ND
C20:4n6 Arachidonic acid	ND	0.037
Ratios		
n6:n3	1.9	19.5
PUFA:SFA	3.7	0.4

MUFA: monounsaturated fatty acid; ND: not detected; PUFA: polyunsaturated fatty acid; SFA: saturated fatty acid.

8.5 Results

A total of 50 participants were enrolled in the Canola-PAD Study with 25 individuals allocated to each dietary intervention. One person withdrew from the study for health reasons unrelated to the intervention, and was replaced. The age of participants ranged from 51 – 87 years, with an average age of 70.7 ± 0.8 years. Thirty-five (70%) participants were male, and 15 (30%) were female.

Physiological Parameters

Body weight, BMI and waist circumference were not different between the Canola Oil group and the Western group at baseline, nor did the dietary intervention have any impact on these parameters (Table 8.2). Diastolic blood pressure was lower in the Canola Oil group compared to the Western group at the end of the intervention; however, this was confounded by a significant difference in baseline values ($p=0.010$). However, subsequent analysis of the delta values (week 8 minus week 0) revealed no differences due to the dietary intervention and that there was a significant effect ($p<0.001$) due to differences among participants at baseline. Systolic blood pressure was not affected by the dietary intervention (Table 8.2).

Vascular Function Parameters

ABI was used both as a diagnostic measure for the presence of PAD and as an outcome measure. It was determined by two techniques (manually and using the VP-1000 apparatus). ABI values did not differ between groups at the end of the study, and ABI values obtained using the two alternate methods were very strongly correlated

(correlation coefficients for right and left ABI at 8 weeks: $r=0.825$, $p<0.001$ and $r=0.867$, $p<0.001$, respectively).

Arterial stiffness was assessed via two complementary approaches. First, baPWV was determined with the VP-1000 apparatus. PWV was not altered by the intervention, except in the Western group, where there was a trend ($p=0.055$) towards an increase in left baPWV over the course of the study (Table 8.2). Secondly, pulse wave analysis was conducted using the SphygmoCor Pulse Wave Monitoring System and SphygmoCor Px Aortic BP Waveform Analysis software. Pulse wave analysis provides an indirect measure of arterial stiffness by quantifying the impact of the reflected pulse wave on central aortic pressure (augmentation index). No differences in the augmentation index or the heart rate-normalized augmentation index were observed between dietary groups (Table 8.2).

Endothelial function was assessed by determining the reactive hyperemia index (RHI). A RHI of 1.66 or higher represents normal endothelial function, while lower values are indicative of endothelial dysfunction. No changes in RHI were observed except for a trend ($p=0.095$) towards increased RHI in the Canola Oil group over the course of the study (Table 8.2). Mean RHI values in this study were above 2.00, suggesting that endothelial dysfunction was not a major contributor towards PAD in the study participants.

Serum Biochemistry

There were few changes observed in fasting serum lipid levels between dietary intervention groups. At 8 weeks, there was a trend ($p=0.056$) for lower triglycerides in the Western group compared to the Canola Oil group, and levels of Apolipoprotein B (the primary apolipoprotein in LDL-cholesterol) were significantly lower in the Western group (Table 8.3); however, baseline values for this marker were significantly different ($p=0.039$), and subsequent analysis confirmed differences among participants at baseline but no effect of the dietary treatments on triglycerides or Apolipoprotein B when delta values were analyzed. Fasting glucose levels increased in the Canola Oil group over the course of the study, but at 8 weeks there was a trend ($p=0.091$) towards higher glycated hemoglobin (HbA1c) values in the Western group compared to the Canola Oil group. An analysis of the diabetic ($\text{HbA1c} \geq 6.5\%$ at week 0) and normoglycemic or prediabetic ($\text{HbA1c} < 6.5\%$) subsets of participants yielded no significant differences in fasting glucose or HbA1c levels due to the intervention; however, we observed that HbA1c levels were significantly higher ($p=0.023$) at baseline in the Western group compared to the Canola Oil group in the diabetic subset. Otherwise, fasting glucose, insulin, HbA1c and advanced glycation end-products in the whole study cohort remained stable (Table 8.3).

Levels of E-selectin, an endothelial cell adhesion molecule important in leukocyte recruitment and associated with progression of atherosclerosis, increased in both dietary groups over the 8-week intervention, and were higher in the Canola Oil group compared to the Western group at the end of the study; however, baseline values were

significantly different between groups ($p=0.020$). Subsequent analysis revealed a participant effect (i.e. the baseline differences accounted for the end-of-study differences by T-test), and that there was no effect of dietary intervention when the delta values for E-selectin were analyzed. There was also a trend towards elevated adiponectin ($p=0.085$) and 4-hydroxynonenal (a marker of oxidative stress; $p=0.097$) in the Canola Oil group compared to the Western group at the end of the study. Four markers of inflammation, adiponectin, IL-1 β , IL-10 and MCP-1, also showed a trend ($p=0.085$, 0.070, 0.056 and 0.092, respectively) towards an increase over the course of the study in the Western group (Table 8.3). For all of these parameters, there were no differences in baseline values between the two dietary groups.

Phospholipid Fatty Acid Composition

The fatty acid composition of the plasma phospholipids reflects the impact of the dietary oil intervention in our study, and serves as an indicator of dietary adherence, since dietary fatty acids have been demonstrated to appear within days of the start of a dietary intervention [21]. In the Canola Oil group, there were minimal changes in some of the less abundant SFAs, but total MUFAs, total n9 MUFAs, and levels of individual MUFAs (C16:1t, C16:1n9, C18:1n9 and C18:1n7), as well as the MUFA:(MUFA+SFA) ratio, were elevated compared to the Western group at the end of the study. Key among these fatty acids is C18:1n9 (oleic acid), which makes up 60% of the fatty acids in conventional canola oil. Total PUFAs did not change, but we observed increased proportions of total n3 PUFAs and C20:5n3 (EPA) in the Canola Oil group over the course of the intervention, and C18:3n3 (ALA) and C22:6n3 (DHA) showed trends ($p=0.096$ and

0.096, respectively) in this direction as well. In the Canola Oil group, the n6:n3 ratio decreased over the course of the study, and the n9:n6 ratio increased, remaining higher than in the Western group at the end of the study (Table 8.4).

In the Western group, the intervention resulted in higher proportions of the SFAs C14:0 (myristic acid, a component of butter and coconut oil) and C24:0 (tetracosanoic acid) compared to the Canola Oil group, and there was also a trend towards increased C20:0 (eicosanoic acid; $p=0.061$). Trends were noted for reduced MUFAs C16:1t and C18:1n9 ($p=0.097$ and 0.073 , respectively), and increased n6 PUFA C20:2n6 (eicosadienoic acid, $p=0.097$) in the Western group over the course of the study (Table 8.4).

Table 8.2 Physiological and Vascular Function Parameters

	Week 0		Week 8		p-Values [†]		
	Canola Oil Group	Western Group	Canola Oil Group	Western Group	Canola Oil Group Week 0 vs 8 [‡]	Western Group Week 0 vs 8 [‡]	Canola vs Western Week 8 [¶]
Physiological Parameters							
Body Weight, kg	81.9 ± 2.7	80.6 ± 2.5	82.1 ± 2.7	81.3 ± 2.5	NS	NS	NS
Body Mass Index, kg/m ²	27.9 ± 0.7	28.6 ± 0.9	28.1 ± 0.7	28.8 ± 0.9	NS	NS	NS
Waist Circumference, cm	100.1 ± 4.0	101.9 ± 2.3	101.5 ± 2.8	103.8 ± 2.4	NS	NS	NS
Systolic Blood Pressure, mm Hg	149 ± 4	143 ± 3	149 ± 4	147 ± 3	NS	NS	NS
Diastolic Blood Pressure, mm Hg	79.4 ± 1.5	72.2 ± 1.6	76.8 ± 1.6	71.9 ± 1.3	NS	NS	0.035^{§¥}
Vascular Function Parameters							
Peripheral Arterial Disease							
R-ABI	0.83 ± 0.04	0.85 ± 0.04	0.82 ± 0.04	0.84 ± 0.04	NS	NS	NS
L-ABI	0.84 ± 0.05	0.90 ± 0.05	0.87 ± 0.03	0.88 ± 0.04	NS	NS	NS
R-Manual ABI	0.84 ± 0.04	0.88 ± 0.04	0.82 ± 0.04	0.85 ± 0.04	NS	NS	NS
L-Manual ABI	0.85 ± 0.04	0.89 ± 0.04	0.85 ± 0.04	0.89 ± 0.04	NS	NS	NS
Arterial Stiffness: Pulse Wave Velocity							
R-baPWV, cm/s	1579 ± 80	1620 ± 91	1565 ± 82	1678 ± 83	NS	NS	NS
L-baPWV, cm/s	1694 ± 102	1947 ± 178	1716 ± 140	2116 ± 203	NS	0.055	NS
Arterial Stiffness: Pulse Wave Analysis							
Aortic AI (AP/PP), %	37.7 ± 1.6	33.4 ± 1.6	38.0 ± 1.4	35.7 ± 1.8	NS	NS	NS
Aortic AI (AP/PP) @ HR75, %	31.2 ± 1.8	27.5 ± 1.4	31.7 ± 1.4	28.9 ± 1.4	NS	NS	NS
Endothelial Function							
Reactive Hyperemia Index	2.03 ± 0.13	2.39 ± 0.17	2.30 ± 0.12	2.42 ± 0.15	0.095	NS	NS

Values are means ± SEM for n=20-25/group. †NS: not significant (p>0.1); p-values for a trend (p=0.05-0.01) and significant differences (p<0.05, in bold) are provided, as assessed by paired[‡] or unpaired[¶] T-tests. Significant difference by T-Test for baseline values are denoted by §, and ¥ denotes a significant difference among participants but no difference between the diet groups when delta values were analyzed. ABI: ankle-brachial index; AI: augmentation index; AP: aortic pressure; baPWV: brachial-ankle pulse wave velocity; L: left; PP: pulse pressure; R: right; rf: relative fluorescence.

