Oils Rich in alpha-linolenic acid independently protect against characteristics of fatty liver disease in the delta-6 desaturase null mouse

By

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Abstract

OILS RICH IN ALPHA-LINOLENIC ACID INDEPENDENTLY PROTECT AGAINST CHARACTERISTICS OF FATTY LIVER DISEASE IN THE DELTA-6-DESATURASE NULL MOUSE

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The biological activity of α-linolenic acid (ALA) is poorly understood and primarily associated with its conversion to eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA). This study used the Δ6 desaturation knockout (D6KO) mouse, which lacks Δ6 desaturase and therefore cannot convert ALA, to evaluate the independent effects of ALA on preventing non-alcoholic fatty liver disease (NAFLD). First, the capacity of very long chain fatty acids to rescue the D6KO lipid profile was established. Next, to evaluate the independent effects of ALA, D6KO or wild-type mice were fed diets containing lard, canola, flaxseed, or fish oil. Following treatment, liver phospholipid fatty acid composition was evaluated and livers were scored for steatosis and inflammation. Glucose tolerance was also evaluated. D6KO mice fed ALA-rich diets had lower liver lipid accumulation, lower hepatic inflammation (8 weeks) and improved glucose tolerance (20 weeks) relative to lard-fed D6KO mice. Overall, this thesis supports an independent biological role for ALA.
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List of Abbreviations

AA: Arachidonic acid
ALA: Alpha-linolenic acid
ApoE: Apolipoprotein E
ARASCO®: Arachidonic acid-rich single celled oil
AUC: Area under the curve
CD: Canola oil diet
CRP: C-reactive protein
D5D: Delta-5 desaturase
D6D: Delta-6 desaturase
D6KO: Delta-6-desaturase knockout
DHA: Docosahexaenoic acid
EPA: Eicosapentaenoic acid
FD: Flaxseed oil diet
HDL: High density lipoprotein
HUFA: Highly unsaturated fatty acids
IL-6: Interleukin-6
KO: Knock-out
LA: Linoleic acid
LD: Lard Diet
LDL: Low density lipoprotein
Lyso-PC: Lyso-phosphatidylcholine
MD: Menhaden oil diet
MetS: Metabolic Syndrome
MUFA: Monounsaturated fatty acid
PC: Phosphatidylcholine
PE: Phosphatidylethanolamine
PI: Phosphatidylinositol
PL: Phospholipid
PPARα: Peroxisome proliferator-activated receptor alpha
PS: Phosphatidylserine
PUFA: Polyunsaturated fatty acid
SF: Safflower oil
SFA: Saturated fatty acid
SM: Sphingomyelin
SO: Soybean oil
SREBP: Sterol regulatory element-binding protein
TG: Triglycerides
TLC: Thin layer chromatography
TNF-α: Tumor necrosis factor alpha
VLDL: Very low density lipoprotein
WT: Wild type
Chapter One

INTRODUCTION
1.0 INTRODUCTION

The increasing prevalence of metabolic diseases has become a serious international concern. In Canada, 59% of the population is considered overweight (Body Mass Index $\geq 25$ kg/m$^2$), while 23% or nearly a quarter of the population is obese (BMI $\geq 30$ kg/m$^2$).\(^1\) Obese individuals are at an increased risk of developing chronic and progressive metabolic disorders including non-alcoholic fatty liver disease (NAFLD) and cardiovascular disease (CVD). Currently, cardiovascular related complications are the leading cause of mortality in Canada and the Western world,\(^2\) while NAFLD is the most prevalent liver disease in North America.\(^3\) More unsettling are reports that paediatric diagnoses of NAFLD are becoming increasingly common in the US, as the childhood obesity rate increases.\(^4\)

N-3 polyunsaturated fatty acids (n-3 PUFA) have gained considerable attention in recent years for their potential to prevent and treat many lifestyle diseases including cancer, CVD and type II diabetes.\(^5\) In particular, the very long chain n-3 PUFA eicosapentaenoic acid (EPA; 20:5 $\Delta 5,8,11,14, 17$) and docosahexaenoic acid (DHA; 22:6 $\Delta 4,7,10,13,16,19$) confer numerous metabolic benefits, from their well-established role in reducing circulating triglycerides (TG),\(^6\) to promoting the resolution of inflammation, preventing platelet aggregation,\(^7\) and stimulating lipid oxidation while inhibiting lipogenesis.\(^8\) Humans can endogenously produce EPA and DHA, but may also obtain these two fatty acids through the consumption of fatty fish products, and fish oil supplements. Unfortunately, fish consumption in North America is low due to many variables including taste, cost, and concern over toxins found in some fish.\(^9\) Thus, the consumption of plant-based n-3 PUFA, which consist primarily of parent n-3 PUFA $\alpha$-linolenic acid (ALA; 18:3 $\Delta 9,12,15$), is one promising alternative to obtaining a sufficient intake of n-3 PUFA. ALA, along with the parent n-6 PUFA linoleic acid (LA) cannot be endogenously synthesized in
mammals but are required for proper growth and development, thus they are considered essential fatty acids.\textsuperscript{10} Once ALA is ingested, mammals possess the requisite enzymes to convert ALA to its primary fatty acid metabolites EPA and DHA.

In contrast to the well-studied n-3 PUFA EPA and DHA, little is known about the biological effects of ALA. Supplementation studies using ALA-rich foods such as flaxseed (59% ALA/total fat)\textsuperscript{11} or chia seed (64% ALA/total fat)\textsuperscript{12} oils have reported that foods rich in ALA can confer similar metabolic benefits to those provided by EPA and DHA. However, the effects of ALA in preventing and treating disease remain controversial, as some studies have reported that ALA-rich diets do not provide any measurable health benefits,\textsuperscript{13} while other studies have argued that the benefits conferred through the consumption of ALA arise solely through ALA’s conversion to EPA and DHA.\textsuperscript{14} This last assertion has been made on numerous occasions, even in light of several tracer studies which have reported that PUFA metabolism is extremely limited, with approximately 0.2% of ALA converted to EPA and <0.1% converted to DHA in humans.\textsuperscript{15} Thus, it is currently unresolved if ALA has direct biological effects in ameliorating disease progression.

One way to firmly establish if ALA can provide health benefits independent of its conversion is to inhibit the metabolic conversion of ALA to EPA and DHA. The first enzyme in the PUFA metabolic pathway is Δ6 desaturase (D6D).\textsuperscript{16} This rate limiting enzyme is highly active in the liver,\textsuperscript{17} the primary site of PUFA metabolism, and responsible for adding an additional double bond to ALA to produce stearidonic acid (SDA; 18:4 Δ6,9,12,15). D6D also desaturates LA to produce γ-linolenic acid (GLA; 18:3 Δ6,9,12). Previously, the partial inhibition of the D6D enzyme was accomplished by pharmacological methods. This resulted in significantly lower EPA and DHA relative to control wild-type mice. However, due to the persistence of EPA and
DHA in tissues and possibility of unwanted metabolic side effects, pharmacological inhibition of D6D is not an optimal method to study the independent effects of ALA. In 2009, the Δ6 desaturase null (D6KO) mouse was developed as a genetic model to study the effects of specific PUFA in health and disease. The D6KO mouse lacks the FADS2 gene which encodes the D6D enzyme and is therefore unable to convert either ALA or LA to the usual n-3 and n-6 metabolites EPA, DHA and AA. If long chain n-6 or n-3 PUFA are not provided in the diet of D6KO mice, all body tissues are nearly void of these long chain fatty acids, making the D6KO mouse a fitting model to study the independent effects of ALA for the first time.

The overall aim of this work was to utilize the D6KO mouse to determine if ALA could independently delay the initiation and progression of risk factors associated with NAFLD. First, classic and recent studies which have evaluated the association between individual PUFA and NAFLD or CVD were reviewed to provide a thorough characterization and critical analysis of the metabolic effects of parent PUFA relative to their downstream metabolites. Following this literature review, the first study is presented. The goal of this study was to minimize diffuse changes in lipid metabolism associated with the removal of the D6D enzyme in order to focus exclusively on the effects of n-3 PUFA. Results of the first study were used to optimize the formula for diets used in the next study. In the second study, the effects of feeding D6KO mice ALA-rich diets on several risk factors for NAFLD, including steatosis, hepatic inflammation, glucose intolerance and abdominal obesity were evaluated. Together, both studies aim to provide a better characterization of the D6KO mouse, and advance scientific understanding of the biological function of ALA in allaying the progression of metabolic abnormalities associated with NAFLD.
Chapter Two

LITERATURE REVIEW

THE EFFECTS OF ESSENTIAL VS. VERY LONG CHAIN POLYUNSATURATED FATTY ACIDS ON NON-ALCOHOLIC FATTY LIVER DISEASE AND CARDIOVASCULAR DISEASE RISK AND PROGRESSION
2.1 Introduction

The biological benefits of polyunsaturated fatty acids (PUFA) have been extensively evaluated over the last several decades. Numerous studies have found that this family of fatty acids are involved in a wide array of cellular processes, from the regulation of gene transcription and lipid metabolism,\textsuperscript{8,19,20} to involvement in intracellular signalling pathways,\textsuperscript{21} and cytokine secretion.\textsuperscript{22} There are two main PUFA classes, the n-3 PUFA family including plant-derived α-linolenic acid (ALA), and ALA’s long chain metabolites eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA); and the n-6 family composed of linoleic acid (LA) and LA’s primary metabolite arachidonic acid (AA). ALA and LA cannot be produced endogenously in mammals and are classified as essential fatty acids (EFA);\textsuperscript{10} however, mammals possess the requisite enzymes to metabolize these EFA to EPA, DHA and AA, respectively.\textsuperscript{23} While both n-3 and n-6 PUFA share the same metabolic pathway, each family of fatty acids has been found to exert distinctly different and sometimes opposing\textsuperscript{24} biological effects.

One area of interest in the field of PUFA research is the association between various PUFA and metabolic diseases. Diseases of metabolic origin, such as cardiovascular disease (CVD), and non-alcoholic fatty liver disease (NAFLD), are highly prevalent in North American society, and account for the highest number of mortalities each year.\textsuperscript{2} However, these diseases are strongly associated with lifestyle, and so may be prevented and even treated through changes in diet, increased exercise, and modifications of other lifestyle factors.\textsuperscript{25} In particular, increased consumption of EPA and DHA has been shown in numerous studies to improve the prognosis of several metabolic diseases including CVD and NAFLD,\textsuperscript{26-31} while raised levels of AA have been associated with cardiovascular complications.\textsuperscript{32,33}
In contrast with the well-studied roles of AA, EPA and DHA in disease initiation and progression, the independent effects of ALA and LA on disease outcomes are relatively unknown. As the average North American does not consume large quantities of EPA and DHA-rich fish products, but does consume substantial amounts of ALA and LA-rich vegetable oils, (Table 2.1) it is of critical importance to further investigate the biological properties of ALA and LA. Currently, it has been surmised that ALA and LA do not act independently, but exert their effects entirely through conversion to their bioactive metabolites. In opposition to this theory, human PUFA conversion efficiency is very low\(^\text{15}\) and most dietary ALA and LA is not converted into EPA, DHA and AA, respectively. Thus, there is a possibility that effects seen in ALA or LA supplementation trials could be due to the direct effects of these EFA, which make up a larger proportion of lipid stores relative to EPA, DHA and AA.

The purpose of this report is to review the existing literature which evaluates the effects of the EFA ALA and LA, and the effects of their downstream metabolites EPA/DHA and AA, on both NAFLD and CVD in human and animal studies. The overall goal is to highlight the shortage of current research which can provide compelling evidence of the independent effects of LA or ALA on NAFLD and CVD while suggesting that these two fatty acids do indeed have biological effects worth future investigation.
### Table 2.1: Key polyunsaturated fatty acids in the North American food chain

<table>
<thead>
<tr>
<th>Polyunsaturated Fatty Acid</th>
<th>Common Sources</th>
<th>AI/AMDR †</th>
<th>Average N. American Daily Intake*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Linoleic Acid (LA)</td>
<td>Safflower oil, Corn oil, Sunflower oil</td>
<td>12-17g/day; 5-10% of Energy</td>
<td>14.5g/day 6.5% of Energy</td>
</tr>
<tr>
<td>Arachidonic Acid (AA)</td>
<td>Eggs, Meat Products</td>
<td>~10% total n-6 intake</td>
<td>0.13g/day 0.06% of Energy</td>
</tr>
<tr>
<td>α-linolenic Acid (ALA)</td>
<td>Flaxseed oil, Walnuts, Chia seeds</td>
<td>1.1-1.6g/day; 0.6-1.2% of Energy</td>
<td>1.4g/day 0.6% of Energy</td>
</tr>
<tr>
<td>Eicosapentaenoic Acid (EPA)</td>
<td>Oily fish, Algal oil, Fortified foods</td>
<td>0.5g/day EPA+DHA** ~10% total n-3 intake</td>
<td>0.04g/day 0.02% of Energy</td>
</tr>
<tr>
<td>Docosahexaenoic Acid (DHA)</td>
<td>Oily fish, Algal oil, Fortified products</td>
<td></td>
<td>0.07g/day 0.03% of Energy</td>
</tr>
</tbody>
</table>

NOTE: †Adequate Intake/Acceptable Macronutrient Distribution Range 2005; *NHANES 2007-2008 ages 2+; % energy based on 2000 calories/day ** Dietitians of Canada/American Dietetic Association 2007

#### 2.2 Polyunsaturated fatty acid metabolic pathway

Mammals are unable to endogenously synthesize ALA (18:3n3) and LA (18:2n6), and must acquire these two essential fatty acids (EFA), exclusively through diet. Once ingested, mammals are capable of metabolizing these two EFA by alternately desaturating and elongating the carbon tail to produce highly unsaturated fatty acids (HUFA) such as AA, EPA and DHA.

Once ALA and LA are digested and absorbed, they can either A) be oxidized to produce ATP via the B-oxidation pathway; B) be used as energy storage molecules (e.g. triglycerides) or for structural purposes (phospholipids, sphingolipids, etc.), or C) they can undergo a series of
desaturations and elongations to produce long chain highly unsaturated fatty acids (HUFA).\textsuperscript{37}

EFA desaturation involves creating an additional double bond in the carbon chain of a FA, and is accomplished by either $\Delta_6$ desaturase (D6D) or $\Delta_5$ desaturase (D5D). EFA elongation is carried out by elongases which extend the carboxyl end of the EFA by an additional two carbons. The n-3 and n-6 fatty acid pathways share the same metabolic pathway, meaning both fatty acid groups compete for the same set of desaturase and elongase enzymes.