Table 8.3 Serum Biochemistry

	Week 0		Week 8		p-Values†		
	Canola Oil Group	Western Group	Canola Oil Group	Western Group	Canola Oil Group Week 0 vs 8‡	Western Group Week 0 vs 8‡	Canola vs Western Week 8¶
Lipid Profile							
Triglycerides, mmol/L	1.56 ± 0.08	1.31 ± 0.09	1.65 ± 0.11	1.33 ± 0.09	NS	NS	0.056
Total Cholesterol, mmol/L	3.30 ± 0.15	3.08 ± 0.17	3.27 ± 0.16	3.19 ± 0.18	NS	NS	NS
LDL-Cholesterol, mmol/L	2.07 ± 0.14	1.94 ± 0.14	1.88 ± 0.12	2.04 ± 0.14	NS	NS	NS
HDL-Cholesterol, mmol/L	1.14 ± 0.05	1.26 ± 0.06	1.14 ± 0.06	1.25 ± 0.07	NS	NS	NS
HDL-Cholesterol/LDL-Cholesterol	1.67 ± 0.13	1.57 ± 0.14	1.59 ± 0.13	1.62 ± 0.12	NS	NS	NS
Apo A1, µg/L	63.5 ± 5.0	74.9 ± 5.7	63.6 ± 5.0	73.2 ± 5.6	NS	NS	NS
Apo B, mg/L	4.23 ± 0.28	3.54 ± 0.24	4.19 ± 0.30	3.52 ± 0.22	NS	NS	0.048 §¥
Apo CII, µg/L	65.26 ± 3.82	58.86 ± 4.17	65.61 ± 4.21	61.72 ± 4.29	NS	NS	NS
Glycemia and Glycation							
Fasting Glucose, mmol/L	5.83 ± 0.17	5.84 ± 0.22	6.00 ± 0.18	5.91 ± 0.24	0.045	NS	NS
Fasting Insulin, mmol/L	81.4 ± 6.4	82.6 ± 9.6	89.0 ± 6.5	88.4 ± 8.8	NS	NS	NS
Glycated Hemoglobin (HbA1c), %	6.14 ± 0.08	6.54 ± 0.23	6.20 ± 0.08	6.56 ± 0.24	NS	NS	0.091
Advanced Glycation End-Products, rf	3.20 ± 0.13	3.24 ± 0.15	3.13 ± 0.12	3.20 ± 0.12	NS	NS	NS
Markers of Inflammation and Oxidative Stress							
Adiponectin, pg/mL	11.1 ± 0.7	13.5 ± 1.3	10.8 ± 0.8	13.3 ± 1.2	NS	NS	0.085
sRAGE, pg/mL	754 ± 72	752 ± 64	739 ± 73	798 ± 71	NS	NS	NS
E-selectin, pg/mL	300 ± 22	229 ± 19	322 ± 23	257 ± 23	0.046	0.007	0.030 §¥
Interleukin-1β, pg/mL	11.4 ± 0.6	10.0 ± 0.5	12.1 ± 0.7	10.9 ± 0.5	NS	0.070	NS
Interleukin-6, pg/mL	23.4 ± 2.0	20.0 ± 1.8	22.9 ± 2.1	21.2 ± 1.6	NS	NS	NS
Interleukin-10, pg/mL	2.01 ± 0.12	1.83 ± 0.07	1.97 ± 0.11	1.97 ± 0.08	NS	0.056	NS
MCP-1, pg/mL	263 ± 12	291 ± 9	269 ± 12	301 ± 10	NS	0.092	NS
4-Hydroxynonenal, µg/mL	2.66 ± 0.12	2.53 ± 0.09	2.73 ± 0.10	2.47 ± 0.10	NS	NS	0.097

Values are means ± SEM for n=20-25/group. †NS: not significant (p>0.1); p-values for a trend (p=0.05-0.01) and significant differences (p<0.05, in bold) are provided, as assessed by paired‡ or unpaired¶ T-tests. Significant differences by T-test in baseline values are denoted by §, and ¥ denotes a significant difference among participants but no difference between the diet groups when delta values were analyzed. HDL: high-density lipoprotein; LDL: low-density lipoprotein; MCP-1: monocyte chemoattractant protein-1; rf: relative fluorescence; sRAGE: soluble receptor for advanced glycation end-products.

Table 8.4 Plasma Phospholipid Fatty Acid Composition

	Week 0		Week 8		p-Values [†]		
Fatty Acid (g/100 g)	Canola Oil Group	Western Group	Canola Oil Group	Western Group	Canola Oil Group Week 0 vs 8 [‡]	Western Group Week 0 vs 8 [‡]	Canola vs Western Week 8 [¶]
Total SFA	47.2 ± 0.7	46.8 ± 0.4	46.4 ± 0.6	46.7 ± 0.3	NS	NS	NS
C8:0 Caprylic acid	0.125 ± 0.010	0.133 ± 0.010	0.142 ± 0.013	0.138 ± 0.012	NS	NS	NS
C14:0 Myristic acid	0.329 ± 0.019	0.377 ± 0.019	0.325 ± 0.018	0.384 ± 0.018	NS	NS	0.025
C16:0 Palmitic acid	27.7 ± 0.5	27.2 ± 0.4	27.0 ± 0.4	27.2 ± 0.4	NS	NS	NS
C17:0 Margaric acid	0.445 ± 0.021	0.458 ± 0.021	0.448 ± 0.019	0.443 ± 0.019	NS	NS	NS
C18:0 Stearic acid	14.7 ± 0.3	14.5 ± 0.3	14.6 ± 0.3	14.5 ± 0.3	NS	NS	NS
C20:0 Eicosanoic acid	0.696 ± 0.025	0.668 ± 0.024	0.729 ± 0.026	0.716 ± 0.026	0.094	0.061	NS
C22:0 Docosanoic acid	1.69 ± 0.07	1.83 ± 0.07	1.70 ± 0.07	1.80 ± 0.07	NS	NS	NS
C24:0 Tetracosanoic acid	1.43 ± 0.06	1.58 ± 0.06	1.38 ± 0.06	1.55 ± 0.05	NS	NS	0.031
Total MUFA	14.0 ± 0.3	14.1 ± 0.4	14.6 ± 0.3	13.6 ± 0.3	0.060	NS	0.018
C16:1t	0.219 ± 0.010	0.224 ± 0.010	0.231 ± 0.009	0.206 ± 0.009	NS	0.097	0.049
C16:1n9 Palmitoleic acid	0.512 ± 0.036	0.481 ± 0.035	0.492 ± 0.031	0.442 ± 0.031	NS	NS	NS
C18:1n9 Oleic acid	8.84 ± 0.29	8.98 ± 0.28	9.08 ± 0.22	8.41 ± 0.22	NS	0.073	0.035
C18:1n7 Vaccenic acid	1.54 ± 0.05	1.47 ± 0.05	1.63 ± 0.05	1.47 ± 0.05	0.026	NS	0.018
C20:1n9 Eicosenoic acid	0.226 ± 0.013	0.214 ± 0.013	0.260 ± 0.016	0.239 ± 0.016	0.002	NS	NS
C24:1n9 Tetracosenoic acid	2.54 ± 0.12	2.55 ± 0.12	2.75 ± 0.13	2.69 ± 0.13	0.025	NS	NS
Total PUFA	37.9 ± 0.6	38.2 ± 0.4	37.9 ± 0.3	38.6 ± 0.3	NS	NS	NS
Total n3 PUFA	9.68 ± 0.31	9.67 ± 0.30	10.36 ± 0.33	9.86 ± 0.32	0.046	NS	NS
C18:3n3 α-Linolenic acid	0.381 ± 0.030	0.382 ± 0.029	0.440 ± 0.028	0.388 ± 0.028	0.096	NS	NS
C20:5n3 Eicosapentaenoic acid	1.00 ± 0.07	1.05 ± 0.07	1.20 ± 0.08	1.02 ± 0.08	0.002	NS	NS
C22:5n3 Docosapentaenoic acid	0.885 ± 0.034	0.914 ± 0.033	0.904 ± 0.035	0.970 ± 0.034	NS	NS	NS
C22:6n3 Docosahexaenoic acid	4.16 ± 0.19	4.20 ± 0.18	4.56 ± 0.24	4.34 ± 0.23	0.096	NS	NS
C20:5n3 + C22:6n3	5.15 ± 0.20	5.25 ± 0.19	5.77 ± 0.25	5.36 ± 0.25	0.022	NS	NS
Total n6 PUFA	35.6 ± 0.5	35.5 ± 0.5	35.3 ± 0.4	36.1 ± 0.4	NS	NS	NS
C18:2n6 Linoleic acid	15.8 ± 0.5	16.3 ± 0.5	15.7 ± 0.5	16.4 ± 0.5	NS	NS	NS
C18:3n6 γ-Linolenic acid	0.199 ± 0.015	0.195 ± 0.015	0.191 ± 0.017	0.184 ± 0.017	NS	NS	NS
C20:2n6 Eicosadienoic acid	0.536 ± 0.017	0.518 ± 0.017	0.519 ± 0.017	0.551 ± 0.016	NS	0.097	NS
C20:3n6 Dihomo-γ-linolenic acid	3.11 ± 0.12	2.95 ± 0.12	2.82 ± 0.12	2.97 ± 0.11	0.003	NS	NS
C20:4n6 Arachidonic acid	11.4 ± 0.4	11.0 ± 0.4	11.1 ± 0.4	11.3 ± 0.4	NS	NS	NS
C22:4n6 Docosatetraenoic acid	0.367 ± 0.019	0.358 ± 0.018	0.384 ± 0.036	0.361 ± 0.036	NS	NS	NS
Total n9 PUFA	12.2 ± 0.3	12.3 ± 0.3	12.7 ± 0.2	11.9 ± 0.2	0.087	NS	0.026

Table 8.4 Cont'd

Fatty Acid (g/100 g)	Week 0		Week 8		p-Values [†]		
	Canola Oil Group	Western Group	Canola Oil Group	Western Group	Canola Oil Group Week 0 vs 8 [‡]	Western Group Week 0 vs 8 [‡]	Canola vs Western Week 8 [¶]
Ratios							
n6:n3	3.74 ± 0.12	3.76 ± 0.12	3.47 ± 0.12	3.76 ± 0.12	0.003	NS	0.081
n9:n6	0.334 ± 0.011	0.350 ± 0.010	0.360 ± 0.009	0.330 ± 0.009	0.038	NS	0.015
PUFA:SFA	0.811 ± 0.017	0.819 ± 0.017	0.824 ± 0.016	0.829 ± 0.016	NS	NS	NS
MUFA:(MUFA+SFA)	0.230 ± 0.005	0.231 ± 0.005	0.240 ± 0.005	0.226 ± 0.005	NS	NS	0.033

Values are means ± SEM for n=20-25/group. †NS: not significant (p>0.1); p-values for a trend (p=0.05-0.01) and significant differences (p<0.05, in bold) are provided, as assessed by paired[‡] or unpaired[¶] T-tests. There were no significant differences in baseline values. MUFA: monounsaturated fatty acid; PUFA: polyunsaturated fatty acid; SFA: saturated fatty acid.

8.6 Discussion

The Canola-PAD Study, an 8-week dietary intervention comparing 25 g/day of conventional canola oil and an equivalent amount of an oil mixture representing the SFA-rich Western diet, had only minimal impact on blood vessel function in adults with PAD. There were trends towards improvement in endothelial function ($p=0.095$) in the Canola Oil group, and worsening L-baPWV ($p=0.055$) in the Western group; however, these changes were not statistically significant. Despite careful efforts to randomize participants at the beginning of the study, we observed a significant difference ($p=0.010$) in diastolic blood pressure between the two diet groups at baseline; however, the dietary interventions did not alter diastolic pressure when the delta values were analyzed. Although only minimal changes in vascular function were observed overall, other researchers conducting similarly powered studies ($n<100$) have demonstrated that dietary oil interventions can have beneficial effects on vascular properties in people with risk factors or established vascular disease. However, whereas our study provided 2.5 g/day of ALA from canola oil, other studies mostly used fish oil or the long-chain n3 PUFAs EPA and DHA. For example, arterial stiffness assessed by aortic blood flow and blood pressure improved in a study of dyslipidemic individuals who consumed 3 g/day of EPA or DHA in capsule form for 7 weeks [16]. Walking distances increased in two 12-week trials of PAD patients consuming either a dairy product enriched with n3 PUFAs (mainly EPA and DHA) [22] or taking 6 g/day of fish oil [14]. Another trial demonstrated an improvement in endothelial function assessed by flow-mediated dilation in hypercholesterolemic individuals who consumed 4 g/day fish oil [15]. In contrast, SFAs

have been associated with impaired endothelial function, as measured after healthy participants consumed a high-SFA meal in an acute study [23]. However, switching from a high-SFA diet to a low-SFA or high-MUFA diet for one month improved endothelial function assessed by flow-mediated dilation in hypercholesterolemic individuals [24].

Participants in the Canola Oil group had elevated levels of fasting glucose compared to the Western group at the end of the study. Although Apolipoprotein B and E-selectin levels were higher at baseline in the Canola Oil group, there were no differences due to the dietary intervention when the delta values were analyzed. The elevated fasting glucose in the Canola Oil group is surprising, given the evidence to support the benefits of replacing SFAs with MUFAs based on lower total cholesterol and LDL-cholesterol, lower fasting insulin levels, and improved insulin sensitivity and post-prandial glycemic control [25-27]. In our study, total MUFAs increased in the plasma phospholipids but the proportion of total SFAs and PUFAs remained stable, as did the PUFA:SFA ratio. The lack of any major change in membrane fatty acid saturation probably accounts for the stability of glycemic index indicators in our participants. This is because the membrane fatty acid composition determines membrane fluidity, and has the ability to regulate signalling events (e.g., insulin signalling) [28].

Jenkins et al [29] conducted a 3-month dietary trial (n=141) using canola oil-enriched bread to achieve a low glycemic index and high ALA and MUFA intervention in type 2 diabetic participants. In this study, fasting glucose declined in both the control (whole wheat bread) and intervention groups, but there was no difference between groups at the end of the study, and total cholesterol, LDL-cholesterol and triglycerides

were all lower in the canola oil group at the end of the intervention [29]. It is possible, therefore, that our study employed a dose of canola oil too low (25 g/day compared to 31 g/day in Jenkins et al's [29] study), or was not long enough to see similar effects on glucose and lipid levels. In addition, only a subset of our participants was diabetic, and perhaps a more severe manifestation of metabolic disease is required to see major changes in these parameters.

In our study, there were no changes in the lipid profile of participants aside from a trend ($p=0.056$) towards elevated triglycerides in the Canola Oil group compared to the Western group. Jenkins et al [29] saw a decrease in serum triglycerides, whereas Gillingham et al [30] saw no changes in their study comparing the Western diet to a diet enriched in high-oleic canola oil and flaxseed oil for 28 days in hypercholesterolemic individuals. While many studies involving dietary oils have focused on LDL-cholesterol levels as an indicator of cardiovascular disease risk and progression, few have addressed the role of dietary fatty acids in the inflammatory process. The literature generally supports a high-MUFA, low-SFA and high n3:n6 PUFA dietary pattern (a Mediterranean-style diet vs the Western diet) as imparting anti-inflammatory effects [31,32]. In Gillingham et al's RCT [30], levels of the inflammatory biomarkers IL-6 and C-reactive protein did not change, nor did levels of the endothelial cell adhesion molecules sVCAM-1 and sICAM-1, but E-selectin levels were lower in the canola/flaxseed oil group at the end of the study. Among markers of inflammation and oxidative stress, we did not observe a change in E-selectin due to dietary intervention. However, there were trends towards increased levels of IL-1 β , IL-10 and MCP-1 in the Western group over the course

of the study and trends towards decreased adiponectin and increased 4-HNE (Canola Oil group). Subsequent analysis showed that the trend in decreased adiponectin (but not 4-HNE) could be attributed to baseline differences between participants and not to dietary intervention. In short, minimal changes to inflammatory markers were observed in our study and others, and many of the inflammatory markers remained stable.

The analysis of the phospholipid fatty acid profile demonstrates the effect the dietary interventions had on the composition of the fatty acids in cell membranes in the serum. Membrane phospholipid fatty acids regulate membrane function and can act as precursors to intracellular signalling molecules, and thus may have far-reaching effects on whole-body functions. The decrease in the n6:n3 PUFA ratio and the increases in total MUFAs, total n3 and n9 fatty acids, EPA, and (EPA+DHA) observed in the Canola Oil group were expected. This is likely because the canola oil in our study is composed of 58% C18:1n9 (oleic acid) and 10% C18:3n9 (ALA). ALA levels were not higher in the Canola Oil group at the end of the study, but ALA is subject to rapid metabolism and/or conversion to EPA and DHA, which may explain why we did not observe any change. In addition, although the dietary fats provided in the intervention altered the types of PUFAs present in the phospholipid fatty acids, the total proportion of PUFAs did not change in either intervention group; this was also expected, since the interventions contained similar amounts of PUFAs (20-30%) [18]. These findings confirm that the study participants adhered to the dietary intervention, despite the relatively minimal changes that were observed downstream of the altered phospholipid fatty acid profile.

Aside from trends towards higher levels of some SFAs at the end of the study, there were fewer changes in phospholipid fatty acid composition in the Western group.

In our study, we used the Western group (57% SFAs, 21% MUFAs, and 22% PUFAs) as a control to the Canola Oil group. Other studies have used similar control or comparator groups in name, but the actual proportions of SFAs, MUFAs and PUFAs vary. In a randomized crossover trial investigating the effects of oils with different proportions of SFAs, MUFAs and PUFAs in obese men and women, the control group consumed a blend of safflower oil and corn oil to achieve a diet high in n6 fatty acids (34% SFAs, 47% MUFAs, and 18% n6 PUFAs) [33]; another trial used the Western diet as a control where the fat component was a blend of butter, olive oil, vegetable lard and sunflower oil (8% SFAs, 19% MUFAs, and 72% n6 PUFAs)[30]. The variability among these control diets speaks to the lack of specificity in describing the Western diet; although it is known to be high in n6 PUFAs and SFAs, the exact fatty acid proportions of the Western diet are ill-defined.

Although only minimal changes to disease parameters were observed in the Canola-PAD Study, other studies have shown it is possible to bring about changes in vascular function and serum biochemistry using dietary interventions in PAD. Carrero et al [22] demonstrated that daily consumption of an n3-enriched dairy product (0.2 g/day EPA and 0.13 g/day DHA) for 8 weeks could improve walking distance and ABI, and Zahradka et al [20] showed that consumption of legumes for 8 weeks could increase ABI and reduce total and LDL-cholesterol. We suspect that either a longer intervention or one with a larger daily dose of the fatty acids of interest would be required to see

significant physiological effects in our study population. At the same time, the associated increase in calories may prove detrimental, and if the amount of fatty acids in the intervention cannot be safely consumed in a realistic diet, there is little value to recommending such a course of treatment. Still, it should be noted that participants in the Canola-PAD Study had no difficulty incorporating the study foods into their usual diet, and they did so without any changes in body weight or BMI. The intervention foods were also well tolerated, with no adverse events relating to the intervention requiring participants to withdraw from the study.

8.7 Conclusions

In summary, this dietary intervention comparing food products containing canola oil to food products containing butter, coconut oil and safflower oil to represent the Western diet, had little impact on blood vessel function in people with PAD. However, further research is required to investigate the effects of canola oil in larger cohorts, over longer periods of time, and in populations without advanced cardiovascular disease, to determine whether the favourable fatty acid profile of canola oil may have more robust benefits.

8.8 Literature Cited

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CHAPTER 9: DISCUSSION

9.1 Discussion

Diet is a major determinant of metabolic and cardiovascular disease. Food and food components may impact human health in three different ways: they may be harmful to health and promote disease progression; they may be neutral, having no effect beyond providing nutritional energy; or they may play a therapeutic role by counteracting the pathophysiological damage brought about by disease conditions. Dietary fat has long been implicated in the development of obesity and cardiovascular disease [1,2], but more recent evidence, including the findings described in this thesis, calls into question our understanding of causality. Our current perspective on dietary fat is that both the type of fat and the amount of fat in the diet are important factors in maintaining metabolic balance. Clinical trials have demonstrated that saturated fats and *trans* fats have strong positive associations with cardiovascular disease, but MUFA- and PUFA-rich diets that include plant-based oils have health benefits [3,4].

The current program of research used pre-clinical and clinical research approaches to examine whether ALA from plant-based oils confers protection against the consequences of metabolic and cardiovascular disease (Figure 3.1). There were three overall objectives: to determine whether plant-based oils, including flaxseed oil and canola oil, can modulate adipose tissue function and inflammation in rodent models of genetic and diet-induced obesity; to determine the effect of n3 fatty acid

supplementation on cardiovascular outcomes in individuals with PAD; and to determine the effect of a dietary intervention with canola oil on parameters of PAD.

The main findings of this research were: (1) a diet enriched in flaxseed oil decreased inflammation in adipose tissue of obese *fa/fa* Zucker rats; (2) high-fat diets containing different proportions of MUFAs and PUFAs altered fatty acid composition, but had little impact on adipose tissue function and broader physiological function in DIO rats; (3) although there is no clear evidence to support the use of n3 PUFA supplementation in people with PAD, further high-quality and appropriately powered RCTs are needed to establish the efficacy of n3 PUFAs in cardiovascular disease; (4) a dietary intervention with canola oil altered the phospholipid fatty acid composition to incorporate higher levels of MUFAs and n3 PUFAs, but was not an effective modulator of blood vessel function in a PAD population with multiple co-morbidities and taking various medications, nor did the intervention have a great impact on physiological parameters or serum biochemistry within the time frame of the study; and (5) analysis of serum metabolites from participants in a canola oil dietary intervention using a non-targeted metabolomics approach yielded several novel molecular targets that could contribute towards developing new therapeutic approaches in PAD.