The n-3 metabolic pathway begins when ALA (18:3n-3) is converted into stearidonic acid (18:4n-3) by D6D (Figure 4.1). An elongase then acts on stearidonic acid to form eicosatetraenoic acid (20:4n-3), followed by further desaturation by D5D. The product of this reaction is eicosapentaenoic acid (EPA, 20:5n-3). EPA can be metabolized by cyclooxygenase (COX) and lipoxygenase (LOX) to form various eicosanoids such as: resolvins, the 3-series prostaglandins and 5-series leukotrienes.\textsuperscript{38} It can also be further metabolized, first undergoing two cycles of elongation to produce tetracosapentaenoic acid (24:5n-3), followed by desaturation to produce tetrahexaenoic acid (24:6n-3), and finally desaturation by Acyl CoA desaturase followed by $\beta$-oxidation to produce docosahexaenoic acid (DHA, 22:6n-3). DHA represents the end of the n-3 fatty acid pathway, but it is a precursor for the production of protectins, resolvins and docosanoids.\textsuperscript{39}

The n-6 pathway begins with LA (18:2n-6) which is desaturated by D6D to produce $\gamma$-linolenic acid (18:3n-6), then elongated to produce homo- $\gamma$-linolenic acid (20:3n-6) (Figure 4.1). Another double bond is formed in homo- $\gamma$-linolenic acid by D5D to produce arachidonic acid (AA, 20:4n-6). Like EPA, AA can also be metabolized by COX and LOX enzymes to produce eicosanoids such as the 2-series prostaglandins and thromboxanes, 4-series leukotrienes, and lipoxins.\textsuperscript{40} AA can also be metabolized by NADPH-dependent cytochrome P450
monooxygenase to produce epoxyeicosatrienoic acids (EETS), 20-hydroxyeicosatetraenoic acids (20-HETE’s), and dihydroxyeicosatrienoic acids (DHET’s). Many AA derived metabolites are produced in response to injury and were classically considered pro-inflammatory, but recent research has suggested that not all AA metabolites can be lumped into this classification. AA can also continue through the fatty acid metabolic pathway, being elongated, desaturated and oxidized to eventually form docosapentaenoic acid (DPA, 22:5n-6).

For the average North American, dietary LA concentrations are much higher than ALA, therefore the production of AA exceeds the production of n-3 downstream products in this competitive pathway. There is also poor conversion of the starting EFAs to their respective HUFAs in humans. Tracer studies have suggested that between 0.2% and 2% of LA is converted into AA. Similar results have been reported in tracer studies with ALA, as ~0.2% is converted to EPA and <0.1% is converted to DHA. One suggestion for this low conversion rate is that the D6D enzyme is rate limiting; therefore, there is a bottleneck in the conversion of the parent fatty acids to their downstream fatty acid products. The rate limiting property of D6D was also shown in rodent studies, but D6D’s efficiency rate varies between species.

2.3 Recommended Fat Intakes in North America

Several organizations regularly publish reports on recommended daily macronutrient intake values. The Acceptable Macronutrient Distribution Range (AMDR) for Energy, Carbohydrate, Fibre, Fat, fatty Acids, cholesterol, Protein and Amino Acids (2005) recommends 20-35% of daily energy for adults is provided by fats. The AMDR for n-6 fatty acids for adults is between 5-10% of total energy, while the DRI for n-3s/ALA is 0.6-1.2%. 10% of total
n-3 and n-6 intake can come from the consumption of long chain PUFA such as eicosapentaenoic acid (EPA, 20:5n-3) and docosahexaenoic acid (DHA, 22:6n-3, and arachidonic acid (AA, 20:4n-6). The American Heart Association recommends that SFA not exceed 7% of energy, while trans-fat intake should be <1% to prevent cardiovascular disease (Table 2.1).

As reported in the latest Canadian Community Health Survey (Cycle 2.2, 2004) adult mean fat intake (% of total energy intake) is 31.4%. Canadians consume, on average, 4.4% LA and between 0.77-0.82% ALA. This is equivalent to 9.2g/day LA and 1.68g/day ALA for women, or 12.5g/day LA and 2.18 g/day ALA for men. Sources of LA in the western diet include vegetable oils such as safflower oil (74g/100g), soybean oil (~53.4g/100g), and sunflower oil (~60.2g/100g), while rich sources of ALA are found in flaxseed oil (~59g/100g), soybean oil (~7.8g/100g), canola oil ~(9.2g/100g), and walnuts (~6.8g/100g).

2.4 Polyunsaturated fatty acids and Non-Alcoholic Fatty Liver Disease
NAFLD encompasses a spectrum of liver pathologies from simple steatosis, to necro-inflammatory steatohepatitis, to severe liver fibrosis and cirrhosis. Steatosis is the most common pathological feature of NAFLD, and is defined as the excessive build-up of lipids within hepatocytes. Dietary fat, de novo lipid synthesis, and the uptake of circulating free fatty acids produced by adipose tissue all contribute to hepatic lipid accumulation in individuals with NAFLD. According to the “two-hit hypothesis” of NAFLD pathology, for simple steatosis to progress to non-alcoholic steatohepatitis (NASH) a second metabolic “hit” consisting of oxidative stress must occur. This oxidative stress is associated with increased peroxidation of
lipids of the hepatocyte membrane and increased release of pro-inflammatory cytokines which initiate hepatic fibrosis. As a result, individuals diagnosed with NASH, the more progressive form of NAFLD, exhibit hepatic inflammation and necrosis in addition to steatosis, in the presence or absence of fibrosis.\textsuperscript{52}

There are several risk factors which can lead to the development of steatosis including obesity, insulin resistance, total parenteral nutrition, rapid weight loss, choline deficiency, and protein malnutrition;\textsuperscript{53} however, increased visceral adiposity and insulin resistance are most commonly associated with the development of NAFLD. Additionally, recent studies have identified a number of gene polymorphisms which are associated with the severity of NAFLD.\textsuperscript{54} Many of these are single nucleotide polymorphisms (SNPs) and are found in areas coding for enzymes associated with fatty acid metabolism, highlighting the important role of fatty acid metabolism in the development of NAFLD.

\textit{2.4.1 EPA and DHA in the prevention and treatment of NAFLD}

The liver is the primary site of lipid metabolism and also produces several inflammatory cytokines. N-3 PUFA directly influence hepatic lipid metabolism through the regulation of gene transcription factors\textsuperscript{19} such as peroxisome proliferator activated receptor alpha (PPAR-\(\alpha\)),\textsuperscript{19} and sterol regulatory element binding protein (SREBP).\textsuperscript{20} DHA and EPA activate PPAR-\(\alpha\)\textsuperscript{8} which up-regulates the expression of genes involved in fatty acid oxidation, desaturation and transport. Indeed, activation of PPAR-\(\alpha\) using the pharmacological agonist Wy 14 643 has been shown to prevent and even reverse experimental NASH.\textsuperscript{55} In the liver, DHA and EPA also inhibit SREBP-1c from up-regulating genes such as fatty acid synthase, and acetyl CoA carboxylase,\textsuperscript{8,56} which are all involved in fatty acid synthesis. Additionally, EPA has been shown to modulate the production of inflammatory cytokines by hepatocytes.\textsuperscript{57} Overall, DHA and EPA increase
fatty acid oxidation, decrease lipogenesis, and reduce inflammatory cytokine production in the liver. Thus, several recent supplementation trials as documented below have been conducted in order to evaluate if the provision of n-3 HUFA can effectively prevent the progression of NASH through modifying lipid profiles, and reducing inflammation.

2.4.2 EPA and DHA: effects on cholesterol and triglycerides

The most well established role of n-3 HUFA is in the reduction of circulating triglyceride (TG) levels\textsuperscript{6,58,59} as EPA and DHA have varied effects on circulating cholesterol, increasing high density lipoprotein (HDL) but also low density lipoprotein (LDL) cholesterol levels.\textsuperscript{59-61} Cross sectional studies of populations with NASH, the progressive form of NAFLD, have reported that individuals with NASH have significantly lower HDL cholesterol.\textsuperscript{62,63} As individuals with NASH have also been found to have lower hepatic HUFA content, several recent supplementation studies have investigated if additional dietary n-3 HUFA can improve lipoprotein profiles in individuals with NAFLD. In one study conducted on 6 individuals with sonography-confirmed NAFLD,\textsuperscript{64} a daily intake of olive oil enriched with 0.83g n-3 HUFA for 12 months resulted in significant increases in HDL levels relative to baseline values and to control NAFLD patients who simply received olive oil supplements. Additionally, n-3 supplementation also resulted in significant reductions in serum TG. Another larger study,\textsuperscript{65} which provided NAFLD patents with an AHA approved diet and 6g/day of seal oil (n=66) found that after 24 weeks of treatment, patents receiving n-3 rich seal oil had significantly decreased LDL and increased HDL levels from baseline. While the same effect on HDL levels was also seen in the control NAFLD group which received an AHA diet alone, the changes in LDL levels were significant only in the n-3 treatment group. NAFLD patients in the n-3 treatment groups
also demonstrated improvements in their overall steatosis score and saw a significant reduction in serum TG levels from baseline and control values. Similarly, two other small scale supplementation trials found that n-3 HUFA supplementation lead to significant improvements in circulating lipoprotein and TG levels,\textsuperscript{66,67} while one study found no effects of n-3 HUFA supplements on lipoprotein levels.\textsuperscript{68} However, the findings of this last study may have been limited by a relatively short treatment duration of eight weeks. Thus, recent evidence from human randomized control trials suggests that increased intake of n-3 HUFA can improve both lipoprotein and TG levels specifically in patents with established NAFLD.

Animal models have also been used to model the NAFLD and NASH phenotypes seen in humans, and to determine if n-3 HUFA supplementation can prevent and treat this disease. Studies using diets depleted of n-3 PUFA have reported resultant increases in cholesterol levels,\textsuperscript{69,70} while studies where n-3 HUFA are supplemented to rats fed high-fat diets initially void of n-3 HUFA have found that n-3 supplementation is sufficient to restore hepatic cholesterol levels to control levels.\textsuperscript{71}

\textbf{2.4.3 EPA and DHA: Effects on inflammation}

Progression of NAFLD is characterized by increased hepatic inflammation. Numerous studies have found EPA and DHA to have anti-inflammatory properties, thus there has been recent interest in the efficacy of DHA and EPA to reduce hepatic inflammatory cell infiltration and cytokine production. Liver biopsies provide the best evidence of the severity of hepatic inflammation but may be challenging to acquire, thus serum markers of inflammation are generally used to determine the effects of dietary supplementation. In one pilot supplementation trial with 23 biopsy proven NASH patents, the provision of 27g/day of a purified EPA
supplement for 12 weeks resulted in significant reductions in serum markers of oxidative stress including TNF-R1 and 2, serum ferritin and thioredoxin as well as a significant decrease in ALT compared with baseline values. Additionally, 6 out of 7 patients who provided liver biopsies at the end of the trial showed improved steatosis and lobular inflammation. This led the authors to conclude that EPA could effectively be used in the treatment of NASH. However, no controls were present in this pilot trial so results should be interpreted accordingly. In another supplementation study with 36 NAFLD patients, 2g/day of PUFA for 6 months resulted in a significant decrease in TNF-α. However, while the trial was based on previous literature which had cited the benefits of n-3 HUFA, the composition of the PUFA supplement was not provided. One other supplementation trial found no changes in serum markers of oxidative stress after one year in 6 NAFLD subjects provided with a daily supplement of n-3 HUFA, however these patients did have a significant increase in adiponectin levels from baseline.

Overall, while n-3 HUFA are considered anti-inflammatory, only a few small trials have evaluated the potential anti-inflammatory and anti-oxidant effects of n-3 HUFA on patients with NAFLD, with mainly positive results. Currently, larger randomized control trials evaluating n-3 PUFA supplementation on the amelioration of NAFLD/NASH are in progress. Results of these trials may provide greater mechanistic insight on the association between long chain n-3 PUFA, hepatic inflammation and NAFLD.

Parallel to human studies, some, but not all rodent NAFLD studies have found that n-3 HUFA protect against and improve hepatic and systemic inflammation. Recent studies using rodent models of induced NAFLD have found that EPA+DHA supplementation can significantly decrease hepatic TNF-α, interleukin-6 (IL-6), and circulating markers of oxidative stress such as ferritin, 8-isoprostane and reduce the formation of reactive oxygen species, when
compared to non-supplemented animals. Additionally, DHA supplementation was found to increase plasma activity of superoxide dismutase (SOD),\textsuperscript{77} a key antioxidant enzyme, when provided to rats on a high-fat choline deficient diet. However, not all studies have found long chain n-3 PUFA to be anti-inflammatory. In one such study using a methionine-choline deficient model of NAFLD, DHA and EPA supplementation was found to decrease steatosis through the activation of PPAR-\(\alpha\), but exacerbated hepatic inflammation via increased lipoperoxide accumulation and related hepatocellular damage.\textsuperscript{78} To explain this contradictory result, it is important to recognize that while n-3 HUFA may stimulate an anti-inflammatory cytokine profile, long chain HUFA molecules are more readily oxidized than shorter chain fatty acids with few or no double bonds. In general, many but not all studies have found that n-3 HUFA can protect against systemic and hepatic inflammation in rodent models of NASH by decreasing pro-inflammatory proteins, while increasing systemic anti-oxidant capacity.

2.4.4 ALA: effects on cholesterol and triglycerides

The effect of ALA-rich oils on cholesterol reduction in individuals with NAFLD has not been described; however, several animal studies have evaluated the effects of ALA-rich diets on hepatic cholesterol. A number of these studies found that flaxseed and flaxseed oil could effectively reduce total serum and/or hepatic cholesterol.\textsuperscript{79} Indeed, a recent mouse study comparing flaxseed oil, fish oil and safflower oil diets found that ALA-rich flaxseed oil could lower total and non-HDL cholesterol to the same degree as fish oil despite the fact that flaxseed-fed mice had significantly less EPA and DHA in most liver lipid fractions.\textsuperscript{11} However, other animal studies have reported that the provision of ground flaxseed had no effect on cholesterol levels,\textsuperscript{80,81} or have suggested that the hypocholesterolemic effects of flaxseed are not associated
with the fat content of flaxseed. Together, animal studies have not provided conclusive evidence to suggest that ground flaxseed and ALA-rich flaxseed oil can effectively reduce hepatic cholesterol synthesis and accumulation in the context of fatty liver disease.