The current study explored the role of dietary fatty acids in adipose tissue function and inflammation using two animal models of obesity. The dietary proportions of n3 and n6 PUFAs have been linked to inflammatory-based pathology in human diseases such as cancer, atherosclerosis and obesity [5-7], a phenomenon that can be at least partially explained by the incorporation of these fatty acids into phospholipid

membranes. The PUFAs in cell membranes influence inflammation through several mechanisms, including membrane fluidity, lipid raft formation, receptor function [8,9], and biosynthesis of potent phospholipid-derived lipid mediators. Many lipid mediators produced from n6 PUFAs have proinflammatory functions, whereas those derived from n3 PUFAs tend to have inflammation-resolving properties [10,11]. Other groups have shown that EPA-derived resolvin E1 and DHA-derived protectin D1 help to prevent inflammation and insulin resistance in high-fat fed mice [12,13]. These long chain n3 PUFAs also modulate adipose tissue inflammation via GPR120, a fatty acid receptor that acts to suppress proinflammatory cytokine secretion from macrophages [14]. The authors also speculate that the incorporation of EPA and DHA into membrane phospholipids at the expense of the n6 PUFA AA may also contribute to the anti-inflammatory effects of n3 fatty acids.

In the studies presented in this thesis, although a diet containing n3 PUFA-rich flaxseed oil was able to attenuate adipose tissue inflammation in a monogenic model of obesity, the same protective effects were not observed in a DIO model, which more closely mimics the typical development of obesity in humans. In fact, the DIO animals seemed to demonstrate an adaptation effect, where the diets containing different proportions of MUFAs and PUFAs altered several parameters over the first few weeks of feeding and the fatty acid composition corresponded with the diet content, but by the end the study there were no differences among groups. The same adaptation effect over time was seen with serum triglycerides from these animals [15]. There was, however, a large difference in the %E from fat between these two studies (8.5%E from

fat in the flaxseed oil study; 55%E from fat in the DIO rat study), suggesting that perhaps the high-fat diet in the latter study simply overwhelmed the metabolism of these animals, making the exact proportions of different fatty acids less important than the overall amount. A diet with 55%E from fat would also be fairly difficult for humans to achieve, since the recommended amount ranges from 20-35%, and is generally not more than about 40% in most people [16].

In the literature, feeding studies in DIO animal models lasting longer than 8 weeks have not yielded consistent results. In a recent study, mice were fed high-fat diets with different proportions of n6:n3 PUFAs (1:1, 5:1, 10:1 and 20:1) using only LA and ALA for 20 weeks. Only the 1:1 ratio resulted in any therapeutic benefit (lower adipose tissue macrophage infiltration compared to the 20:1 diet group), but all of the high-fat diets generally led to similar levels of adiposity and adipose tissue inflammation [17]. However, another study showed rats fed high-fat diets with a 1:1 ratio of n6:n3 PUFAs (using primarily GLA and ALA) for 16 weeks had lower body and visceral fat weight, decreased expression of circulating inflammatory markers, lower blood lipids and improved glycemic markers, compared to rats fed a high-SFA diet [18].

A number of rodent models of obesity exist, including the monogenic *fa/fa* Zucker rat used in Chapter 4, but polygenic models are closer to the etiology of human obesity and metabolic syndrome, and therein lies the strength of the high-fat DIO model. The strains most often used in DIO studies are Sprague Dawley, Wistar, Long Evans or Lewis rats [19]. It is generally accepted that high-fat diets can be used in rodents to induce characteristics of obesity that are commonly seen in humans,

including insulin resistance, hypertriglyceridemia and compromised β -cell function [19]. In general, high-fat feeding produces organ-specific effects that are comparable to changes seen in human obesity, including changes to adipocyte morphology and metabolism, increased production of inflammatory mediators in the adipose tissue, and hepatic steatosis [19]. However, neither the exact fat content nor the exact fat composition of the diets employed is standardized, and can vary between 20%E – 60%E from fat, and fat sources (animal or plant) can also be different; this has led to considerable variability in the findings reported among studies [20].

Our DIO animal study results seem to indicate that the amount of fat in the diet matters more than the exact fatty acid composition with respect to developing obesity and its sequelae. In this regard, our findings are in line with those of Buettner et al [19], who showed similar trends in obesity development in rats fed high-fat diets containing different proportions of SFAs, MUFAs and PUFAs (except for animals fed marine n3 fatty acids, as these diets tended to inhibit weight gain). In general, we observed significant changes only when the n6:n3 PUFA ratio was very low (1:1), as was the case in the *fa/fa* Zucker rat flaxseed oil diet (improved adipose tissue function, Chapter 4) and in the DIO study flaxseed-canola oil blend diet (lower lipid content in the liver [21]), but more subtle changes in the n6:n3 ratios and PUFA:SFA ratios among isocaloric diets had little effect on overall metabolism. And notably, no changes in adiposity (body weight) among groups were observed as a result of the diets.

What can we learn from this pre-clinical work with regards to human obesity and dietary fats? The literature on the effects of n3 PUFAs on human adiposity [22,23] and

inflammation [24] tends to be minimal and inconclusive, but the variability of outcomes can be attributed largely to limitations in study design. Much of the research in obese humans has targeted populations with one or more co-morbidities, including cardiovascular disease, T2D, or other features of the metabolic syndrome. Given the contributions of obesity and obesity-related inflammation towards these co-morbidities, it may prove fruitful to isolate the role of n3 PUFAs in people who are obese but not suffering from other pathological conditions, i.e. “metabolically healthy obesity” – although, in our experience with clinical trials, this population would likely prove to be difficult to identify and recruit, and such a study may not be feasible. Thus, the pre-clinical studies presented here set the stage for future investigations to resolve the mechanisms by which n3 PUFAs (both long-chain and precursor fatty acids) act in human tissues, and lay the groundwork for RCTs to better evaluate whether varying proportions of fatty acids in the diet have an impact on the obese state.

Obesity is a major risk factor for many other chronic diseases, including cardiovascular diseases affecting the major blood vessels of the body [25]. In recent years, a number of systematic reviews and meta-analyses have tackled the relationship between cardiovascular disease and n3 PUFAs. The results have been difficult to interpret, in part because the research question “Are n3 fatty acids protective in cardiovascular disease?” is more complex than it initially seems. Some trials reported no association between n3 PUFA consumption and cardiovascular risk [26-28], while others saw a benefit in at least some endpoints [29-34]. The outcomes of such trials likely depend on the population (age, gender, ethnicity), type of cardiovascular disease,

disease stage and severity, co-morbidities, degree of pharmacological management, intervention duration and follow-up period, n3 PUFA type and dose, and method of n3 PUFA delivery (diet or capsule), to name only a few factors. The choice of endpoints in these trials is also an important consideration. Patient-centred outcomes such as adverse cardiovascular events or death, and functional outcomes like walking distance, should probably be the focus for researchers designing RCTs evaluating n3 PUFA supplementation, rather than surrogate outcomes such as inflammatory biomarkers. Of course, longer intervention periods and extensive follow-up are needed to assess rare outcomes like cardiovascular events, and this increases the cost of such trials. But at this stage of the research, the ambiguous language used to describe the findings (i.e., “does not clearly support”, “may protect”, and “the evidence is not clear-cut”) reinforces the notion that the answer to this question is far from settled, and perhaps longer, well-designed trials are now warranted

A similar outcome was seen when we conducted a systematic review and meta-analysis of the literature examining the effect of n3 PUFA supplementation in PAD [35]. It is thought that n3 PUFAs may have remedial effects in PAD due to their haematology-altering properties [36]. In general, diet-based therapeutic approaches to PAD management have not been well studied. The few RCTs that have been conducted in this population are typically short in duration, variable in their intervention designs, lacking in scientific rigour (e.g., use of inappropriate comparators), and each measure only a few of a wide range of outcomes.

There are variations in the lengths of the interventions described in this thesis. In the animal studies, the interventions were 8 and 12 weeks in the Zucker rats (Chapter 4) and the DIO rats (Chapter 5), respectively. In considering the design of these studies, it was important to choose durations that were relevant to the outcomes measured (markers of inflammation, adipose tissue function, and hepatic steatosis). These were based on previously published work using comparable models and dietary treatments that demonstrated changes in similar outcomes [37,38]. We studied interventions 12 weeks or longer in the systematic review (Chapter 6), and the Canola-PAD Study intervention was 8 weeks in duration (Chapters 7 and 8). The systematic review included trials that examined both patient-centred outcomes (such as cardiac events, stroke and death) and surrogate outcomes (e.g., walking distance, BMI and biochemical markers). Among these, patient-centred outcomes are the most clinically relevant, but are more costly to investigate, since observing these rare events requires longer intervention and follow-up periods as well as large participant populations. As an alternative, appropriate validated surrogate markers can offer valuable data about the health of a population, and changes are typically evident within a shorter period of time. For example, pulse wave velocity served as a measure of arterial stiffness in the Canola-PAD Study. Quantification of pulse wave velocity is widely considered to be the most useful marker of arterial stiffness, and is predictive of coronary events [39,40].

It should be noted that a Cochrane systematic review update [41] on n3 PUFA supplementation and intermittent claudication was published just prior to the completion of this work. The Cochrane review [41] included nine trials, four of which

overlapped with the five included in our review. The main reason for the discrepancy was the breadth of outcomes investigated: the Cochrane review collected data on serum biochemistry (lipids and inflammatory markers) in addition to clinical endpoints, while our review was focused primarily on patient-centred outcomes, such as cardiovascular events and walking distance; additionally, we included one trial [42] the Cochrane review rejected that defined PAD by $ABI \leq 0.9$ (not by the presence of intermittent claudication), and we also set more stringent inclusion criteria for intervention duration (12 weeks vs 4 weeks). Despite these differences, the findings of the two reviews are remarkably similar: based on the evidence of these reports, n3 PUFA supplementation is not recommended for routine use by people with PAD. However, given the underpowered studies and the significant statistical heterogeneity among the trials included, both reviews also highlight a need for more research in this area.

The Canola-PAD Study, a double-blind RCT testing the effect of a dietary intervention containing either canola oil or a Western diet oil mixture, aims to address this research gap. The Canola-PAD Study is unique in several aspects: i) while there have been dietary fatty acid intervention studies investigating the effects of MUFAs or ALA on blood vessel function [43,44], to the best of our knowledge, this is the first controlled intervention using canola oil as the primary lipid source; ii) the study employed new technologies (e.g., EndoPAT, AGE Reader) to efficiently conduct non-invasive vascular assessments; and iii) the study provides valuable data on sample size calculation.

The Canola-PAD Study design emphasis was on keeping the intervention as realistic to usual dietary and lifestyle patterns as possible. Previous interventions with n3 PUFAs have generally used capsules or supplements to deliver the n3 PUFA dose. Although capsules provide a simple, standardized way of delivering treatments, many people may prefer to consume n3 fatty acids in a more natural form, and it would not be possible to provide the recommended amount of MUFAs in capsules. By providing the study oils in food items comprising the main meal of the day, the Canola-PAD Study was able to manipulate one-third to one-half of the dietary fat intake each day, but in a way that could easily be incorporated into the participants' usual diets. In addition, the Canola-PAD Study recruited individuals with multiple co-morbidities (e.g., obesity, T2D, hypertension), and as such, most participants were on multiple medications to manage their conditions. This ensures that our study sample is representative of the PAD population, and can be generalized to other PAD patients. (Never mind that recruiting a sample of individuals diagnosed exclusively with PAD and not taking any pharmaceuticals would be near-impossible). Both of these design features may have contributed to the lack of changes we observed in the study, since the effect of the dietary intervention may have been lost in the milieu of other metabolic and pathophysiological processes. Nevertheless, a truly robust change should be observable even in this type of population, as we have already seen using a different intervention [45].

The calculation of an optimum sample size is also required to arrive at ethically and scientifically valid results. The sample size for the Canola-PAD Study was

determined from the best available data from a parallel study: the ABI values in another dietary intervention that measured the effect of pulse varieties (beans, peas, chickpeas and lentils) on vascular function in a PAD population [45]. Based on the mean endothelial function values in the current study with power = 0.80 at an alpha level = 0.05, a sample size of 100 participants (n=50/group; twice the number enrolled in the Canola-PAD Study) would be required to detect changes. This new sample size calculation may also help to explain why so few changes in PAD parameters were detected in our study. The findings of the pulse varieties study also suggest that the bioactive constituents in pulses (e.g., dietary fibre, flavonoids) may simply be more potent in altering vascular function in PAD than changing the type of fat in the diet.

In terms of effect size, a 5% change in ABI was observed in the pulse varieties study [45]. This was a large enough change to alter some patients' diagnosis of PAD, and thus has considerable clinical relevance. However, there is little research demonstrating the rate of change for other surrogate measures of PAD, such as pulse wave velocity or endothelial function in response to dietary interventions. The findings of the Canola-PAD Study indicate, however, that 8 weeks at the given dose may not be long enough to evaluate these changes.

As a means of summarizing the findings from the Canola-PAD Study, we conducted a correlation analysis of some of the key parameters (Table 9.1). Correlation analysis is a powerful tool to detect relationships among individuals or samples because the data from each arm are pooled together, effectively increasing the study population size; in addition, correlation analysis is useful for identifying associations between

variables when confounding elements may be present, such as multiple co-morbidities. Significant positive associations were detected between BMI and total n6 PUFAs, and between total n6 PUFAs and total SFAs. A significant negative correlation was observed between phospholipid total n3 PUFAs and serum LDL-cholesterol.

The results of the correlation analysis raise a couple of important points. First, the direct correlation between n6 PUFAs and BMI is a novel finding, as most PUFA research has focused on the role of n3 PUFAs (not n6 PUFAs) in adiposity. Some research supports the concept that a high dietary n6:n3 PUFA ratio contributes to low-grade chronic inflammation and promotes the development of many chronic diseases, including obesity [7,18,46]. Our results in Chapter 8 and Chapter 5 do not agree with this position, and suggest instead that it is the absolute amount of fat in the diet that impacts obesity and not the precise n6:n3 PUFA ratio. Other studies have also indicated that n6 PUFAs may not confer cardioprotective effects as previously believed, and may in fact be harmful [47]. In the Canola-PAD Study, the correlation between n6 PUFAs and BMI, but the *lack* of correlation between BMI and ABI, indicates that adiposity is not an important determinant of PAD severity, whereas previously it had been expected that obesity, as a common risk factor for cardiovascular disease, would contribute to PAD development and progression.

The association between SFAs and n6 PUFAs in our study is not surprising, given the prevalence of both of these types of fat in the Western diet intervention. However, a negative association between n3 PUFAs and LDL-cholesterol was perhaps unexpected.

LDL-cholesterol has long been touted as an important indicator for cardiovascular disease risk. Over the past three decades, clinical guideline recommendations for LDL-cholesterol levels have been developed on the basis of observational studies and some RCTs with the aim of reducing this risk factor [48]. However, the latest RCT-based evidence questions the importance of cholesterol for cardiovascular disease risk assessment [49,50], and the report from the American College of Cardiology/American Heart Association states that the expert panel was unable to find RCT evidence to support titrating cholesterol-lowering drug therapy to achieve LDL-cholesterol to non-HDL-cholesterol levels to reduce atherosclerotic cardiovascular disease events [51]. Rather than chasing LDL-cholesterol targets, the new guidelines released in 2013 instead focus on the intensity of statin therapy for specific populations [51]. Much of the scientific and medical community remains firmly entrenched in the belief that LDL-cholesterol is a key disease risk indicator, and so we continue to include it as a parameter in our studies. The finding that LDL-cholesterol levels are negatively correlated to total n3 PUFAs provides further support for the overall benefits derived from an n3 PUFA-rich diet. However, whether LDL-cholesterol continues to be an important indicator of disease risk in years to come remains to be seen.

The parameters measured in each of the dietary intervention studies discussed are summarized in Table 9.2, allowing for a broad overview of the work completed and a relatively simple way to compare and contrast the findings across studies, models and disease conditions. Due to the complexity of the study designs, changes in parameters are indicated with reference to the diet with the lowest n6:n3 PUFA ratio in each study.

One of the overarching trends among the studies presented is the generally consistent reflection of the dietary fatty acid composition in the phospholipid fatty acids. This indicates that the diets are altering cellular physiology; however, the magnitude of downstream effects varies in each of the studies. Many of the parameters we assessed did not change as the result of the interventions; this speaks to the limited potency of dietary plant-based oils in changing whole-body metabolism in the context of established multi-factorial diseases.

In summary, the novel findings of this thesis include: (1) dietary flaxseed oil was able to decrease proinflammatory biomarkers, attenuate T-cell infiltration and reverse adipocyte hypertrophy in *fa/fa* Zucker rats despite no change in adiposity and with no harmful effects observed, demonstrating the potential of high-n3 PUFA diets to resolve obesity-related pathologies; (2) dietary n6:n3 ratios greater than 2:1 had little impact on parameters of obesity, but a 1:1 ratio was effective at altering levels of some adipokines, suggesting that n6 and n3 PUFA proportions matter little unless they are equal or very close to equal; and (3) a dietary plant-based oil intervention using n6:n3 ratios of 2:1 compared to 20:1 was not an effective modulator of vascular function in individuals with PAD, nor did the intervention have a great impact on physiological parameters or serum biochemistry in this population.

Table 9.1 Canola-PAD Study Correlation Analysis

	BMI		ABI		LDL-Chol		Total n3		Total n6		Total SFA	
	<i>r</i>	<i>p</i>	<i>r</i>	<i>p</i>	<i>r</i>	<i>p</i>	<i>r</i>	<i>p</i>	<i>r</i>	<i>p</i>	<i>r</i>	<i>p</i>
BMI	-	-	-0.002	0.988	-0.035	0.827	0.156	0.330	0.356	0.022	-0.058	0.719
ABI	-0.002	0.988	-	-	0.227	0.154	0.011	0.947	0.027	0.867	0.022	0.893
LDL-Chol	-0.035	0.827	0.227	0.154	-	-	-0.366	0.019	0.017	0.916	0.128	0.423
Total n3	0.156	0.330	0.011	0.947	-0.366	0.019	-	-	0.020	0.899	-0.206	0.196
Total n6	0.356	0.022	0.027	0.867	0.017	0.916	0.020	0.899	-	-	-0.539	<0.001

Bolded values indicate a significant ($p<0.05$) correlation. ABI: ankle-brachial index; BMI: body mass index; LDL-Chol: low-density lipoprotein cholesterol; n3: n3 polyunsaturated fatty acid; n6: n6 polyunsaturated fatty acid; *r*: Pearson's correlation coefficient; SFA: saturated fatty acid.

Table 9.2 Effects of the Dietary Intervention with the Lowest n6:n3 Ratio in the Study

	<i>fa/fa</i> Zucker Rats (FaFlax vs. other groups as indicated)	High-Fat DIO Rats (Canola-Flaxseed Oil vs. other groups as indicated)	People with PAD (Canola vs. Western at 8 weeks, or Canola group over time as indicated)
Anthropometrics			
Body weight	No Δ vs <i>fa/fa</i> control \uparrow vs lean	\downarrow vs lard, soybean	No Δ
Adipose tissue weight	No Δ vs <i>fa/fa</i> control \uparrow vs lean	No Δ	
Body mass index			No Δ
Waist circumference			No Δ
Adipose Tissue Physiology			
Adipocyte size	\downarrow vs <i>fa/fa</i> control \uparrow vs lean		
Macrophage infiltration	No Δ		
T-cell infiltration	\downarrow		
Adipokines			
Adiponectin	\downarrow vs lean	No Δ	\downarrow (trend)
Leptin	\downarrow vs <i>fa/fa</i> control	No Δ	
IL-1 β			No Δ
IL-6	No Δ		No Δ
IL-10	\uparrow vs lean		No Δ
IL-18	\downarrow vs <i>fa/fa</i> control		
TNF- α	\downarrow vs <i>fa/fa</i> control		
MCP-1	\downarrow vs <i>fa/fa</i> control	No Δ	No Δ
Other Markers of Inflammation and Oxidative Stress			
E-selectin			No Δ^*
Haptoglobin	\uparrow vs lean	\downarrow vs <i>high-oleic canola</i>	
PAI-1		No Δ	
sRAGE			No Δ
4-Hydroxynonenal			\uparrow (trend)

Table 9.2 Cont'd

	<i>fa/fa</i> Zucker Rats (FaFlax vs. other groups as indicated)	High-Fat DIO Rats (Canola-Flaxseed Oil vs. other groups as indicated)	People with PAD (Canola vs. Western at 8 weeks, or Canola group over time as indicated)
Vascular Parameters			
Systolic blood pressure		No Δ	No Δ
Diastolic blood pressure		No Δ	No Δ^*
ABI			No Δ
Pulse wave velocity			No Δ
Pulse wave analysis			No Δ
Endothelial dysfunction			↑ (trend in Canola group)
Lipid Profile			
Triglycerides		↓ <i>vs high-oleic canola, lard</i>	↑ (trend)
Total cholesterol		No Δ	No Δ
LDL-cholesterol			No Δ
HDL-cholesterol			No Δ
HDL-cholesterol/LDL-cholesterol			No Δ
Apolipoprotein AI			No Δ
Apolipoprotein B			No Δ^*
Apolipoprotein CII			No Δ
Glycemic Index			
Glycated hemoglobin			↓ (trend)
Fasting glucose		↓ <i>vs lard</i>	↑ (Canola group)
Fasting insulin		No Δ	No Δ
HOMA-IR		No Δ	
Advanced glycation end-products			No Δ