2.4.5 ALA: effects on inflammation

Observational studies have found inverse correlations between ALA and plasma markers of inflammation, leading to an interest in ALA’s association with inflammation in the context of metabolic disorders. In one small study examining the anti-inflammatory effects of ALA in healthy young subjects, provision of flaxseed oil for four weeks lead to a significant decrease in TNF-α and IL-6β production in mononuclear cells. However, four additional weeks of supplementation with fish oil produced a more profound decrease in production of both inflammatory proteins. This led the study authors to conclude that the beneficial effects of flaxseed oil supplementation were due to associated increases in cellular EPA content, as a dose response effect could be observed between cellular EPA and inflammatory protein production. In another study, evaluating the effects of a mixed PUFA diet, a 17% PUFA diet containing ~2:1 ratio of LA:ALA was associated with a significant reduction in C-reactive protein (CRP) compared to a diet with a Western style PUFA ratio. However, as the PUFA content and sources were mixed (walnuts, walnut oil and flaxseed oil were used), it is difficult to attribute effects exclusively to ALA.
2.4.6 **EPA/DHA vs. ALA and NAFLD**

While there is solid evidence that long chain n-3 PUFA can lower TG accumulation in the liver and circulation, the role of individual n-3 PUFA in modulating lipoprotein levels and inflammation in the liver is less clear. Many human studies have provided evidence that long chain n-3 PUFA can improve lipoprotein profiles; however, the efficacy of ALA-rich oils to improve lipoprotein profiles in humans has not yet been evaluated. Recent interest in the anti-inflammatory and lipid-lowering effects of n-3 HUFA has led to several small pilot studies which have evaluated n-3 HUFA supplementation in individuals with NAFLD/NASH. Many of these studies have concluded that n-3 HUFA can directly modify liver lipid metabolism and inflammation. In contrast, while an inverse association between ALA status and certain inflammatory markers has been reported, there is a lack of evidence which supports a direct effect of ALA in preventing or resolving inflammation. Furthermore, studies that have found beneficial effects of ALA in the context of NAFLD often suggest that these effects are solely due to the conversion of ALA to EPA and DHA. Finally, recent reviews which have attempted to determine the effects of n-3s on NAFLD and other associated metabolic diseases have focused primarily on the effects of marine-derived long chain n-3 PUFA. \(^\text{86,87}\) Thus, the direct effects of ALA in preventing NAFLD, while challenging to discern, are in need of careful evaluation to validate claims that all n-3 PUFA may beneficially modulate lipid profiles and inflammation.

2.4.7 **n-6 PUFA in the prevention and treatment of NAFLD**

Few studies have evaluated the effects of n-6 fatty acids on the development and progression of NAFLD. Studies evaluating the hepatic fatty acid levels of patients with NASH have determined that these patients have a hepatic LA content comparable with healthy
individuals, but have lower hepatic levels of AA.\textsuperscript{62,88,89} NASH patients then have lower n-6 product/precursor ratios compared with a healthy population, trends driven mainly by the change in AA levels.

This shift in hepatic n-6 content does not seem to be due to differences in diet, as in one study, the self-reported dietary intake of LA and AA in NASH and non-NAFLD populations did not differ.\textsuperscript{62} To further validate reported dietary intake, RBC fatty acid content was also measured, and did not differ significantly between study populations. The study authors suggested that discrepancies between dietary and hepatic fatty acid content in NASH patents could be caused by dysfunction of D6D or D5D leading to perturbations in PUFA metabolism. Enzyme activities were not measured in this study; therefore, future studies are required to determine the mechanisms responsible for the changes in PUFA levels observed in NASH patients.

The decrease in hepatic AA seen in NASH patients is especially surprising, as AA is generally associated with inflammation, and inflammatory cell infiltration differentiates NASH from simple steatosis. The findings of these cross sectional studies then appear to contradict the traditionally held belief that AA levels are associated with increased inflammation.

Several animal studies have attempted to evaluate the effects of dietary LA and AA in NAFLD. One study utilized mice lacking the liver-specific cytochrome P450 reductase gene which consequently led to a NAFLD-like phenotype. When fed a standard chow diet, these mice accumulated hepatic LA and ALA, while a decrease in long chain HUFA was observed.\textsuperscript{90} This observation is similar to what is observed in human subjects with NAFLD.
In another rodent study, the accumulation of saturated fat via exogenous sources (high SFA diet) or increased de novo synthesis (high sucrose diet) played a significant role in the development of fatty liver disease, while the provision of a high LA diet did not lead to the development of NAFLD. In contrast, a study evaluating the effects of a high monounsaturated fatty acid (MUFA) diet, a high n-6 (LA) diet, or a high n-3 (EPA+DHA) diet on hepatic fibrosis in hypercholesterolemic rabbits found that only the MUFA and n-3 rich diets were able to prevent fibrosis. However, animals fed a high n-6 or high n-3 diet had significantly lower total hepatic cholesterol and cholesterol esters, and tended to have lower circulating cholesterol levels compared with rabbits on the MUFA diet. In this study there was no clear distinction made between long chain and short chain PUFA, but all three unsaturated fat diets resulted in equivalent liver AA contents, suggesting that the effects of the n-6 diet were primarily due to LA. Overall, the individual effects of LA vs. AA, or ALA vs. EPA/DHA could not be firmly determined from either study.

Few studies have separately evaluated LA and AA intake in relation to NAFLD progression. One such mouse study attempted to determine if AA supplementation could prevent steatosis induced by conjugated linoleic acid supplementation. A control diet enriched in LA was also evaluated. The provision of either an LA-enriched diet, or a CLA-enriched diet supplemented with AA resulted in the prevention of liver lipid accumulation which otherwise occurs when mice consume diets enriched exclusively in CLA. Thus, this study demonstrated that AA could effectively prevent steatosis; however, clear conclusions could not be drawn about the preventative effects of LA.
2.4.8 AA vs. LA and NAFLD

The individual effects of LA and AA supplementation on NAFLD have not undergone extensive evaluation; therefore, firm conclusions on the association between n-6 PUFA and NAFLD cannot be drawn. Human studies have found statistical associations between changes in n-6 product to precursor ratios which suggest abnormal PUFA metabolism/desaturation, while animal studies have provided evidence that LA-enriched diets are more protective against NAFLD compared with diets rich in carbohydrates or SFA, but not in comparison with n-3-enriched diets.

2.5 Polyunsaturated Fatty Acids and Cardiovascular Disease

While NAFLD has only emerged in the last few decades as a nationwide concern, CVD has long been the leading cause of mortality in the West. Individuals diagnosed with NAFLD have an increased risk of developing CVD, as similar metabolic risk factors are responsible for the development of both diseases. CVD is an umbrella term for the many diseases which affect the heart and blood vessels. The major cause of CVD in the Western world, atherosclerosis, is characterized by the narrowing of the arterial lumen caused by the build-up of oxidized lipoproteins and cholesterol on the blood vessel walls. This fatty build-up leads to the movement of inflammatory cells and matrix proteins into the arterial intima, and smooth muscle cell proliferation within the vessel wall.

Classical risk factors for CVD include mean serum cholesterol levels, high blood pressure and smoking. Some newer risk factors and biomarkers associated with CVD include development of NAFLD, hypertriglyceridemia, glucose intolerance and insulin resistance.
raised levels of pro-inflammatory cytokines, and increased levels of various soluble adhesion molecules.

The relation between lipoproteins and CVD has also been well established, and serum levels of each fraction are regularly used to determine CVD risk. Serum HDL concentrations are inversely associated with atherosclerosis. An HDL level of <40mg/dl in men and <50mg/dl in women is considered one risk factor for metabolic syndrome (MetS) and CVD. Conversely, LDL particles, and especially small dense LDL are considered atherogenic. This is mainly because LDL particles are more susceptible to oxidation, to arterial proteoglycan binding, and to permeation of the arterial endothelium, critical steps in the progression of atherosclerotic plaques. Very low density lipoproteins (VLDL), and especially large VLDL are also positively associated with cardiovascular disease.

Since the quality of an individual’s diet plays an important role in the occurrence and development of CVD, many studies have evaluated the relationship between dietary fat and the incidence and progression of CVD. One area of interest and ongoing controversy has been in determining the effects of specific n-6 and n-3 fatty acids on CVD risk.

2.5.1 EPA and DHA in the prevention and treatment of CVD

The cardiovascular benefits of long chain n-3 PUFA EPA and DHA have been thoroughly evaluated through numerous observational studies and clinical supplementation trials. In one large observational study of 11 323 adults with previous histories of myocardial infarction, it was concluded that 1g/day n-3 PUFA was associated with a distinct reduction in total mortality, and CVD-associated mortality. A follow-up study on patients with chronic heart

22
failure found similar results. To further explore the effects of n-3 HUFA, 20 healthy volunteers were infused with either an n-6 or n-3 HUFA emulsion for 48 hours in a randomized crossover designed study. The n-3 infusion resulted in significantly lower TNF-α and IL-8 release from monocytes, and suppressed monocyte recruitment process. N-3 HUFA infusion was then able to suppress aspects of the endothelial inflammatory response associated with the development of atherosclerosis. Several other intervention and population studies have found that consuming either DHA/EPA supplements or n-3 rich fatty fish have anti-inflammatory and cardio-protective effects.

Controversy on the exact benefit of long chain n-3s does exist, as several studies have found no effect of DHA and/or EPA supplementation on CVD endpoints. Indeed, a recent large systematic review of over 80 clinical trials and cohort studies on the efficacy of n-3 PUFA to prevent CVD, cancer and all-cause mortality concluded that there was no clear protective effect of n-3 PUFA. However, several methodological concerns exist about data pooling and study inclusion criteria used in this review. Contrary to this first meta-analysis, another concurrent systematic review conducted by Wang et al. found that n-3 HUFA did reduce rates of CVD-related mortality and all-cause mortality. More recent meta-analyses have added to the debate about the effects of n-3 HUFA-rich foods or supplements on lowering CVD-related mortality, as many have found significant effects, while others have found moderate or negligible association. Overall, strong evidence exists that long chain n-3 HUFA can improve several risk factors associated with CVD, but controversy exists whether EPA and DHA can directly contribute to decreased CVD-related mortality.

Animal studies have provided strong evidence of the cardio-protective effects of DHA/EPA. In both genetic and experimentally induced rodent models of CVD or MetS, n-3
HUFA supplementation has been shown to reduce circulating TG levels,\textsuperscript{116,117} total cholesterol,\textsuperscript{116-118} and weight gain.\textsuperscript{118,119} The modulation of circulating lipid levels then represents a key mechanism by which long chain n-3 PUFA can prevent the development of CVD.

Additionally, studies with rodent models have supported the anti-inflammatory effects of n-3 HUFA seen in human studies. In one study,\textsuperscript{120} the addition of EPA to a Western style diet provided to Apolipoprotein E knockout (ApoE KO) mice resulted in lower production of several macrophage recruitment and adhesion molecules. This then led to lower macrophage count in aortic lesions, and significantly less aortic plaque formation compared to ApoE KO mice on a standard Western diet. The effects of EPA were suppressed when the expression of PPAR-\(\alpha\) was silenced, suggesting that EPA provides anti-inflammatory effects through activation of PPAR-\(\alpha\). Other rodent studies have found that EPA/DHA can reduce lipid peroxidation,\textsuperscript{116} and increase the release of adiponectin,\textsuperscript{22} an anti-inflammatory adipokine. Thus, several studies utilising rodent models have found that EPA and DHA reduce vascular inflammation, and so provide protection against the development and progression of atherosclerosis.

\textbf{2.5.2 ALA in the prevention and treatment of CVD}

As with studies evaluating the association between ALA and NAFLD, many cardiovascular studies on the benefits of ALA suggest that this fatty acids protective effects derive simply through its conversion to EPA/DHA,\textsuperscript{39,121} or though modulating the production of AA. However, recent arguments that ALA may also have effects independent of its conversion
have emerged, but studies clearly demonstrating the independent effects of ALA on the prevention and treatment of CVD are lacking.

In the 1990s, authors of the Multiple Risk Factor Intervention Trial (MRFIT) reported that increased ALA in the serum cholesterol ester and phospholipid (PL) fractions was independently associated with a significant decrease in the risk of stroke in middle aged men at high risk of CVD. Other epidemiological studies have reported similar findings. Randomized control trials evaluating the effects of ALA on inflammation and lipid regulation in the context of CVD have yielded mainly positive results. A number of supplementation trials using ALA-rich oils have found that ALA intake is associated with reduced risk of acute cardiovascular events and lower concentrations inflammatory mediators such as CRP and IL-6. Supplementation studies using whole flaxseeds have also found that groups receiving the supplement have lower total and LDL cholesterol, but as whole flaxseeds are also rich in lignans and fibre, it is difficult to attribute results directly to the effect of ALA. However, a few studies have reported no change on measures of CVD risk after ALA supplementation and when compared with a control LA rich diet. Thus, while the majority of evidence suggests that increased intake of ALA-rich foods can confer cardiovascular benefits, controversy remains about the direct benefits of ALA.

Animal studies, especially those utilizing flaxseed as the source of ALA, have generally determined that ALA supplementation is associated with reduced CVD risk. In one study conducted in hypercholesterolemic rabbits, flaxseed supplementation was found to significantly reduce the rate of aortic plaque formation after 8 and 12 weeks of supplementation, but these effects diminished after 16 weeks. The same research group also reported that in LDL-r null mice, which are prone to developing atherosclerosis, adding ground flaxseed reduced fatty
streak formation, inflammation, and lowered serum cholesterol. A recent study, also utilizing the LDL-r null mouse determined that it was specifically the ALA contents of flaxseed that protected against the development of atherosclerosis when mice were challenged with a diet rich in cholesterol and trans-fatty acids. While this last study design allowed the authors to attribute results of flaxseed supplementation to ALA, animal studies, like human trials, are not able to provide evidence for the direct effects ALA in the presence of EPA and DHA.

2.5.3 EPA/DHA vs. ALA and CVD

In summary, long chain n-3 PUFA beneficially modify several risk factors associated with CVD, including vascular inflammation and the profile of circulating lipids. ALA-rich foods have also been associated with favorable cardiovascular outcomes, both in epidemiological studies and clinical trials. However, it remains unknown about how ALA may provide cardiovascular benefits, whether through similar mechanisms as EPA and DHA, or through conversion to EPA and DHA.

2.5.4 LA and CVD

In the last century LA consumption has risen in the western world as vegetable oils replaced the use of animal fats in cooking. While LA is an n-6 PUFA, which is generally not recommended for individuals at risk of cardiovascular complications, many studies have found inverse associations between circulating LA and CVD outcome events.

Studies assessing the association between LA and lipoprotein concentrations have determined that, in general, LA beneficially modifies lipoprotein levels, but causes increased
LDL oxidation. Using serum from 519 participants from the Framingham Heart Study and other studies, Siguel measured total cholesterol, HDL levels and plasma PUFA concentrations. The study authors found that both n-6 and n-3 PUFA were inversely associated with the total cholesterol/HDL ratio; however, the association between individual n-6 PUFA and cholesterol concentrations was not discussed. In the ERA-JUMP study serum samples from 1098 males of American, Japanese American, Japanese and Korean heritage were used to assess the association between LA and different classes of lipoproteins. LA was found to be inversely associated with concentrations of large VLDL, total LDL, small LDL, and LDL size; and positively associated with concentrations of large HDL, a finding supported by other studies. Overall, this study suggested that LA was positively associated with healthy lipoprotein levels, across multiple ethnicities. LA has also been found to be inversely associated with plasma TG levels. However, several studies have found a positive association between serum LA and oxidation velocity of atherogenic VLDL and LDL.