Table 9.2 Cont'd

	<i>fa/fa</i> Zucker Rats (FaFlax vs. other groups as indicated)	High-Fat DIO Rats (Canola-Flaxseed Oil vs. other groups as indicated)	People with PAD (Canola vs. Western at 8 weeks, or Canola group over time as indicated)
Phospholipid Fatty Acids			
Total SFA	No Δ	No Δ	No Δ
C8:0 Caprylic acid			No Δ
C14:0 Myristic acid			↓
C16:0 Palmitic acid		No Δ	No Δ
C18:0 Stearic acid		No Δ	No Δ
C20:0 Eicosanoic acid			↑ (trend in Canola group)
C22:0 Docosanoic acid			No Δ
C24:0 Tetracosanoic acid			↓
Total MUFA	No Δ	↑ vs safflower, soybean	↑
C16:1t			↑
C18:1n9 Oleic acid		No Δ	↑
C18:1n7 Vaccenic acid		↑ vs safflower, soybean, lard	↑
C20:1n9 Eicosenoic acid			↑ (Canola group)
C24:1n9 Tetracosenoic acid			↑ (Canola group)
Total PUFA	No Δ	↑ vs lard, high-oleic canola	No Δ
n3 PUFA	↑	↑ vs safflower	↑ (Canola group)
C18:3n3 Alpha-Linolenic acid	No Δ	No Δ	↑ (trend in Canola group)
C20:5n3 Eicosapentaenoic acid	↑	No Δ	↑ (Canola group)
C22:5n3 Docosapentaenoic acid	↑	No Δ	No Δ
C22:6n3 Docosahexaenoic acid	No Δ	No Δ	↑ (trend in Canola group)
n6 PUFA	No Δ	↓ vs safflower, soybean	No Δ
C20:3n6 Dihomo-GLA			↓ (trend in Canola group)
C18:2n6 Linoleic acid	No Δ	↓ vs safflower, soybean ↑ vs high-oleic canola	No Δ
C20:4n6 Arachidonic acid	No Δ	No Δ	↑ (trend in Canola group)
n9 PUFA	No Δ		↑
n6:n3	↓	↓ vs safflower	↓ (trend)
n9:n6			↑
PUFA:SFA	No Δ		No Δ

Table 9.2 Cont'd

*There was a difference in baseline values, but no change in delta values due to dietary intervention.

Data in italic text (DIO rats) is from Hanke et al's study [15] on the same animals.

In the *fa/fa* Zucker rat study, adipokines were measured in adipose tissue; in all other studies, adipokines and inflammatory markers were measured in serum. Changes in parameters in all studies are indicated between groups at the end of the study (i.e. at 8 weeks in the *fa/fa* Zucker rat study, at 12 weeks in the DIO study, and at 8 weeks in the Canola-PAD Study). Phospholipid fatty acid composition was measured in splenocytes, adipose tissue and plasma in each of the three studies, respectively.

Δ: delta (change); HOMA-IR: homeostatic model assessment – insulin resistance.

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CHAPTER 10: CONCLUSION

10.1 Summary and Implications

In summary, the findings reported in this thesis indicate that dietary fatty acids from plant-based oils play many roles in metabolic and vascular disease. Dietary fatty acids have been shown to alter the composition of cell membrane phospholipids, modulate the activity of immune cells, alter lipid metabolism and storage, and improve adipose tissue function. Flaxseed oil had the highest proportion of n3 PUFAs among the plant-based oils tested, which also included oils rich in MUFAs (high-oleic canola oil and conventional canola oil), n3 PUFAs (canola oil) or n6 PUFAs (safflower oil or soybean oil), and was demonstrated to be the most effective at bringing about changes in obesity. At this time, there is no evidence to suggest that diet interventions enriched with MUFAs and n3 PUFAs impact cardiovascular outcomes in individuals with PAD, but further studies with appropriate statistical power and well-designed interventions will help to clarify the true potential of n3 PUFAs in primary and secondary prevention. From our findings, it appears that the n6:n3 PUFA ratio must be lower than 2:1 to bring about changes in metabolic and vascular disease.

New approaches to prevent and manage metabolic and cardiovascular disease are required to reduce the burden of these widespread public health problems. The findings of this thesis have contributed, through the use of animal models and human studies, to the current state of knowledge regarding the potential role of n3 dietary fats from plant-based oils in these disease conditions. Research focused on the potential

protective and/or harmful effects of foods and food components enhances our understanding of the role of diet in the development and progression of diseases like obesity and PAD. This, in turn, helps shape nutrition guidelines and health claims, ultimately results in the development of new food products, and places emphasis on dietary patterns to help support better health.

10.2 Strengths and Limitations

Strengths:

Animal Studies

- Use of two different animal models of obesity: 1) monogenic model, the obese *fa/fa* Zucker rat, 2) polygenic high-fat DIO model, the Sprague Dawley rat.
- Use of several complementary biochemical techniques to analyze adipose tissue function (protein and mRNA analysis, histochemical analysis, serum biochemistry, and fatty acid analysis).
- Meticulous diet design in both animal studies.

Systematic Review and Meta-Analysis

- Rigorous methodology in conducting the systematic review and meta-analysis with guidance from experts in the field.

Clinical Trial

- The Canola-PAD Study had several novel features: 1) first controlled intervention using canola oil as the primary lipid source to examine blood vessel function, 2) used non-invasive technologies used to conduct vascular assessments, 3) provides sample size data for future investigations.
- Incorporated realistically attainable fatty acid proportions and amounts, and used portion sizes that were reasonable.
- Use of validated, non-invasive vascular testing methods that were direct measures of vascular function.

- Application of advanced statistical methods to examine different aspects of the data.
- High rate of compliance and 0% drop-out rate.
- The food items were well tolerated, easy to consume as part of the usual diet, and participants appreciated the ability to tailor the selection of food items to their individual preferences.
- Data and samples for a broad scope of assessments were collected: vascular function measurements, biochemical markers, serum metabolites, DNA (saliva), mobility (treadmill testing), and cognitive function – allowing for potential further analysis and conclusions.

Limitations:

Animal Studies

- The findings gleaned from animal models of obesity cannot be generalized to human health without exercising caution, especially since two of the primary measures for human obesity (BMI and waist circumference) are meaningless in animals.

Systematic Review and Meta-Analysis

- The scope of the systematic review and meta-analysis was limited by the need to complete the review during a 4-month graduate course.

Clinical Trial

- The effect of the Canola-PAD Study dietary intervention may have been confounded by the other foods the participants were consuming in their usual diet or by their medications.
- The Western group served as a control group in the Canola-PAD study, but in Manitoba, canola oil consumption is generally already quite high, and this might not have been overcome by the Western diet food items provided in the study, perhaps confounding the results. We could also have added a group without PAD.
- While the metabolomics analysis provided fodder for future studies, it yielded no concrete causal links between the dietary intervention and the altered metabolites.

10.3 Future Directions

- Investigation of the efficacy of dietary flaxseed oil in reversing adipocyte hypertrophy in humans (as we demonstrated in *fa/fa* Zucker rats, Chapter 4) by taking adipose tissue biopsies.
- The link between n6 PUFA intake and BMI should be further explored. A small animal model system for blood vessel function in PAD would be an asset in this area.
- Future dietary oil interventions in the PAD population will benefit from the findings of the Canola-PAD Study, as a more accurate sample size can now be calculated.
- Some data analysis remains in the Canola-PAD Study: cognitive tests, functional assessments (walking distance) and a more comprehensive correlation analysis, which was deemed beyond the scope of this thesis.
- Dietary intervention studies in healthy volunteers are required to obtain evidence for health claims through Health Canada.
- The metabolomics analysis sets the stage for further investigation of the metabolites of interest in cell culture or animal models.

Appendix A.

An Exploratory Comparative Metabolomics Analysis: Novel Metabolites of Interest Following a Dietary Canola Oil Intervention in Adults with Peripheral Arterial Disease

Introduction

Metabolomics is the study of the metabolome, the complete collection of endogenous small molecules that are transformed during metabolism and are the products of cellular processes [1]. Metabolites form a large web of metabolic reactions, linking products from many different metabolic reactions in a complex network of pathways. Metabolites are considered the key link between genes and phenotypes, because they provide direct signatures of biochemical activity [2]. The term metabolome was coined in analogy with genome, proteome, and transcriptome; like these entities, the metabolome is highly dynamic, and can change from second to second.

Metabolomics is useful for profiling the metabolic products of cellular processes. Conducting a metabolomics analysis using a non-targeted approach provides a complete overview of the metabolites in a biological fluid (such as serum), allowing these molecules to be separated and identified without bias regarding the nature of the compounds that may be associated with a particular physiological or pathophysiological process. Molecules originating from food integrate into a vast network of metabolites

that may have far-reaching effects on the function of cells and organ systems, and thus, metabolomics may also be used to study the effects of dietary interventions.

We used a non-targeted metabolomics analysis to examine serum samples from a dietary canola oil intervention in PAD patients (described in Chapter 7 and 8) to examine diet-specific changes in the study participants' metabolite profiles. The goal of this exploratory study was to identify metabolites of interest in vascular disease which may also serve as potential future targets for cardiovascular disease research.

Materials and Methods

Chemicals

HPLC grade acetonitrile, methanol, and spectroscopic grade formic acid were purchased from Sigma/Aldrich Ltd (Oakville, ON, Canada). Double-distilled water was collected at 18 Ω from a Barnstead E-Pure system (Dubuque, Iowa, USA). The electrospray ionization low (ESI-L) concentration tuning mix and atmospheric pressure interface time of flight (API-TOF) reference mass solution were purchased from Agilent Technologies (Mississauga, ON, Canada).

Extraction of Metabolites

Fasting blood samples were collected at baseline and after eight weeks from participants of the Canola-PAD Study (n=50), a dietary intervention trial that supplied adults with PAD with food products containing 25 g/day of either canola oil or an oil mixture representing the Western diet. Participants consumed these products as part of their usual diet. The details of the design of this trial are described in Chapter 7 and

have been published elsewhere [3]. Blood samples were allowed to clot at room temperature for 45-60 min, and centrifuged at $1,000 \times g$ for 10 min at room temperature. The serum layer was aliquoted into 1.5 mL microcentrifuge tubes and stored at -80°C .

Prior to extraction, serum samples were thawed on ice. Serum (100 μL) was added to 200 μL acetonitrile (Acn), and vortexed for 30 sec. The samples were centrifuged at $10,000 \times g$ for 10 min at 4°C . The supernatant (250 μL) was transferred to a new microfuge tube and dried under an N-EVAP™111 nitrogen evaporator (Organomation Associates, Berlin, MA, USA). The sample was reconstituted for LC-MS in 200 μL of 4:1 Acn:ddH₂O, and placed in a glass insert in a brown LC vial. The 4:1 Acn:ddH₂O mixture was used as a blank throughout this study.

Chromatography

A 1260 Rapid Resolution system (Agilent Technologies, Santa Clara, CA, USA) containing a binary pump and degasser, well-plate auto-sampler with thermostat (1290), and thermostat column compartment was used for all analyses.