Animal studies have also suggested that LA-rich oils may not be atherogenic, especially in relation to SFA-rich or MUFA-rich diets. In one study, LDL-receptor null mice fed a safflower oil-enriched diet developed significantly smaller atherosclerotic plaques relative to mice on a high SFA-diet. Another rodent study comparing the effects of either LA or oleic acid enriched diets in LDL-receptor null mice found that treatment with LA resulted in lower lipid accumulation and smaller vascular lesions, but increased oxidative stress.

Cell studies have developed possible mechanisms for an LA induced increase in HDL. In a study by Pandey et al., PL enriched with one or more LA molecules were able to promote production of apolipoprotein A-1 (ApoA-1), the major component of HDL particles, from primary human hepatocytes. The LA enriched PL seemed to work through mitogen-activated
protein kinase stimulation of nuclear receptor peroxisome proliferator activated receptor alpha (PPAR-α), which would then activate increased synthesis of apoA-1.

Several studies have found LA levels to be inversely related to markers of systemic inflammation. Studies have found that LA was inversely associated with CRP, and directly associated with anti-inflammatory adiponectin.\textsuperscript{150} Of interest, one recent supplementation study reduced the dietary LA component of 22 subjects from 12\% to 4\% of total calories at a constant ALA intake and changes in inflammatory markers were evaluated at 4 weeks. In this study, no changes were observed in inflammatory profiles. Other studies have also concluded that circulating LA is not associated or inversely associated with inflammation in the context of CVD.\textsuperscript{136}

A significant argument against dietary LA is that increased consumption of this essential fatty acid may outcompete ALA and result in decreased production\textsuperscript{151} and incorporation of EPA and DHA into cellular PL.\textsuperscript{152,153} This in turn might inhibit the cardio-protective effects otherwise conferred by DHA and EPA. Subsequent studies have shown this is not the case. For example, a fish oil supplementation study determined that addition of LA to the diet did not affect the TG lowering properties of fish oil.\textsuperscript{154} In fact, studies have found that LA may in fact act synergistically with ALA and its metabolites to reduce the risk of inflammation, atherosclerosis and cardiovascular disease.\textsuperscript{155,156}

In summary LA, the primary n-6 PUFA, does not seem to directly increase the risk of CVD, nor has strong evidence been produced that LA speeds the progression of CVD. Additionally, most studies which have evaluated LA content or intake have not concurrently accounted for AA content.
2.5.5 AA and CVD

There have been few AA supplementation trials with human subjects. One early trial, conducted by Seyberth et al. provided a large dose of 6g/day ethyl arachidonate to 4 healthy male volunteers to determine the effects of an increase in dietary AA. After 3 weeks this dosage led to a significant increase (from 10% to 60%) in in vitro of platelet aggregation, leading the study authors to prematurely conclude the study. This pilot study led to an avoidance of human AA feeding trials for many years due to ethical concerns. More than two decades after this initial study, a new AA feeding study was initiated. In this study, a dose of 1.7g/day AA was provided to 10 healthy young men, which was several fold higher than the mean US intake of 0.13g/day AA. After 150 days, study subjects showed a significant increase in the production of both pro-inflammatory thromboxane A2 (TXA2) and anti-inflammatory PGI2. This finding was contrary to the findings of Seyberth et al.; however, the AA dosage during this trial was also significantly lower than the AA supplement provided to the subjects in Seyberth’s study. Finally, 1.7g/day of AA did not cause a significant change in blood cholesterol and lipoprotein distribution. Overall, the authors concluded that modest supplementation of AA does not have adverse health effects on healthy young to middle aged men.

While this supplementation study found that AA had no effect of cholesterol, several epidemiological studies have found positive associations between AA and LDL levels. Additionally, many studies have also found significant positive correlations between serum or RBC AA and myocardial infarction, stroke mortality, and hypertension.

Animal studies have also provided evidence of AA-associated increases in total cholesterol and TG. In one study with male hamsters, AA supplementation was found to be
associated with increased circulating TG levels, while supplementation with other FA such as LA or EPA had no effect on TG concentration. A more recent FA supplementation study\textsuperscript{163} in mice found that mice consuming a diet enriched with 15.5\% AA by volume had higher total cholesterol, TG, free fatty acids and LDL levels compared with mice on control LA-rich soybean oil diets. These mice also showed increased transcription of the \textit{ob} gene, and increased weight gain, positively linking increased AA consumption with metabolic disorders.

2.5.6 AA vs. LA and CVD

N-6 fats as a family should not be labeled as pro-inflammatory, but instead the effects of individual n-6 fatty acids should be considered. Overall, greater evidence points to the long chain AA, as a mediator of inflammation, while there is little evidence correlating levels of LA to CVD outcome measures. Indeed, early studies which had found a link between LA and increased risk of CVD did not concurrently evaluate AA concentrations, or simply evaluated total n-6 fatty acid concentrations, and not individual n-6 fatty acids.

2.6 The Δ6 Desaturase knock-out mouse: A new model to study the independent effects of LA and ALA

The Δ6 desaturase knock-out mouse is a newly developed genetic model which can be utilized to study the biological effects of individual PUFA. The homozygous D6D knock-out mouse does not have a functional copy of the FADS2 gene, which encodes the D6D protein. Therefore, D6D knock-out mice are unable to convert LA or ALA to their primary metabolites AA, EPA and
DHA. In 2009, two research groups independently developed D6D knock out mouse models: the FADS2 -/- model, created by Stoffel and colleagues at the University of Cologne; and the D6KO mouse developed by Nakamura and colleagues at the University of Illinois. Both groups confirmed the absence of FADS2 gene expression and verified that a D6D isozyme was not present, effectively demonstrating that the conversion of LA and ALA to AA, EPA and DHA was effectively blocked in their mouse models.

Nakamura and colleagues provided detailed characterization of the phenotype, and reproductive capacity of D6KO mice fed either HUFA-restricted diets, or diets supplemented with AA or DHA. When provided with a purified diet containing adequate LA and ALA, but void of AA, EPA and DHA (AIN93G), D6KO mice developed ulcerative dermatitis, intestinal ulceration, hypertriglyceridemia, and enlarged spleens and larger livers. Additionally, several phenotypic differences were present between male and female D6KO mice, as female mice had reduced thymus glands but improved weight gain relative to male D6KO mice. The addition of 0.4% w/w AA to the diet was sufficient to prevent dermatitis and intestinal ulceration in D6KO mice. Male D6KO mice were also rendered functionally sterile due to impairments in the later stages of spermatogenesis. However, dietary supplementation with 0.2% DHA was sufficient to fully restore male reproductive ability, while provision of 0.2% AA partially restored spermatogenesis. D6KO female reproductive organs were also altered. Similar to the D6KO mouse, FADS2 -/- mice were also found to be functionally sterile, while supplementation with AA, EPA and DHA rescued the fatty acid profile and prevented sterility. In summary, sterility could be effectively prevented in both D6D knock-out mouse models through HUFA supplementation, and additionally, in the D6KO mouse provision of AA was sufficient to prevent several other phenotypic abnormalities.
Fatty acid composition of several tissues of the FADS2 -/- model and D6KO mouse were also reported. While very long chain PUFA were entirely absent from the liver, serum, retina, adrenals, and gonads of FADS2 -/- mice, Nakamura and colleagues reported the persistence of long chain PUFA in all tissues of the D6KO mouse. In this regard, the fatty acid profile of the D6KO model seemed to better reflect previous PUFA-restriction studies, as HUFAs persist in mouse tissues after up to 2 generations of PUFA restriction.\(^{165}\) Of interest, tissue AA, EPA and DHA were reported to decline at different rates in D6KO mice. In the liver, the main site of PUFA metabolism,\(^{166}\) AA and DHA contents declined by 95%, while in the brain, AA decreased by 45% and DHA decreased by merely 15-33%.\(^{18}\) The presence of three previously absent fatty acid products was also reported in D6KO mice; these fatty acids were identified as 20:3(Δ7, 11, 14); 20:4(Δ7, 11, 14, 17) and 22:4(Δ9, 13, 16, 19). It was deduced that these three fatty acids were the products of the metabolism of LA and ALA by elongases and D5D, the enzyme usually associated with the metabolism of 20-carbon PUFA. In the FADS2 -/- mouse, presence of novel fatty acid products was not reported.

Beyond the initial studies which provided an initial characterization of the reproductive capacity and/or phenotype of FADS2 -/- and D6KO mice, only one other study utilizing peritoneal macrophages from D6KO mice has been published. In this cell culture study, macrophages from D6KO mice fed an AA-sufficient diet were found to have lower cholesterol biosynthesis and increased antioxidant enzyme (paraoxonase-2) expression relative to macrophages from mice fed AA-deficient diets.\(^{167}\) As higher levels of cholesterol and lower paraoxonase-2 production\(^{168}\) are associated with the initiation of atherosclerosis, in this study AA-depletion was associated with early atherogenesis.
In conclusion, a D6D knock-out mouse would make a suitable model to study the effects of ALA or LA separate from the effects of their very long chain fatty acid metabolites EPA, DHA and AA. While the phenotypes of both the FADS2 -/- and D6KO models have been characterized, a more thorough evaluation and description of fatty acid composition has been provided for the D6KO mouse model developed by Nakamura and colleagues. The D6KO mouse is then suitable for use in future studies which aim to assess the effects of ALA or LA on initiation and progression of lifestyle diseases such as CVD or NAFLD.

2.7 Conclusion: Evaluating the individual effects of essential vs. very long chain PUFA on NAFLD and CVD

Individual classes of PUFA have differential effects on the development and progression of NAFLD and CVD. The n-3 HUFA EPA and DHA lower circulating TG, may improve cholesterol levels and have anti-inflammatory effects. Thus, the metabolic benefit of these two long chain fatty acids has been most clearly established. In contrast AA, is mainly pro-inflammatory and positively associated with circulating cholesterol, TG concentrations and overall CVD risk; however, further study is required to determine the association between AA and NAFLD risk. The effects of the parent PUFA ALA and LA have not been clearly established. There is evidence that ALA has similar but less potent effects on circulating lipids and inflammation as its metabolites. As such, while diets rich in EPA and DHA are highly recommended for those with MetS-associated diseases, the replacement of cooking oils with oils rich in ALA may provide additional benefit. Overall, more research into the specific effects of ALA is required to fully understand how this fatty acid modifies NAFLD and CVD risk. Unlike
ALA, LA does not seem to share the same biological effects as its primary metabolite AA. The metabolic effects of LA seem the most controversial, as many studies have suggested that supplementation with this fatty acid can improve cholesterol, and LAs effects on inflammation are unclear. Overall, this review has provided evidence that the EFAs ALA and LA may have metabolic effects which are distinct from those of their long chain metabolites. Further evaluation of these potential effects would be valuable, as these two fatty acids compose the bulk of total PUFA consumed in the Western diet. Additionally, greater understanding of the potential therapeutic effects of ALA and LA could lead to better diet plans prescribed to those at risk of, or suffering from diseases associated with MetS.
Chapter Three

RATIONALE, OBJECTIVES, HYPOTHESIS
and EXPERIMENTAL DESIGN
3.1 Rationale

Non-alcoholic fatty liver disease (NAFLD) is currently the most prevalent liver disease in North America and represents an early manifestation of the Metabolic Syndrome. Hepatic steatosis is the hallmark trait of NAFLD, while an inflammatory component is associated with all progressive forms of NAFLD. Over the last few decades there has been increased interest in the protective effects of the n-3 PUFA α-linolenic acid (ALA), and its metabolites, eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), as increased intake of n-3 PUFA has been linked to reduced hepatic lipid accumulation and inflammation. The effects of EPA and DHA have been widely investigated, but little is known about the direct effects of ALA. While mammals have the enzymes to convert ALA to EPA and DHA, they cannot synthesize ALA; therefore, mammals must attain this essential fatty acid through dietary sources such as flaxseeds and walnuts. The first enzyme in the n-3 metabolic pathway is Δ6 desaturase (D6D). As there is always some conversion of ALA in mammals, is it then difficult to determine the direct biological effects of ALA independent of its metabolites EPA and DHA. This barrier has recently been addressed with the development of the Δ6 desaturase knock out (D6KO) mouse. The purpose of this thesis is to first further characterize this newly developed mouse model, and next to utilize the D6KO mouse model to determine the independent role of ALA in the prevention of hepatic steatosis and inflammation associated with NAFLD. Overall, the findings from this study have the potential to enhance our fundamental understanding of the specific role of ALA in health and disease.
3.2 Hypothesis

The overall hypothesis of this thesis is that ALA, similar to EPA and DHA, can independently prevent the development of hepatic steatosis and inflammation associated with NAFLD. Specifically, in the first study it is hypothesized that:

1. Provision of downstream PUFA can restore the hepatic lipid profile of D6KO mice.

In the second study it is hypothesized that:

2. Hepatic phospholipids of D6KO mice fed diets high in ALA and void of EPA and DHA will be void of long chain n-3 PUFA, and enriched in ALA.

3. Liver lipid accumulation will be prevented to the same extent in D6KO mice fed ALA-rich oils and menhaden oil, as compared to mice fed a diet void of n-3 PUFA.

4. Similar to D6KO mice fed fish oil, D6KO mice fed diets containing ALA-rich oils will have low levels of hepatic inflammation relative to D6KO mice fed diets void of n-3 PUFA.
3.3 Objectives

The overarching objective of this thesis was to use the D6KO mouse to determine if the provision of ALA can prevent the development and progression of non-alcoholic fatty liver disease, independent of ALA’s conversion to EPA and DHA.

The primary objective of the first study was:

1. To determine if the provision of downstream PUFA can effectively rescue the D6KO hepatic fatty acid profile

The primary objectives of the second study were to use the D6KO mouse to determine the independent effects of ALA-rich oils on:

2. Phospholipid fatty acid composition of the liver, the primary site of lipid metabolism, at 8 and 20 weeks.
3. The accumulation of lipids in the liver at 8 and 20 weeks.
4. Hepatic and systemic inflammation at 8 and 20 weeks.

The secondary objectives of this study were to determine the effects of ALA on glucose tolerance, weight gain, and to determine if the effects of ALA were dose dependent.
3.4 Experimental Design

D6KO heterozygous male and female mice on a C57BL/6 background were crossed. To characterize the D6KO mouse model, 28 day old D6KO and WT male and female mice were placed on one of three diets: 1) safflower oil (LA rich); 2) soybean oil (LA + ALA); or 3) menhaden oil + safflower oil (EPA + DHA) for 28 days (n = 3-7/group). Following dietary treatment, animals were euthanized and livers and serum were collected. Liver and serum phospholipids were analyzed to further characterize fatty acid distribution the model, and to determine if provision of preformed HUFA had rescued the D6KO phenotype.

To determine the independent effects of ALA, 28 day old male D6KO and WT offspring were placed on one of four high fat diets: 1) lard diet (0% n-3 PUFA, negative control); 2) canola diet (~8% ALA); 3) flax diet (~55% ALA); or fish oil diet (high EPA/DHA; positive control). Mice were fed and weighted weekly. After either 8 weeks (n = 6-8/treatment group), or 20 weeks (n = 8-11/treatment group) on diet, mice were fasted overnight, and an intraperitoneal glucose tolerance test was performed. Mice were then euthanized, and liver and other tissues were collected. Phospholipid fatty acid analysis of liver was performed using thin layer chromatography followed by gas chromatography. Additionally, liver sections underwent histological evaluation and were scored for steatosis and inflammation. To determine systemic inflammation, serum C-reactive protein (CRP) levels were quantified using enzymatic methods.
Chapter Four

MENHADEN OIL, BUT NOT SAFFLOWER OR SOYBEAN OIL, AIDS IN RESTORING THE POLYUNSATURATED FATTY ACID PROFILE IN THE DELTA-6 DESATURASE KNOCKOUT MOUSE

As published in Lipids in Health and Disease 2012, 11: 60 – “Menhaden oil, but not safflower or soybean oil, aids in restoring the polyunsaturated fatty acid profile in the novel Δ6-desaturase null mouse” by J Monteiro, FJ Li, A Rabalski, MH Moghasian, MT Nakamura, and DWL Ma
4.1 Abstract

**Background:** Polyunsaturated fatty acids (PUFA) have diverse biological effects, from promoting inflammation to preventing cancer and heart disease. Growing evidence suggests that individual PUFA may have independent effects in health and disease. The individual roles of the two essential PUFA, linoleic acid (LA) and α-linolenic acid (ALA), have been difficult to discern from the actions of their highly unsaturated fatty acid (HUFA) downstream metabolites. This issue has recently been addressed through the development of the Δ-6 desaturase knock out (D6KO) mouse, which lacks the rate limiting Δ-6 desaturase enzyme and therefore cannot convert LA or ALA into AA, EPA and DHA. However, a potential confounder in this model is the production of novel Δ-5 desaturase (D5D) derived fatty acids when D6KO mice are fed diets containing LA and ALA, but void of arachidonic acid. **Objective:** The aim of the present study was to characterize how the D6KO model differentially responds to diets containing the essential n-6 and n-3 PUFA, and whether the direct provision of downstream HUFA can rescue the phenotype and prevent the production of D5D fatty acids. **Methodology:** Liver and serum phospholipid (PL) fatty acid composition was examined in D6KO and wild type mice fed i) 10% safflower oil diet (SF, LA rich) ii) 10% soy diet (SO, LA + ALA) or iii) 3% menhaden oil +7% SF diet (MD, HUFA rich) for 28 days (n=3-7/group). **Results:** Novel D5D fatty acids were found in liver PL of D6KO fed SF or SO-fed mice, but differed in the type of D5D fatty acid depending on diet. Conversely, MD-fed D6KO mice had a liver PL fatty acid profile similar to wild-type mice. **Conclusions:** Through careful consideration of the dietary fatty acid composition, and especially the HUFA content in order to prevent the synthesis of D5D fatty acids, the D6KO model has the potential to elucidate the independent biological and health effects of the parent n-6 and n-3 fatty acids, LA and ALA.
4.2 Background

Δ6-desaturase (D6D) is the first enzyme in the metabolic conversion of α-linolenic acid (ALA), an n-3 polyunsaturated fatty acid (PUFA), to eicosapentaenoic acid (EPA), and docosahexaenoic acid (DHA). D6D is also responsible for initiating the conversion of the n-6 PUFA, linoleic acid (LA), to arachidonic acid (AA). D6D is encoded by the FADS2 gene which is present in all mammals \(^{16}\) and is most highly expressed in the liver \(^{17}\), the main site of PUFA metabolism \(^{166,169,170}\). While mammals can endogenously produce a limited amount of AA, EPA and DHA from dietary LA and ALA, mammals do not have the ability to produce LA/ALA endogenously. Thus, both these fatty acids must be attained solely through dietary means and are considered essential fatty acids (EFA) \(^{10}\).

HUFAs, such as AA, EPA and DHA are primarily deposited in membrane phospholipids (PL) as opposed to intracellular triglyceride stores, and contribute to a wide array of cellular functions such as eicosanoid synthesis, gene expression \(^{23}\), cell signalling, and protein function \(^{21}\). Liver phospholipids are differentially enriched in select fatty acids; phosphatidylcholine (PC) is the main PL and therefore contains the greatest quantity of fatty acids, while hepatic phosphatidylinositol (PI) and phosphatidylethanolamine (PE) are enriched in the eicosanoid precursor AA \(^{171}\), and lyso-phosphatidylcholine (lyso-PC) is preferentially enriched with DHA for delivery to the brain \(^{172}\). Thus, it is of interest to determine the fatty acid composition of each PL fraction in order to fully explore the effects of perturbations in essential fatty acid metabolism.

Select n-6 and n-3 PUFA have garnered attention from the scientific community and general public for their roles in the prevention and treatment of lifestyle diseases such as
cardiovascular disease, Type II diabetes and certain cancers. Many studies \(^{173-175}\) have aimed to assess the protective or therapeutic effects of either ALA or LA through dietary intervention. However, these studies cannot attribute results directly to either ALA or LA as there is always some conversion to more highly unsaturated fatty acids (HUFA), such as AA, EPA and DHA. Thus, any outcomes observed in such studies could also be due to the effects of AA, EPA and DHA which are all considered biologically active metabolites. Similarly, studies aiming to determine the effects of restricting a particular HUFA such as DHA or AA have not been able to do so without simultaneous removal of ALA or LA from the diet \(^{164,176}\). A model in which the conversion of ALA and LA to EPA, DHA and AA is inhibited would then be of great utility in determining if LA and ALA are independently able to prevent or alleviate disease.

This hurdle has recently been addressed with the development of the novel \(\Delta-6\) desaturase knock out (D6KO) mouse \(^{18,40}\). The homozygous D6KO mouse lacks a functional copy of the FADS2 gene, and is therefore unable to produce the D6D protein (Fig. 1). This renders the D6KO mouse completely deficient in EPA/DHA/AA if these HUFA are not provided in the diet, since no alternative pathway for the production of these long chain fatty acids exists.

Previous studies with the D6KO mouse have focused on the effects of HUFA restriction through the provision of LA and ALA-enriched soybean oil on macrophage activity \(^{167}\), dermatitis, and intestinal ulceration \(^{18}\). In contrast, the effects of a diet enriched only in LA on PUFA metabolism and HUFA synthesis in the D6KO mouse are currently unknown. Dietary studies have also been carried out on D6KO mice using diets enriched with purified AA (ARASCO\(^{\text{®}}\)) and DHA (DHASCO\(^{\text{®}}\)) oils \(^{18,164}\). However, the effect of HUFA-enriched oils more commonly found in human diets, such as fish oil, have not been explored in this novel mouse model.
The objective of the current study was to determine the differential effects of LA and ALA-enriched diets in D6KO mice, with special focus on phospholipid PUFA composition in the liver, the primary site of PUFA metabolism. Secondly, to evaluate if adding a natural source of HUFA (menhaden oil) to the diet can effectively restore the hepatic phospholipid HUFA content of D6KO mice to levels seen in wild-type (WT) mice. Results of this study further support the use of the D6KO model for future studies aiming to determine the independent effects of LA and ALA in ameliorating disease.

**Figure 4.1 Regular and modified n-6 and n-3 polyunsaturated fatty acid metabolic pathways** A) The normal n-6 and n-3 fatty acid metabolic pathway. B) In the absence of Δ6 desaturase, Δ5 desaturase and elongases metabolize LA and ALA to produce 3 novel fatty acids: 20:3(Δ7, 11, 14); 20:4(Δ7, 11, 14, 17), and 22:4(Δ9, 13, 16, 19)
4.3 Methods

4.3.1 Animals and diet

Heterozygous D6KO mice backcrossed to a C57BL/6 background\textsuperscript{177}, were transferred from University of Illinois at Urbana-Champaign to the University of Guelph where a colony of heterozygous mice was established. Experimental mice consisting of D6KO and WT mice were obtained from the breeding of harems consisting of one heterozygous male and 2-3 females. Breeders were fed standard laboratory mouse chow (Harlan Teklad #9004), and pups were continued on this diet until 28 days old. Mice were housed within ventilated cages at 22°C in humidity-controlled environment on a 12 hour light: 12 hour dark cycle for the study duration.

At 21 days mice were weaned, and tail snips were obtained for DNA extraction and PCR analysis to determine genotype\textsuperscript{18}. PCR primers D6D WT forward (CGGTGGGAGGAGGAGTAGAAGAC); D6D WT reverse (CCTCTCCCTGGTTACCTCCCTTC); D6D KO forward (GCTATGACTGGGCACAACAG); and D6D KO reverse (TTCGTCCAGATCATCCTGATC) were used.

Twenty-eight-day old homozygous D6KO and WT mice were placed on one of three experimental diets (Table 4.3; modified AIN93G diet; Research Diets): 10% Soy diet (SO; cat# D09072305) (n=5 WT and 3 KO), 10% safflower diet (SF; cat# D04092701) (n=7 WT and 7 KO), or 3% menhaden + 7% safflower diet (MD; cat# D04092703) (n=4 WT and 4 KO). Mice were fed experimental diets for 28 days. At 56 days of age, the experimental mice were euthanized using CO\textsubscript{2}, and final body weights recorded. This investigation was approved by the University of Guelph Animal Care Committee in accordance with the requirements of the Canadian Council on Animal Care.
Table 4.3: Diet fatty acid composition of 10% safflower oil, 10% soybean oil or 3% menhaden + 7% safflower oil diets.

<table>
<thead>
<tr>
<th>Fatty Acid</th>
<th>10% Safflower n-6 (LA)</th>
<th>10% Soy n-3 (ALA)</th>
<th>3% fish + 7% safflower n-3 (HUFA)</th>
</tr>
</thead>
<tbody>
<tr>
<td>14:0</td>
<td>0.2</td>
<td>0.2</td>
<td>2.6</td>
</tr>
<tr>
<td>16:0</td>
<td>6.3</td>
<td>10.8</td>
<td>10.4</td>
</tr>
<tr>
<td>16:1</td>
<td>0.1</td>
<td>0.2</td>
<td>3.2</td>
</tr>
<tr>
<td>18:0</td>
<td>2.6</td>
<td>4.3</td>
<td>3.0</td>
</tr>
<tr>
<td>18:1n9</td>
<td>14.6</td>
<td>21.1</td>
<td>12.2</td>
</tr>
<tr>
<td>18:1c11</td>
<td>0.7</td>
<td>1.5</td>
<td>1.4</td>
</tr>
<tr>
<td>18:2n6</td>
<td>73.6</td>
<td>52.8</td>
<td>53.7</td>
</tr>
<tr>
<td>18:3n6</td>
<td>0.0</td>
<td>0.0</td>
<td>0.1</td>
</tr>
<tr>
<td>18:3n3</td>
<td>0.2</td>
<td>6.9</td>
<td>0.6</td>
</tr>
<tr>
<td>18:4n3</td>
<td>0.0</td>
<td>0.0</td>
<td>1.1</td>
</tr>
<tr>
<td>20:0</td>
<td>0.4</td>
<td>0.4</td>
<td>0.4</td>
</tr>
<tr>
<td>20:1</td>
<td>0.1</td>
<td>0.3</td>
<td>0.2</td>
</tr>
<tr>
<td>20:2n6</td>
<td>0.0</td>
<td>0.0</td>
<td>0.2</td>
</tr>
<tr>
<td>20:3n6</td>
<td>0.1</td>
<td>0.0</td>
<td>0.1</td>
</tr>
<tr>
<td>20:4n6</td>
<td>0.0</td>
<td>0.0</td>
<td>0.4</td>
</tr>
<tr>
<td>20:3n3</td>
<td>0.0</td>
<td>0.0</td>
<td>0.1</td>
</tr>
<tr>
<td>20:5n3</td>
<td>0.0</td>
<td>0.0</td>
<td>3.9</td>
</tr>
<tr>
<td>22:0</td>
<td>0.3</td>
<td>0.6</td>
<td>0.4</td>
</tr>
<tr>
<td>22:1</td>
<td>0.1</td>
<td>0.2</td>
<td>0.1</td>
</tr>
<tr>
<td>22:4n6</td>
<td>0.0</td>
<td>0.0</td>
<td>0.1</td>
</tr>
<tr>
<td>22:5n6</td>
<td>0.0</td>
<td>0.0</td>
<td>0.2</td>
</tr>
<tr>
<td>22:5n3</td>
<td>0.0</td>
<td>0.1</td>
<td>0.8</td>
</tr>
<tr>
<td>24:0</td>
<td>0.2</td>
<td>0.2</td>
<td>0.1</td>
</tr>
<tr>
<td>22:6n3</td>
<td>0.0</td>
<td>0.0</td>
<td>4.0</td>
</tr>
<tr>
<td>24:1</td>
<td>0.0</td>
<td>0.0</td>
<td>0.3</td>
</tr>
<tr>
<td>sum</td>
<td>100%</td>
<td>100%</td>
<td>100%</td>
</tr>
</tbody>
</table>

Data are the means of analysis in duplicate and represent percentage of total fatty acids in each diet.
4.3.2 *Fatty acid analysis*

Upon termination, liver samples were collected, flash frozen in liquid nitrogen, and stored at -80°C. For analysis, 0.1 g of liver tissue was weighed and homogenized in 2.5 ml of 0.1 M potassium chloride. 10 ml chloroform (Fisher, Cat #C298-4): methanol (Fisher, Cat #A452-4) solution (2:1 v/v) was then added to the homogenized liver samples, according to Folch et al. Samples were vortexed, flushed with nitrogen to prevent oxidation, and stored at 4°C overnight [33]. Samples were then centrifuged at 577xg for 10 minutes to separate phases. The lipid-containing chloroform layer was extracted into a pre-weighted acid-washed screw cap test tube with Teflon lined cap, dried down under nitrogen gas, and weighed to determine the total mass of lipids. 6 ml chloroform was added back into homogenized liver sample, and the extraction process was repeated. Dried lipid samples were reconstituted at 10 mg/ml chloroform for thin layer chromatography (TLC).