Chromatographic separations of serum samples were performed on an Agilent ZORBAX SB-Aq column (2.1 mm \times 100mm, 1.8 μm) at a column temperature of 45°C . The mobile phases were water and Acn with 0.1% formic acid. The gradient duration was 10 min with 0-6 min 2% Acn; 6-8 min 60% Acn; 8-8.5 min 60% Acn; 8.5-8.6 min 2% Acn; and 8.6-10 min 2% Acn. The post-run time between injections was 2 min. The auto-sampler temperature was maintained at 6°C and the flow rate was 0.7 mL/min. In order to minimize the carryover of samples during the successive injections, the injection needle

was washed in two separate vials (5 rinses per vial) before each injection. An aliquot (1 μ L) of serum extract was injected for individual analysis.

Mass Spectrometry

MS was performed on an Agilent 6538 Q-TOF mass spectrometer equipped with a dual ESI source in positive mode. The capillary voltage, the fragmentor, the skimmer and the OCT 1 RFVpp were set to 4000, 80, 50, and 750 V, respectively. The drying gas (N_2) was set to 11 L/ min at 300°C and the nebulizer at 50 psig. Spectra were acquired over the m/z 50-1,000 range. Reference masses of 121.0508 and 922.0097 were used for all runs. The Molecular Feature Extraction (MFE) algorithm was applied to all replicates and features with abundance >4000 were extracted. Average values for RT and m/z values of each feature were calculated.

Data Processing and Statistical Analysis

The workflow employed for data processing comprised several algorithms used by Agilent MassHunter Qualitative (MHQ, B.05) and Mass Profiler Professional (MPP, 12.6). The raw data files were first acquired and stored as “*.d” files using Agilent MassHunter Acquisition software (B.05) ready to be processed in MHQ. A naïve extraction procedure (MFE) was the first algorithm applied to the total ion chromatograms (TIC) files. The MFE parameters were set to allow the extraction of detected features with an absolute abundances >4,000 counts providing information regarding $[M + H]^+$ isotopes and their corresponding Na^+ adducts.

The resulting extracted ions were treated as single features for which potential formula were generated. The collected information summarizing retention time (RT), exact masses and ion abundances were converted into compound exchange format ("*.cef") and were exported to MPP for further comparative and statistical analyses. Using alignment and normalization procedures, individual "*.cef" files were binned and combined to generate new "*.cef" files. These new files were reopened in MHQ for further data mining using a 'find by ion' algorithm. This targeted feature algorithm helped with minimizing the false positive and negative features found by MFE procedure. A second series of individual "*.cef" files were created from original individual "*.d" files and exported into MPP for statistical and differential analysis. A frequency filtration was used to only accept features that were detected in at least 55% of samples. This filtration step was employed to ensure an elimination of the potential feature extraction artifacts. Other MPP filtering procedures such as number of detected ions (set to '2') and charge states (set to 'all charge states permitted') were also applied. The RT compound alignment parameters were set to 0.15 min with a mass tolerance of 0.002 Da. The data were normalized using a percentile shift algorithm set to 75 and were baselined to the median of all samples. Moderated T-test ($p < 0.01$) and paired T-test ($p < 0.05$) analyses were conducted using the MPP software.

Heatmapping was conducted using GENE-E software [4]. Metabolites were first clustered by class and chemical structure similarities, and ordered by abundance at Week 0 using a log 2 normalized scale. Relative abundance in the canola oil group and

the Western diet group at Week 8 were calculated and mapped according to increased abundance (green) or decreased abundance (red).

Results

Using the molecular feature extraction and ‘find by ion’ algorithms, followed by application of a frequency filter that eliminated metabolites present in <55% of the samples, a total of 169 metabolites were found. To gain an overall picture of the types of metabolites present in the serum, these 169 metabolites were grouped by chemical structure and heat-mapped to measure abundance at Week 8 in each of the dietary intervention groups relative to baseline (Figure A1). Metabolites detected included glycerophospholipids and sphingolipids, glycosylated lipids (glycans), fatty acyl derivatives, di- and tripeptides, lipid-based plant hormones, and various pharmacological agents.

The dietary intervention altered the serum levels of several metabolites (Table A1), including several derivatives of sphingolipids (ceramides) and phospholipids (phosphatidylethanolamines). Over the course of the study, levels of Cer(d18:2/14:0) and Cer(d18:1/24:1) decreased by 40% and 30%, respectively, in the study participants in the Canola Oil group. Notably, although ceramides were detected in baseline samples, none were present in the serum of participants in the Western group by the end of the intervention. Phosphatidylethanolamine levels generally remained stable in both dietary intervention groups, except for a 60% increase in PE(18:3/19:0) in the serum of participants in the Canola Oil group over the course of the study.

Several metabolites were differentially detected in participants who were diabetic (HbA1c > 6%) and those who were normoglycemic or pre-diabetic (HbA1c ≤ 6%) at baseline (Table A2). Metabolites involved in jasmonate biosynthesis were detected in both diet groups. At the end of the study, methyl jasmonate was elevated by 10% in diabetic Western group. Dihydrophaseic acid, a derivative of abscisic acid, decreased by half in the serum of normoglycemic participants in the Canola Oil group over the course of the study.



Figure A1. Heatmap of metabolites in serum from Canola-PAD Study participants.

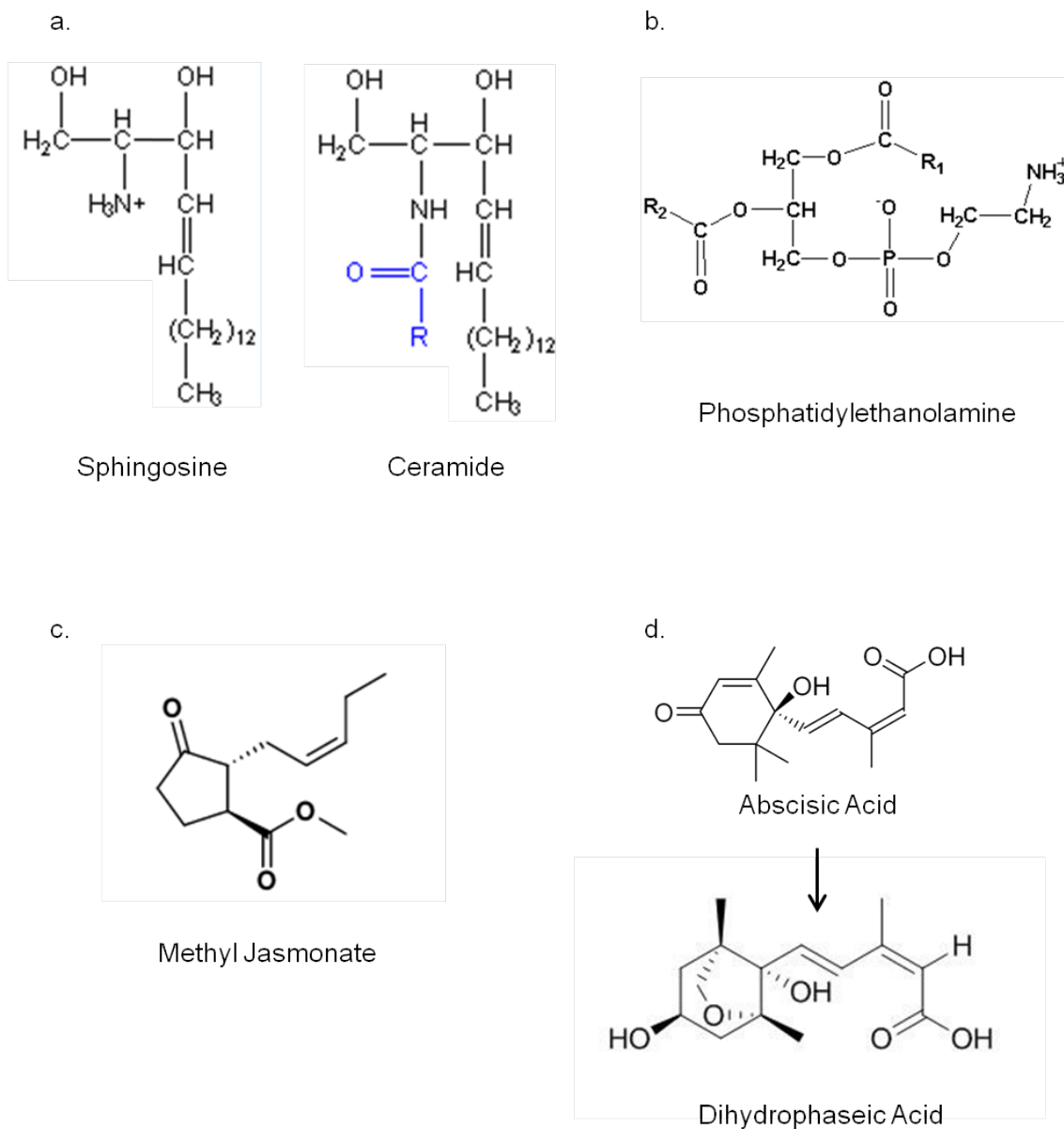


Figure A2. Chemical structures of metabolites detected in serum: a. Sphingosine and ceramide (-R represents a fatty acid); b. Phosphatidylethanolamine (-R₁ and -R₂ represent fatty acids); c. Methyl jasmonate; d. Absciscic acid and dihydrophaseic acid. These images are in the public domain.