Phospholipid fractions were then separated using TLC. Briefly, silica H plates (VWR 5721-7) were activated by heating at 100°C for 1 hour. 100 μl of each liver lipid sample were spotted along 2 cm lanes scored onto the activated plates. Samples were run alongside known phospholipid standards for 2.5 hours in chloroform/ methanol/ 2-propanol (Fisher A416-1) /KCl (0.25%w/v)/triethylamine (Sigma Cat# CT0886) (30:9:25:6:18). Fractions were visualized using 0.1% 8-anilino-1-naphthalene sulfonic acid (w/v) (Fluka # 10417) under UV light. Bands corresponding to PC, PE, PS, PI, SM and lyso-PC were identified and individually scraped into acid washed test tubes already containing 1.0 μl (PC) or 0.1 μl (PE, PS, PI, SM, Lyso PC) 17:0 FFA as an internal standard and 2 ml hexane. 2 ml 14% boron triflouride-methanol (Sigma, cat #B1252) was next added, and samples were methylated for 1.5 hours at 100°C. Samples were allowed to cool (RT), then 2 ml distilled water was added to each sample to halt methylation, and
samples were centrifuged at 577xg for 10 minutes. The hexane layer containing methylated fatty acids was extracted into a gas chromatography vials. Samples were then dried down under a gentle stream of nitrogen gas and reconstituted in 30µl (100µl for PC) hexane in glass gas chromatography inserts [33].

PL fatty acid methyl esters were quantified using an Agilent 6890N gas chromatograph equipped with a flame ionized detector and separated on a Supelco SP-2560 fused silica capillary column (100 m, 0.2µm film thickness, 0.25 mm i.d.; Sigma cat #24056 ) [179]. Hydrogen was used as the carrier gas and set at a constant flow rate of 30 mL/min. Samples were injected in splitless mode. The injector and detector ports were set at 250°C. Fatty acid methyl esters were eluted through the column using temperature program of: 0.2 min at 60°C, then increasing 13°C/min until a temperature of 170°C was reached. 170°C was held for 4 min, then increased 6.5°C/min to 175°C, increased 2.6°C/min to 185°C, increased 1.3°C/min to 190°C, and finally increased 13°C/min to 240°C and held at this temperature for 13 min. The run time per sample was 37.77 min. Fatty acids were identified by comparing peak retention times with those of a known standard (Nu-Chek-Prep, Elysian, MN). Fatty acid peak areas were determined using EZChrom Elite software (Version 3.3.2). Fatty acid values were expressed as percent area in each PL fraction.

Serum lipid fraction analysis was carried out under the same conditions as liver tissue samples above using 50µl serum. Briefly, serum lipids were double extracted, dried down and reconstituted in 150µl chloroform. 100 µl of reconstituted lipid sample was spotted onto an activated TLC H-plate, and phospholipid bands were scraped, methylated for an hour, and analyzed using Agilent 7890 gas chromatograph under the same temperature program as liver samples.
4.3.3 **Statistical analysis**

Data were analyzed using SAS (v. 9.1) statistical software. 2-way analysis of variance test was performed on data, followed by Tukey post-hoc tests on main effects when the interaction term was not significant; Least Squares Means was used when interaction values were significant. Statistical significance was set at p < 0.05.

4.4 **Results**

4.4.1 **Animals**

Mean mouse body weight and food intake across all treatment groups was not significantly different at the end of the study (Table 4.1). Data derived from D6KO groups were compared directly with WT groups, as heterozygous D6KO hepatic PL profiles did not differ from WT liver PL profiles.

**Table 4.1:** Mean weight gain and diet consumption

<table>
<thead>
<tr>
<th>Diet</th>
<th>Genotype</th>
<th>n</th>
<th>Mean Weight Gain (g)*</th>
<th>Mean Diet Consumed (g)*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Safflower</td>
<td>WT</td>
<td>7</td>
<td>6.2 ± 1.9</td>
<td>71.8 ± 4.6</td>
</tr>
<tr>
<td></td>
<td>KO</td>
<td>7</td>
<td>4.7 ± 1.7</td>
<td>70.16 ± 5.45</td>
</tr>
<tr>
<td>Soy</td>
<td>WT</td>
<td>5</td>
<td>4.6 ± 2.2</td>
<td>71.3 ± 5.7</td>
</tr>
<tr>
<td></td>
<td>KO</td>
<td>3</td>
<td>5.1 ± 1.0</td>
<td>71.6 ± 6.0</td>
</tr>
<tr>
<td>Menhaden</td>
<td>WT</td>
<td>4</td>
<td>6.6 ± 1.6</td>
<td>75.6 ± 4.1</td>
</tr>
<tr>
<td></td>
<td>KO</td>
<td>1</td>
<td>5.6 ± 1.9</td>
<td>78.9**</td>
</tr>
</tbody>
</table>

Note: *Weight gain is calculated from day on which mice were placed on experimental diet until day mice were terminated. Diet consumption was calculated from day mice were placed on experimental diet to Day 25. **Diet consumption data available for one mouse
Figure 4.2 Novel Δ-5 desaturase (D5D) products are found in soybean oil (SO) fed D6KO mice. Representative chromatogram of the PC fraction obtained from liver samples of SO fed D6KO sample (BLUE) superimposed on a MD-fed D6KO (GREEN). D5D products [20:3(Δ7,11,14); 20:4(Δ7,11,14,17); 22:4(Δ9,13,16,19)] are noted in black, major HUFA are noted in red.
Table 4.2: Novel D5D product composition in the serum phosphatidylcholine fraction

<table>
<thead>
<tr>
<th>Fatty Acid</th>
<th>Menhaden 4</th>
<th>Safflower 7</th>
<th>Soy 5</th>
<th>Menhaden 4</th>
<th>Safflower 7</th>
<th>Soy 3</th>
<th>Interaction p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>WT</td>
</tr>
<tr>
<td>20:3(Δ7,11,14)</td>
<td>0.1 ± 0.09</td>
<td>0</td>
<td>0</td>
<td>0.1 ± 0.04</td>
<td>4.0 ± 1.74a</td>
<td>2.6 ± 0.69a</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td></td>
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<td></td>
<td></td>
<td></td>
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<td>KO</td>
</tr>
<tr>
<td>20:4(Δ7,11,14,17)</td>
<td>0.1 ± 0.07</td>
<td>0.1 ± 0.08</td>
<td>0.2 ± 0.05</td>
<td>0.1 ± 0.03</td>
<td>0.2 ± 0.06</td>
<td>0.1 ± 0.02</td>
<td>0.23</td>
</tr>
<tr>
<td>22:4(Δ9,13,16,19)</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0.2 ± 0.05a</td>
</tr>
<tr>
<td>Total D5D Products</td>
<td>0.2 ± 0.20</td>
<td>0.1 ± 0.08</td>
<td>0.2 ± 0.05</td>
<td>0.2 ± 0.04</td>
<td>4.2 ± 1.80a</td>
<td>2.9 ± 0.70a</td>
<td>&lt;0.01</td>
</tr>
</tbody>
</table>

Note: Data were analyzed by 2-way ANOVA and are expressed as means ± SD. Means with letters represent a significant difference from wild-type values within a row (p < 0.05)
4.4.2 *The production of D5D novel fatty acids and their selective distribution in the liver*

Based on previously published results\(^\text{18}\), three peaks corresponding to three D5D-derived fatty acids \([20:3n6 (\Delta7, 11, 14), 20:4n3 (\Delta7, 11, 14, 17)\) and \(22:4n3 (\Delta9, 13, 16, 19)\)] were found in the liver PL fractions of D6KO mice fed LA-rich safflower oil (SF) and LA+ALA-enriched soybean oil (SO) diets (Fig. 2; Fig. 3). These three fatty acids were absent from all three experimental diets, and were also absent in the chromatographs of WT SF and SO fed mice. All three novel fatty acids were found to be differentially distributed among the six PL fractions (PC, phosphatidylcholine; PE, phosphatidylethanolamine; PS, phosphatidylserine; PI, phosphatidylinositol; SM, sphingomyelin; Lyso-PC, lyso-phosphatidylcholine). The PI fraction contained the highest percentage of 20:3n6 (\(\Delta7, 11, 14\)) (SO = 23.5 ± 4.4%; SF = 30.6 ± 1.6%) followed by the PS (17.7 ± 1.6%), PE (11.7 ± 1.7%), SM (8.4 ± 3.2%), Lyso-PC (4.8 ± 1.0%), and PC factions (4.1 ± 0.6%) in SO-fed D6KO mice; and followed by PS (20.9 ± 2.2%), PE (13.4 ± 0.7%), PC (5.4 ± 0.4%), SM (5.0 ± 3.7%), and Lyso-PC fractions (4.0 ± 1.7%) in SF-fed D6KO mice (Fig. 3A). In the PI fraction of D6KO mice fed SF, 20:3n6 (\(\Delta7, 11, 14\)) comprised almost a third of all fatty acids, and in the PI fraction of SO-fed D6KO mice, this novel fatty acid accounted for almost a fourth of total fatty acids. 20:4n3 (\(\Delta7, 11, 14, 17\)) and 22:4n3 (\(\Delta9, 13, 16, 19\)) were also selectively distributed across PL fractions, with the highest levels (relative to total fatty acids) found in PE, PI and PS fractions (Fig. 3B,C).

Trace amounts of all three novel fatty acids were found in the PL fractions of menhaden (MD)-fed D6KO mice, but in much lower levels in comparison to D6KO mice fed SO and SF (Fig. 3). In the PC fraction of MD-fed mice total D5D fatty acids composed 0.23 ± 0.03% of total fatty acids; in comparison, the PC fraction of SF and SO-fed mice contained 5.40 ± 0.35% and 4.77 ± 0.69%, respectively. Similar trends were observed in other PL fractions (Fig. 3).
D5D fatty acids were not detected in WT mice fed SF and SO diets, while PL fractions of WT mice fed MD were all found to contain less than 1% D5D fatty acids. Novel D5D fatty acid content of all PL fractions was significantly different (p < 0.001) between WT and D6KO mice fed either SO and SF, but % D5D fatty acids were similar between WT and D6KO mice fed MD (Fig. 4F).

4.4.3 Comparison of liver LA and ALA levels across treatment groups

LA content increased in most PL fractions of the three D6KO treatment groups. In all PL fractions of SO-fed D6KO mice LA content increased, and this increase was significant in most fractions. In particular, in the PC fraction of SO-mice LA content almost doubled from a mean level of 21.12 \pm 3.38\% in WT mice to 38.83 \pm 0.34\% in D6KO mice (p < 0.0001) (Fig. 4A); while in the PE fraction LA content showed a 3-fold increase. A similar effect was seen between D6KO and WT mice fed SF diet (Fig. 4A). In contrast, in MD-fed mice, LA content also increased in 5 of the 6 PL fractions, but this increase was less than 2-fold in any fraction.

In all PL fractions of SF-fed and SO-fed mice ALA content remained unchanged or increased. The increase in ALA content was especially evident in SO-fed mice, as the SO diet contains greater levels of ALA than the SF diet. Across all PL fractions of D6KO mice on a SO diet there was an approximately 3-fold or greater increase in ALA content when compared with WT mice fed SO-diets. In particular, the PC fraction of SO-fed D6KO mice contained 1.75 \pm 0.11\% ALA compared with only 0.61 \pm 0.10\% in WT mice (p < 0.0001) (Fig. 4B). Unlike the results seen in SF and SO fed mice, ALA levels remained constant or showed non-significant decreases in MD-fed D6KO in comparison to their WT counterparts.
Figure 4.3 Preferential distribution of 3 novel D5D fatty acids among the liver phospholipid fractions of D6KO mice across dietary treatment groups. A) Distribution of 20:3 (Δ7,11,14); B) distribution of 20:4 (Δ7,11,14,17); C) distribution of 22:4 (Δ9,13,16,19)
Figure 4.4 Comparison of select fatty acid values found in the liver PC fraction (shown as percent of total PC fatty acids) between genotypes and across dietary treatment groups: A) linoleic acid; B) α-linolenic acid; C) arachidonic acid; D) eicosapentaenoic acid; E) docosahexaenoic acid; F) total novel Δ-5 fatty acids. *indicates significant difference (p < 0.05) between D6KO and WT pairs; differences between means determined by 2-way ANOVA. MD, menhaden oil, SF, safflower; SO, soy
4.4.4 Highly unsaturated fatty acids

Levels of AA were significantly lower in most PL fractions of SF and SO-fed D6KO mice compared with their WT counterparts (Fig. 4C). There was also a small decrease in AA content in D6KO mice fed MD compared with WT mice which did not reach significance in most PL fractions.

EPA content remained similar or decreased in D6KO mice fed SO or SF diets, while EPA levels actually increased in MD-fed D6KO mice. After 4 weeks of feeding D6KO mice a soy-based diet, EPA content decreased non-significantly in the PC (Fig. 4D) and PE fractions. In the PE fraction of MD-fed D6KO mice, EPA levels increased from 4.76 ± 0.23% to 8.89 ± 1.07% (p < 0.05), and a similar increase was seen in most other fractions.

There was a decrease in DHA across all PL fractions in both SF-fed and SO-fed D6KO mice. In the PL fractions of MD-fed D6KO mice, DHA content remained equivalent to WT mice, or declined (Fig. 4E), but to a lesser extent than was seen between D6KO and WT SF or SO treatment groups.

4.4.5 N6/N3 ratio

The n6/n3 fatty acid ratio significantly increased 2-6 fold in the PC and PE fractions of SO and SF-fed D6KO mice (SO WT ratio = 4.04 ± 0.74 vs. SO D6KO ratio =15.44 ± 0.56; SF WT ratio = 25.29 ± 11.32 vs. SF D6KO ratio = 69.66 ± 23.71; p = 0.05), while there was only a slight non-significant increase in this ratio in MD-fed mice (MD WT ratio = 1.59 ± 0.18 vs. MD D6KO ratio = 1.89 ± 0.33).
4.4.6 Novel fatty acid distribution in serum lipid fractions

Serum phospholipid fractions were analyzed and it was determined that all three novel D5D products were present in the PL factions of D6KO mice. The distribution of serum PL D5D fatty acids was similar to the distribution found in liver PL fractions (Table 4.2).

4.5 Discussion

4.5.1 Change in liver lipid composition of D6KO mice fed HUFA-deficient diets

The novel D6KO model has the potential to enhance our fundamental understanding of the role of the essential fatty acids ALA and LA in health and disease. However, careful examination of the phenotype under different experimental conditions is needed in order to ascertain its potential utility and limitations. Previous research has used diets containing n-6 and n-3 fatty acids from soy\textsuperscript{18}, however, the effect of feeding only an LA-enriched diet on the fatty acid composition of liver phospholipid fractions in D6KO mice is currently unknown. Given that n-6 and n-3 fatty acids share this same pathway, it is important to determine if feeding D6KO mice a diet enriched in LA will result in a different hepatic fatty acid profile than mice fed a diet containing both LA and ALA\textsuperscript{40}. Therefore, the first objective of the current study was to characterize the liver phospholipid composition of D6KO mice fed a diet rich in LA but void of ALA and HUFAs.

In agreement with previous studies, three novel fatty acid peaks were present in the phospholipid fractions of D6KO mice. The first two fatty acids were identified as 20:3\textit{n}6(Δ7, 11, 14) and 20:4\textit{n}3(Δ7, 11, 14, 17) and are the products of desaturation by D5D\textsuperscript{18}. Additionally, a third novel fatty acid, 22:4\textit{n}3(Δ9, 13, 16, 19) was identified as a product of an elongase acting
on 20:4n3(Δ7, 11, 14, 17). A diet rich in LA, such as a diet containing safflower oil, results in the production of 20:3n6(Δ7, 11, 14), with trace quantities of the other two D5D fatty acids in the tissues of D6KO mice. Feeding D6KO mice a diet containing a source of ALA, such as soybean oil, results in the production of all three novel D5D fatty acids, with increased production of the two novel n-3 D5D products compared to a SF diet.

Mean weight gain and food intake was not significantly different among treatment groups, which indicates that mice had roughly equivalent caloric intake. The absence of any gross changes in the health status of D6KO mice may also suggest that, over a short duration, the provision of the parent PUFA LA and ALA may be sufficient to maintain regular biological functioning, even as HUFA stores are depleted. Additionally, the similar fatty acid profile of heterozygous D6KO mice and WT mice suggests that even one functional copy of the FADS2 gene is sufficient to maintain normal fatty acid levels. However, treatment duration was relatively short, and future research is required to determine if D6KO mice will differ from WT mice in response to diets of varied fatty acid composition over a longer time frame.

We confirmed that D6KO mice fed SF and SO diets had considerably less EPA, DHA, and AA in all liver PL fractions, as compared to WT mice. The n-6/n-3 fatty acid ratio was also greatly increased in the liver tissues of D6KO mice on SF and SO diets. The n-6/n-3 ratio is one method of assessing tissue n-3 content relative to n-6 fatty acid content. A high n6/n3 ratio, such as the ~15:1 ratio of the average American diet, has been associated with increased disease risk, while decreasing this ratio to the recommended ~4:1 has been shown to protect against disease. While FADS2 loss of function is rare in humans, several SNPs in the FADS2 gene have been discovered. Through the modification of desaturase activity, certain FADS2 SNPs have also been associated with disease risk. The results of this study then suggest
that a decreased ability to process ALA or LA via FADS2 polymorphisms\textsuperscript{188} or loss of function can lead to a higher PUFA ratio, which may increase disease susceptibility. Additionally, future studies can use the D6KO mouse as a suitable model to study FADS2 SNPs which lead to decreased D6D activity.

4.5.2 The selective distribution of novel fatty acids

The distribution of AA across phospholipid fractions is tightly regulated, with each PL fraction containing varied amounts of this n-6 fatty acid. In agreement with other studies\textsuperscript{171,189}, we found that PI and PE fractions were enriched in AA (data not shown). 20:3n6(Δ7, 11, 14), the major D5D fatty acid, was shown to be selectively distributed among the PL fractions, composing almost a third of PI fatty acids in SF-fed D6KO mice, and also contributing largely to the PS and PE fatty acid pool. Thus, the distribution of this fatty acid seems to follow a similar distribution to AA. This could be associated with selective PL remodeling seen with AA. For example, in a previous study it was determined that while the initial incorporation of AA into PC was high, over time there was a greater incorporation of AA into PE and PI\textsuperscript{171}. This finding, and others\textsuperscript{190,191} suggest that some enzymes associated with PL remodeling show specificity for AA. It is plausible that these same enzymes could also have a similar affinity for 20:3n6(Δ7, 11, 14), causing the distribution of this novel n-6 fatty acid to mimic the selective distribution of AA across PL fractions.

Novel D5D fatty acids were also discovered in serum phospholipids of D6KO mice, suggesting that once formed, D5D fatty acids are not retained by the liver, but make their way
into the circulation. The effect of these D5D fatty acids on extra-hepatic tissues is currently unknown, but certainly could be a potential confounder.

4.5.3 A diet supplemented in preformed HUFA partially rescues D6KO phenotype

The MD diet used in this study contained 3% (w/w) menhaden oil and 7% safflower oil, amounting to approximately 0.4% (w/w) DHA, 0.04% AA, and 0.39% EPA (based on % composition values in Table 4.3). Previous studies have shown that 0.2% w/w DHA (DHASCO) supplementation is sufficient to restore reproductive ability in D6KO mice\textsuperscript{164}, while 0.4% w/w AA supplementation could prevent the dermatitis and intestinal ulcers\textsuperscript{18} otherwise seen in D6KO mice fed HUFA deficient diets. Whether the provision of only AA, EPA/DHA, or both n-6 and n-3 HUFA is sufficient for optimal rescue in these disease models requires further study. Towards understanding this important question, this study demonstrates the efficacy of menhaden oil, a natural oil containing a mixture of n-6 and n-3 HUFA, in rescuing the D6KO phenotype. This finding is highly relevant and demonstrates the utility of menhaden oil as an important control group in future investigations using this model.

The addition of menhaden oil to the diet of D6KO mice had a profound effect on PUFA levels in the PL fractions of the liver. In contrast with D6KO mice on SF or SO diets, D6KO mice fed MD diets had liver lipid profiles that resembled their WT counterparts. While the PC fraction of MD-fed D6KO mice did contain significantly more LA compared with their WT counterparts, the difference in LA content was much greater between WT and D6KO mice in the SO and SF treatment groups. D6KO mice fed MD had EPA levels which exceeded levels found
in WT mice, while DHA levels approached WT levels. ALA levels in MD-fed D6KO and WT mice were nearly equivalent.

MD-fed D6KO mice also showed similar novel D5D fatty acid profiles to the MD WT treatment group, possibly through the action of HUFA to stimulate and inhibit certain transcription factors. HUFA are well-known inhibitors of SREBP-1c (sterol regulatory element binding protein-1c) which increase the transcription of D6D\textsuperscript{16,192,193} and D5D\textsuperscript{194}. Studies have also concluded that during EFA deficiency or starvation, PPARα (peroxisome proliferator-activated receptor alpha) is activated through the endogenous release of PUFA and up-regulates the transcription of D6D and D5D\textsuperscript{194,195}. Thus, in conditions where HUFA are restricted, SREBP-1c and PPARα, two otherwise opposing enzymes- may act to increase the transcription of D5D, resulting in increased production of D5D fatty acid products. While gene expression was not specifically evaluated in the current study, the study results support this hypothesis of D6D and D5D regulation by PPARα and SREBP-1c, as the hepatic PC fraction of MD-fed D6KO mice contained less than 0.25% novel D5D fatty acids (of total fatty acids), while SO and SF-fed D6KO mice PC fractions contained between 4-6% novel fatty acids.
4.6 Conclusion and Future Directions

The present study demonstrates that the conversion of LA and ALA to AA, EPA, and DHA is abolished in D6KO mice. This is readily observed by the presence of three novel D5D fatty acids in both liver and serum phospholipid fractions. However, since the function of these D5D fatty acids is unknown, their unwanted production in D6KO mice represents a limitation of this mouse model. The implications of this study demonstrate that this limitation can be overcome through the provision of dietary HUFAs, which inhibit the alternate metabolism of ALA and LA by D5D. The major dietary n-3s and n-6s consumed in a Western diet are ALA and LA, yet minimal conversion of these fatty acids into their long chain metabolites occurs in humans. Little is known about the independent biological actions of LA and ALA, thus with the aid of a fully characterized D6KO model, future dietary studies can provide better insight into the independent biological roles of LA and ALA in health and disease.
Chapter Five

OILS RICH IN $\alpha$-LINOLENIC ACID INDEPENDENTLY PROTECT AGAINST CHARACTERISTICS OF NON-ALCOHOLIC FATTY LIVER DISEASE IN THE $\Delta6$-DESATURASE NULL MOUSE
5.1 Abstract

Non-alcoholic fatty liver disease (NAFLD) is the primary liver disease in North America. The n-3 polyunsaturated fatty acids (PUFA) eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) have been shown to prevent and treat features of NAFLD, but little is known about the direct biological effects of their essential fatty acid precursor α-linolenic acid (ALA). In mammals, Δ6 desaturase (D6D) initiates the metabolism of linoleic acid (LA) and ALA to arachidonic acid (AA), EPA and DHA, respectively. The D6D knock-out (D6KO) mouse lacks D6D, and is therefore unable to convert ALA or LA into EPA, DHA or AA. The aim of this study was to use the D6KO mouse to evaluate the effects of ALA-rich oils on hepatic phospholipid (PL) fatty acid composition, and to determine if ALA can independently prevent hepatic steatosis and inflammation in a dose dependent manner. D6KO and wild-type mice were placed on one of four high fat (14% w/w) diets: 1) lard diet (LD, 0% n-3 PUFA), 2) canola + ARASCO diet (CD, ~8% ALA), 3) flax +ARASCO diet (FD, ~55% ALA), 4) menhaden oil diet (MD, 30% EPA/DHA). ARASCO was added to CD and FD to prevent AA deficiency. Animals were euthanized after 8 weeks (n = 6-8/treatment group), or 20 weeks (n = 8-11/treatment group) on diet and liver PL fatty acid composition and liver lipid weight were determined. Livers were histologically examined for steatosis and inflammation, and serum C-reactive protein (CRP) levels and glucose tolerance were assessed. Livers of D6KO mice consuming CD and FD were void of very long chain n-3 PUFA and enriched in ALA. D6KO fed FD had lower liver lipid accumulation, lower hepatic inflammation at 8 weeks, while serum CRP concentrations did not differ between treatment groups at 20 weeks. After 20 weeks FD KO animals also had higher glucose tolerance relative to LD KO mice. In conclusion, this study is the first to provide evidence that ALA may act independently on risk factors associated with NAFLD.
5.2 Introduction

In recent years, non-alcoholic fatty liver disease (NAFLD) has emerged as the leading liver disease in North America, and is garnering increased international attention.\textsuperscript{196} NAFLD is defined by the accumulation of lipids in hepatocytes in the absence of chronic alcohol consumption and viral infection, and is prevalent in individuals with metabolic abnormalities associated with Metabolic Syndrome (MetS).\textsuperscript{197} Both MetS and NAFLD are closely associated with obesity and insulin resistance,\textsuperscript{3} although NAFLD has also been characterized in individuals who are not obese but display other metabolic abnormalities.\textsuperscript{198}

Fat accumulation in the liver, or steatosis, marks the initiation of NAFLD. According to the commonly cited “two hit hypothesis” of NAFLD progression,\textsuperscript{199} In the presence of a metabolic “second hit” such as oxidative stress, simple steatosis can progress into steatohepatitis, cirrhosis, and in the most extreme cases, hepatocellular carcinoma. Histological scoring systems, such as the NAFLD activity score,\textsuperscript{200} have been developed to maintain clinical consistency in determining the presence and severity of NAFLD, with scores assigned for grade of steatosis, inflammatory cell infiltration, fibrosis and tissue destruction. Similar scoring systems have also been utilized in animal models of NAFLD.\textsuperscript{201}

Poor lifestyle and dietary habits are closely linked with the development of MetS and NAFLD. In particular, low polyunsaturated fatty acid (PUFA) intake is associated with increased prevalence of NAFLD.\textsuperscript{202} Indeed, n-3 PUFA are potent ligands of several transcription factors involved in fatty acid metabolism.\textsuperscript{8} Thus, dietary supplementation with eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), the principle n-3 highly unsaturated fatty acids (n-3 HUFA), has emerged as one promising method to prevent and treat liver lipid accumulation and inflammation associated with NAFLD.\textsuperscript{31,65,203,204}
Both n-3 and n-6 PUFA share the same metabolic pathway involved in essential fatty acid metabolism, and therefore compete for the same set of enzymes (Figure 4.1). Δ6 desaturase (D6D) is the rate limiting enzyme which initiates the metabolism of ALA and linoleic acid (LA). \(^{16}\) EPA and DHA are the principle products of several rounds of desaturation and elongation of ALA, while arachidonic acid (AA) is the primary metabolite of LA. Of interest, the partial pharmacological inhibition of D6D results in increased liver lipid content. \(^{205}\) Other studies have also reported that a partial or complete inhibition of elongases and desaturases result in fat accumulation in the liver. \(^{206}\) Thus, in addition to diet studies, the inhibition of select enzymes in essential fatty acid metabolism can also provide valuable insight into the association between dietary PUFA intake, endogenous PUFA production and the development of NAFLD.

While many studies have concluded that EPA and DHA can aid in the prevention of NAFLD and other metabolic conditions, little is known about the effects of the parent essential fatty acid α-linolenic acid (ALA), in treating NAFLD. A detailed characterization of the biological functions of ALA is needed, as ALA is the most widely consumed n-3 PUFA in the Western diet. North Americans consume 1.4g/day ALA on average, \(^{35}\) which amounts to ~0.6% of total daily energy requirements, and which meets the current Dietary Reference Intake of 1.1-1.6g/day ALA. \(^{34}\) In contrast, consumption of EPA and DHA combined is approximately 0.11g/day. Additionally, increased consumption of several ALA-rich oils such as flaxseed and chia seed oil has been associated with improvements in lipid abnormalities \(^{78,79}\) and inflammation; \(^{14,85}\) However, as all mammals have the capacity to convert ALA to EPA and DHA, beneficial effects associated with the consumption of ALA-rich foods cannot be directly attributed to ALA (Figure 4.1).
The recent generation of the Δ6 desaturase knock-out (D6KO) mouse has made it possible to study the independent effects of ALA for the first time. The D6KO mouse cannot synthesize the D6D enzyme and so is incapable of converting ALA or LA to their usual downstream fatty acid metabolites EPA, DHA or AA.\textsuperscript{18,164} All tissues of the D6KO mouse are deficient of very long chain PUFA if these fatty acids are not provided in the diet. Previous studies have utilized the D6KO mouse to evaluate the effects of PUFA restriction on reproduction, macrophage activity and intestinal and epidermal lesions.\textsuperscript{18,167} In the absence of D6D, Δ5 desaturase can metabolize LA and ALA to produce three novel fatty acids with unknown biological effects; however, our previous study\textsuperscript{207} reported that the production of these three fatty acids could be effectively inhibited through the provision of downstream PUFA in the diet. Thus, fatty acid metabolism in response to a variety of dietary regimens has been characterized in the D6KO mouse.\textsuperscript{18,164,207}

In the present study the D6KO mouse was utilized to determine the effects of ALA-rich diets on the PL fatty acid composition in the liver, the main organ involved in PUFA metabolism, and also to determine if ALA can independently prevent hepatic steatosis and inflammation associated with NAFLD. To further characterize the metabolic changes associated with NAFLD, glucose tolerance, systemic inflammation and weight gain were also evaluated. It was hypothesized that, similar to EPA and DHA, ALA-rich diets could prevent the development of risk factors associated with the development of NAFLD in a dose dependent manner. Results of this study support the use of ALA-rich oils to aid in the prevention of NAFLD.
5.3 Methodology

5.3.1 Experimental Animals and Diet

D6KO mice were obtained as a gift from Dr. Nakamura (University of Illinois at Urbana-Champaign), and backcrossed for 10 generations to a C57BL/6 background. Heterozygous D6KO mice were bred to generate experimental mice. Harems were maintained on an AIN76A diet (Research Diets) and pups were continued on this diet until 28 days old. The AIN76A diet is low in n-3 PUFA, thus reducing maternal transfer of n-3 PUFA to offspring during gestation and lactation. Mice were housed within ventilated cages at 22°C in a humidity-controlled environment on a 12 hour light: 12 hour dark cycle for the study duration. Mice were housed 1-4 per cage, monitored daily and weighed weekly.