Table A1. Serum Metabolites Altered by the Dietary Intervention

Metabolites	Formula	Mass	m/z	ABUNDANCE		
					<u>Canola Oil</u>	<u>Western</u>
				Week 0	Week 8	Week 8
<i>Ceramides</i>						
C-6 Ceramide	C ₂₄ H ₄₇ NO ₃	397.3564	398.3844	20 ± 2.8	18 ± 0.9	NP
Cer (d18:2/14:0)	C ₃₂ H ₆₁ NO ₃	507.4651	508.4711	47 ± 2.2	33 ± 2.2	NP
Cer (d18:2/15:0)	C ₃₃ H ₆₃ NO ₃	521.4808	522.4881	41 ± 1.9	32 ± 2.4	NP
Cer (d18:2/16:0)	C ₃₄ H ₆₅ NO ₃	535.4964	536.5037	28 ± 2.6	27 ± 1.9	NP
CerP (d18:1/24:1(15Z))	C ₄₂ H ₈₂ NO ₆ P	727.5879	728.6049	47 ± 1.2	36 ± 1.9	NP
<i>Phosphatidylethanolamines</i>						
PE (14:1(9Z)/22:1(13Z))	C ₄₁ H ₇₈ NO ₈ P	743.5465	744.5538	660 ± 300	510 ± 120	599 ± 99
PE (18:3(6Z,9Z,12Z)/19:0)	C ₄₂ H ₇₈ NO ₈ P	755.5465	756.5471	1100 ± 520	1800 ± 430	708 ± 256
PE (18:3(9Z,12Z,15Z)/19:1(9Z))	C ₄₂ H ₇₆ NO ₈ P	753.5309	754.5381	940 ± 360	780 ± 280	1460 ± 380
PE (18:3(9Z,12Z,15Z)/20:2(11Z,14Z))	C ₄₃ H ₇₆ NO ₈ P	765.5309	766.5381	270 ± 100	310 ± 60	326 ± 61
PE (18:3(9Z,12Z,15Z)/20:3(8Z,11Z,14Z))	C ₄₃ H ₇₄ NO ₈ P	763.5152	764.5225	470 ± 230	570 ± 120	566 ± 115
PE (19:0/22:6(4Z,7Z,10Z,13Z,16Z,19Z))	C ₄₆ H ₈₀ NO ₈ P	805.5622	806.5694	1500 ± 670	1500 ± 410	1636 ± 327
PE (22:4(7Z,10Z,13Z,16Z)/17:1(9Z))	C ₄₄ H ₇₈ NO ₈ P	779.5465	780.5538	710 ± 300	740 ± 270	1178 ± 332

Abundance is measured as ion counts per minute × 10³ based on output from mass spectrometry. Cer: ceramide; PE: phosphatidylethanolamine; NP: not present. Bold type denotes significant differences (p<0.05) between 0 and 8 weeks by paired T-test. Week 0 values for both dietary groups were averaged (n=50).

Table A2. Metabolites Differentially Detected in Diabetic and Normoglycemic Participants

Metabolites	Formula	Mass	m/z (ESI+)	ABUNDANCE				
				Week 0	Week 8 Canola Oil		Week 8 Western	
					DB	NG	DB	NG
<i>Jasmonates</i>								
(9S,13S)-1a,1b-dihomo-jasmonic acid	C ₁₄ H ₂₂ O ₃	238.1569	239.1642	87 ± 63	NP	74 ± 13	NP	83 ± 12
Epi-4'-hydroxyjasmonic acid	C ₁₂ H ₁₈ O ₄	226.1205	227.1278	12 ± 2.7	NP	21 ± 2.4	15 ± 1.2	15 ± 1.2
(-)-11-hydroxy-9,10-dihydrojasmonic acid	C ₁₂ H ₂₀ O ₄	228.1362	229.1434	340 ± 190	550 ± 120	210 ± 380	320 ± 48	320 ± 48
Methyl jasmonate	C ₁₃ H ₂₀ O ₃	224.1406	225.1479	90 ± 5.4	NP	NP	100 ± 1.3	89 ± 6.5
<i>Abscisic Acid Derivatives</i>								
Abscisic acid	C ₁₅ H ₂₀ O ₄	264.1362	265.1434	NP	NP	NP	NP	NP
Dihydrophaseic acid	C ₁₅ H ₂₂ O ₅	282.1467	283.1517	210 ± 42	157 ± 52	100 ± 27	187 ± 43	146 ± 37

Abundance is measured as ion counts per minute × 10⁻³ based on output from mass spectrometry. DB: diabetic (HbA1c > 6%); NG: normoglycemic (HbA1c ≤ 6%); NP: not present. Bold type denotes significant differences (p<0.05) between 0 and 8 weeks by paired T-test. Week 0 values for both dietary groups and HbA1c indices were averaged (n=50).

Discussion

This study examined the effect of a dietary intervention with food products containing either canola oil or an oil mixture typical of the Western diet on the serum metabolite profile of people with PAD. One hundred sixty nine metabolites of interest were identified through filtering and searching the KEGG database for molecular feature similarities. These metabolites included glycerophospholipids and sphingolipids, glycosylated lipids (glycans), fatty acyl derivatives, vitamins, di- and tripeptides, lipid-based plant hormones, and various pharmacological agents that were likely derived from the study participants' medications. Although many more metabolites (>16,000) than the 169 entities depicted in Figure A1 were initially detected by LC-MS, most of these did not meet the criteria set by our data processing protocol. For example, we eliminated metabolites that appeared in <55% of our samples by reasoning that compounds not present in most of the samples were unlikely to be related to the intervention, and thus would not be of great significance for future investigation. Additionally, many of the metabolites we detected had no match or a very poor match in the KEGG database when we searched for feature similarities using the molecular feature extraction and 'find by ion' algorithms. Although the KEGG database is growing rapidly as researchers deposit their findings, we are limited by the information that is currently available via this resource. We could not confirm the identity of all metabolites without further targeted MS/MS analysis, which was deemed beyond the scope of this study. However, among the metabolites that we were able to match in the database, we detected some statistically significant changes over the course of the study.

Levels of several ceramide metabolites decreased in participants in the Canola Oil group, and were not detected at all in participants in the Western diet group at week 8. Ceramides belong to the sphingolipid family and are composed of sphingosine and a fatty acid molecule (Figure A2a). They serve as structural elements in the cell membrane and can participate via signalling pathways in a variety of cellular processes, including cell differentiation, apoptosis and proliferation [5]. They are formed locally in the walls of blood vessels and released into the bloodstream [6,7], where they play a role in the regulation of vascular tone, growth and maturation through their paracrine actions at sphingosine-1-phosphate receptors [8]. Ceramides are enriched in low density lipoproteins extracted from atherosclerotic lesions [9,10], where they promote subendothelial aggregation of lipoproteins and formation of foam cells [11]. In rodent models of lipid oversupply, targeted inhibition of ceramide biosynthesis attenuates atherosclerotic lesion formation [12,13] and partially reverses endothelial dysfunction [14], thus providing evidence for arterial ceramide accumulation as a precipitator of cellular dysfunction in part by impairing nitric oxide bioavailability. Although only a trend in improved endothelial function was observed in the Canola Oil group of the Canola-PAD Study ($p=0.095$; Table A2), the decline in ceramide metabolite levels during the intervention indicates a possible benefit of the Western diet, since ceramides appear to have harmful effects on blood vessels in atherosclerotic disease. However, since ceramides and their derivatives have been investigated primarily in cell and animal models, the role of ceramides in human vascular disease requires further study.

A large number of phosphatidylethanolamines were detected in the serum of study participants. Phosphatidylethanolamines are glycerophospholipids in which a phosphoethanolamine occupies a glycerol substitution site (Figure A2b). Phosphatidylethanolamines comprise 25% of all phospholipids in mammalian cell membranes, where they serve as precursors or substrates in several metabolically important signalling pathways [15]. In the heart, phosphatidylethanolamines play a protective role following cell damage from ischemia [16], and are involved in regulating the process of coagulation [17]. In our study, the abundance of most phosphatidylethanolamines remained stable during the dietary intervention, except for PE (18:3/19:0), which increased by 60% in the participants in the Canola Oil group. This increase may reflect the higher content of ALA (18:3n3) in the canola oil diet compared to the Western diet, and is in agreement with the finding that the n6:n3 serum phospholipid fatty acid ratio decreased in the Canola Oil group relative to the Western group (Table 8.4).

Both diet groups in the Canola-PAD Study included participants who were diabetic ($\text{HbA1c} > 6\%$) and normoglycemic ($\text{HbA1c} \leq 6\%$), which allowed for differential analysis of changes in metabolite levels. We observed that methyl jasmonate increased over the course of the study in the diabetic participants in the Western group. Jasmonates are a family of plant oxylipin stress hormones. In plant cells, jasmonates are derived from polyunsaturated fatty acids released from cellular lipid pools by lipases, and are subsequently oxygenated by lipoxygenases to form hydroperoxide derivatives (cyclopentanones) (Figure A2c) [18,19]. They occur ubiquitously in plants (including both

canola (*Brassica napus*) [20] and safflower (*Carthamus tinctorius*) [21]) and their production is induced by different types of environmental stressors; in response, they mediate signalling pathways that trigger defense mechanisms [22], such as inducing production of reactive oxygen species and plant secondary metabolites, including phenolic compounds used as chemical defenses [22-25]. In relation to vascular function, methyl jasmonate has been demonstrated to induce endothelial cell death and suppress angiogenesis in vivo [26]. The patterns of jasmonate metabolites we observed in our samples are challenging to explain, given that research on jasmonates has focused mainly on plant processes, and very little has been done that can be related directly to human health. However, this research gap represents an opportunity for future mechanistic studies in animal and cell models to elucidate the putative role of jasmonates in human health and disease.

Finally, we observed a decrease in dihydrophaseic acid in the serum from normoglycemic participants in the canola oil group. Dihydrophaseic acid is a catabolic product of abscisic acid, a plant hormone formed from the products of glycolysis in the chloroplasts of vascular plants, including canola [27] and safflower [28] (Figure A2d). Abscisic acid functions as a buffer against the deleterious effects of plant stressors (heat, salinity, chilling, and herbicide toxicity) [29], and is involved in regulating numerous physiological processes during seed development [30]. Very little is known about the possible role of abscisic acid and its metabolites in humans or mammalian model systems, as they have been studied primarily in plant culture. Our results indicate that

these molecules may play a role in the regulation of glycemic control, although it is difficult to determine their impact from these initial findings.

A couple of important considerations stem from this work. Although the findings presented here are preliminary, metabolic screens such as the present study represent a novel approach for investigating the medicinal properties of dietary oils. Although there is still controversy regarding the efficacy of different oils in providing beneficial effects in health and disease, many of the compounds we obtain by consuming these oils (such as jasmonate and abscisic acid) are not natural to our systems, and they may be responsible for health benefits seen in dietary interventions. We also need to consider that the current study may have been limited by the exclusion criteria set by our data processing protocol. By eliminating molecular candidates that appeared in fewer than 55% of our samples, we may have missed key metabolites appearing in specific individuals because of other factors related to their health status, which would make them valuable in the development of personalized medicine. Additionally, the sensitivity of this type of metabolomics analysis is both an advantage and a limitation. It allows the discovery of a vast array of molecules at detection levels lower than any other available technology; yet, whether these compounds have any physiological relevance at this concentration is not known and cannot be deciphered from the current results without further research. Therefore, approaches to be taken in future studies include using an analogous dietary intervention at the preclinical level to study the role and significance of the metabolic targets identified here; and examining the associations between these

molecular candidates and disease parameters to gain insight into the potential for developing tailored therapies for specific disease conditions.

Conclusion

In summary, this non-targeted metabolomics analysis identified several metabolites that were significantly altered in abundance by the dietary treatment; however, none of these have previously been well-studied or even recognized as playing a role in dietary plant-based oil interventions. This then represents an opportunity for exploring new pathways of dietary lipid metabolism and how they might be associated with pathological states such as PAD and T2D. Experiments in model systems will help to determine the function of new metabolites, which can then contribute to the development of personalized nutrition, tailored to the biological state of an individual with a view to maximizing the health benefits of a diet and minimizing the negative effects.

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