Mice were weaned at twenty-one days old, and tail snipped for DNA extraction and PCR analysis to determine genotype. As phenotypic differences were reported between male and female D6KO mice, only male mice were used in this study. Twenty-eight day old D6KO and WT mice were placed on one of four modified AIN93G experimental diets (Table 5.1) for eight or twenty weeks (30% energy from fat, 2% cholesterol, 0.2% cholic acid; Research Diets): lard diet (LD, cat# D09071101), canola diet (CD, cat# D09071105), flaxseed diet (FD, cat# D0907110) or menhaden diet (MD, cat# D09071104). Both FD and CD diets contained 0.4% (w/w) ARASCO to provide sufficient dietary arachidonic acid to maintain the n-6 PUFA pathway so the n-3 PUFA pathway could be isolated for study. Additionally, providing sufficient AA, which is a fatty acid product downstream of D6D, prevented the alternate metabolism of LA and ALA by Δ5 desaturase. LD and MD diets naturally contained AA. Dietary fat, cholesterol and cholic acid content were based on previous studies of diet-induced
steatosis. Water and diet were provided *ad libidum* and refreshed weekly. Diets were stored sealed at 0°C and diet fatty acid composition was verified through gas chromatography (Table 5.2). This investigation was approved by the University of Guelph Animal Care Committee in accordance with the requirements of the Canadian Council on Animal Care.
Table 5.1: Composition of 30% lard, menhaden, canola, or flax modified AIN-93G rodent diet (provided by manufacturer, Research Diets)

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<td>200 800</td>
<td>200 800</td>
<td>200 800</td>
</tr>
<tr>
<td>L-Cystine</td>
<td>3 12</td>
<td>3 12</td>
<td>3 12</td>
<td>3 12</td>
</tr>
<tr>
<td>Corn starch</td>
<td>258 1032</td>
<td>258 1032</td>
<td>258 1032</td>
<td>258 1032</td>
</tr>
<tr>
<td>Maltodextrin 10</td>
<td>132 528</td>
<td>132 528</td>
<td>132 528</td>
<td>132 528</td>
</tr>
<tr>
<td>Sucrose</td>
<td>100 400</td>
<td>100 400</td>
<td>100 400</td>
<td>100 400</td>
</tr>
<tr>
<td>Cellulose, BW200</td>
<td>50 0</td>
<td>50 0</td>
<td>50 0</td>
<td>50 0</td>
</tr>
<tr>
<td>Lard</td>
<td>132 1188</td>
<td>0 0</td>
<td>0 0</td>
<td>0 0</td>
</tr>
<tr>
<td>Canola Oil</td>
<td>0 0</td>
<td>128.2 1154</td>
<td>0 0</td>
<td>0 0</td>
</tr>
<tr>
<td>Flaxseed Oil</td>
<td>0 0</td>
<td>0 0</td>
<td>128.2 1154</td>
<td>0 0</td>
</tr>
<tr>
<td>Menhaden Oil</td>
<td>0 0</td>
<td>0 0</td>
<td>0 0</td>
<td>132 1188</td>
</tr>
<tr>
<td>ARASCO, 40% ARA</td>
<td>0 0</td>
<td>3.8 34</td>
<td>3.8 34</td>
<td>0 0</td>
</tr>
<tr>
<td>t-Buthydroquinine</td>
<td>0.0264 0</td>
<td>0.0264 0</td>
<td>0.0264 0</td>
<td>0 0</td>
</tr>
<tr>
<td>Mineral Mix</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>S10022G</td>
<td>35 0</td>
<td>35 0</td>
<td>35 0</td>
<td>35 0</td>
</tr>
<tr>
<td>Vitamin Mix V10037</td>
<td>10 40</td>
<td>10 40</td>
<td>10 40</td>
<td>10 40</td>
</tr>
<tr>
<td>Choline Bitarate</td>
<td>2.5 0</td>
<td>2.5 0</td>
<td>2.5 0</td>
<td>2.5 0</td>
</tr>
<tr>
<td>Cholesterol</td>
<td>19 0</td>
<td>19 0</td>
<td>19 0</td>
<td>19 0</td>
</tr>
<tr>
<td>Sodium Cholic Acid</td>
<td>1.9 0</td>
<td>1.9 0</td>
<td>1.9 0</td>
<td>1.9 0</td>
</tr>
<tr>
<td>Total</td>
<td>943 4000</td>
<td>943 4000</td>
<td>943 4000</td>
<td>943 4000</td>
</tr>
</tbody>
</table>
Table 5.2: Fatty acid composition of 30% lard, menhaden, canola, or flax modified AIN-93G rodent diets

<table>
<thead>
<tr>
<th>Fatty Acid ( % total FA )</th>
<th>Lard</th>
<th>Canola + ARASCO</th>
<th>Flax + ARASCO</th>
<th>Menhaden</th>
</tr>
</thead>
<tbody>
<tr>
<td>14:0</td>
<td>1.2</td>
<td>0.2</td>
<td>0.1</td>
<td>7.7</td>
</tr>
<tr>
<td>16:0</td>
<td>20.1</td>
<td>4.6</td>
<td>5.6</td>
<td>19.2</td>
</tr>
<tr>
<td>16:1</td>
<td>1.7</td>
<td>0.3</td>
<td>0.1</td>
<td>11.2</td>
</tr>
<tr>
<td>18:0</td>
<td>11.1</td>
<td>2.3</td>
<td>3.5</td>
<td>3.7</td>
</tr>
<tr>
<td>18:1c9</td>
<td>35.0</td>
<td>56.7</td>
<td>16.2</td>
<td>6.5</td>
</tr>
<tr>
<td>18:1c11</td>
<td>2.5</td>
<td>3.3</td>
<td>0.8</td>
<td>3.2</td>
</tr>
<tr>
<td>18:2n6</td>
<td>22.3</td>
<td>18.8</td>
<td>15.3</td>
<td>1.9</td>
</tr>
<tr>
<td>18:3n6</td>
<td>0.0</td>
<td>0.1</td>
<td>0.1</td>
<td>0.4</td>
</tr>
<tr>
<td>18:3n3</td>
<td>1.0</td>
<td>7.5</td>
<td>54.9</td>
<td>1.7</td>
</tr>
<tr>
<td>18:4n3</td>
<td>0.3</td>
<td>0.0</td>
<td>0.0</td>
<td>3.5</td>
</tr>
<tr>
<td>20:0</td>
<td>0.3</td>
<td>0.7</td>
<td>0.3</td>
<td>0.4</td>
</tr>
<tr>
<td>20:1</td>
<td>0.7</td>
<td>1.2</td>
<td>0.2</td>
<td>1.0</td>
</tr>
<tr>
<td>20:2n6</td>
<td>0.6</td>
<td>0.0</td>
<td>0.0</td>
<td>0.2</td>
</tr>
<tr>
<td>20:3n6</td>
<td>0.2</td>
<td>0.0</td>
<td>0.2</td>
<td>0.4</td>
</tr>
<tr>
<td>20:4n6</td>
<td>0.5</td>
<td>1.5</td>
<td>1.3</td>
<td>1.4</td>
</tr>
<tr>
<td>20:3n3</td>
<td>0.4</td>
<td>0.0</td>
<td>0.2</td>
<td>0.3</td>
</tr>
<tr>
<td>20:5n3</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
<td>15.2</td>
</tr>
<tr>
<td>22:0</td>
<td>0.0</td>
<td>0.6</td>
<td>0.3</td>
<td>0.4</td>
</tr>
<tr>
<td>22:1</td>
<td>0.1</td>
<td>0.4</td>
<td>0.0</td>
<td>0.4</td>
</tr>
<tr>
<td>22:4n6</td>
<td>0.1</td>
<td>0.1</td>
<td>0.0</td>
<td>0.3</td>
</tr>
<tr>
<td>22:5n6</td>
<td>0.1</td>
<td>0.3</td>
<td>0.1</td>
<td>0.7</td>
</tr>
<tr>
<td>22:5n3</td>
<td>0.2</td>
<td>0.0</td>
<td>0.0</td>
<td>3.1</td>
</tr>
<tr>
<td>24:0</td>
<td>0.1</td>
<td>0.2</td>
<td>0.2</td>
<td>0.0</td>
</tr>
<tr>
<td>22:6n3</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
<td>14.9</td>
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<td>24:1</td>
<td>0.2</td>
<td>0.3</td>
<td>0.1</td>
<td>0.9</td>
</tr>
<tr>
<td>sum</td>
<td>99</td>
<td>99</td>
<td>100</td>
<td>99</td>
</tr>
</tbody>
</table>

| % Saturated              | 32.8 | 8.6            | 10.0          | 31.4 |
| % Monounsaturated        | 40.2 | 62.2           | 17.4          | 23.2 |
| % n-3 Polyunsaturated    | 1.9  | 7.5            | 55.1          | 38.7 |
| % n-6 Polyunsaturated    | 23.8 | 20.8           | 17.0          | 5.3  |

NOTE: Values are the means of analysis in duplicate and represent percent of total fatty acids in each diet. Lipids were extracted by the methods of Folch et al.,\textsuperscript{178} methylated and total lipids were analyzed using gas chromatography.
5.3.2 DNA extraction and Genotyping by PCR

At weaning a 0.3 cm section of tail was collected in an eppendorf tube and frozen at -20°C. To extract DNA, 500 µl of tail buffer and 35 µl of 10 mg/ml Protease K (Invitrogen #25530-015) were added to tail snips, which were then incubated at 55°C overnight in a dry heat block. Tail buffer was composed of 50Mm Tris, pH 8.0; 100 mM NaCl; 1% sodium dodecyl sulfate (SDS); and 25 mM EDTA, pH 8.0.

Following overnight incubation, 500 µl buffer saturated phenol (Invitrogen UltraPure™ # 15513-039) was added to each digested sample, vortexed, and centrifuged at 496 xg at 4°C for 10 minutes (ThermoScientific Sorvall Legen Micro 21R) to separate phases. The aqueous phase was extracted and added to a new eppendorf tube containing 1 ml ice cold 95% ethanol. Samples were gently tipped to mix contents, and chilled for 20 min at -20°C. Samples were then centrifuged to pellet DNA at 496 xg, 4°C for 10 min. Supernatant was discarded, and 1 ml 70% ethanol was added to pelleted samples. Samples were again centrifuged at 496 xg at 4°C for 10 min, supernatant was discarded, and 1 ml 99% ethanol was added to rinse DNA pellets. Samples were spun down at 496 xg at 4°C for 4 min, and supernatant was carefully aspirated. DNA pellet was suspended in 25 µl Tris-EDTA (TE) buffer and mixed thoroughly.

For PCR determination of genotype, 0.5 µl suspended DNA sample was added to 24.5 µ of appropriate master mix and mixed well. Master mix was composed of: DNase/RNase-free PCR H₂O (Invitrogen, #10977-015); 10X PCR buffer, 50 mM MgCl₂, and 0.2 µl/reaction Platinum Taq Polymerase (Invitrogen #10966-034), 10 mM dNTP’s (Fermentas #R0192) and appropriate primers. Master mix solution containing DNA samples were then inserted into a thermocycler (Applied Biosystems Veriti 96 Well Thermal Cycler) to amplify DNA.
Thermocycler parameters were: 95°C for 2 min; 35 cycles of 95°C for 30 s, 60°C for 30 s, 72°C for 90 s; 5 min at 72°C, then cooled to 4°C. 12 µl amplified DNA was separated by gel electrophoresis (110 V, 45 min, BioRad Powerpac) in a 150 ml 2% agarose gel (Sigma #A5093-100G) with ethidium bromide (Fisher #BP1302-10). DNA samples were mixed with 2 µl 6x loading dye and run along a 100 bp DNA ladder (Fermentas #SM0241). Gels were visualized with a Fluorchem HD2 imaging system using UV light and an EtBr filter.

5.3.3 Intra-peritoneal Glucose Tolerance Test

After either 8 or 20 weeks of experimental diet, mouse weights were recorded (final mass) and mice were fasted overnight (~14 h). Mice were placed into a new cage containing only wood shavings to remove all potential food sources. Fresh water was provided ad libidum to all mice during the fast and subsequent glucose tolerance test procedure. Following the overnight fast, mice were weighted (morning mass) and were given intra-peritoneal injections of 30% glucose solution (MP Biomedicals, #194024) at a dose of 0.75g/kg (morning mass). Tail vein blood glucose was measured before glucose injection, and at 10, 20, 30, 40, 50, 60 and 90 minutes following injection (Contour blood glucose meter; Bayer). Following IPGGT, experimental mice were euthanized with CO₂ gas. Glucose values were plotted against time after injection, and area under the curve was calculated using the trapezoidal method.²⁰⁹
5.3.4 Tissue Sample Collection

Immediately following euthanization, blood was collected via cardiac puncture, allowed to coagulate at room temperature for 20 minutes in an uncoated eppendorf tube, and then centrifuged (5 min at 10 xg) to separate serum from solid blood components. Serum was snap frozen and stored at -80°C. Livers were weighed, and half of the medial lobe was fixed in 10% formalin for sectioning and staining, while remaining liver tissue was snap frozen and stored at -80°C for fatty acid analysis. Epididymal adipose tissue was collected, weighed; snap frozen and stored at -80°C.

5.3.5 Fatty Acid Analysis

PL fatty acid analysis of liver tissue was carried out as detailed in Chapter 4.3.2 for both 8 week and 20 week time-point mice. Total fatty acid composition of each diet was determined using Agilent 7890A GC.

5.3.6 Histology

Following fixation in 10% formalin, liver segments were sectioned and stained for histological evaluation of steatosis and inflammatory cell infiltration. Liver sections were stained with hematoxylin and eosin (H&E) and trichrome, and light microscopy was used to evaluate each stained section for microvesicular and macrovesicular fatty acid changes, inflammatory (lymphocyte) cell infiltration and fibrosis. A score from 0-3 was assigned for
steatosis (0=<5%; 1= 5-33%; 2= 33-66%; 3= >66%) and inflammation (0= no inflammation; 1= 1-2 foci; 2= 2-4 foci; 3= >4 foci) using the standardized clinical NAFLD activity score.200

5.3.7 Biochemical Analysis

The circulating CRP concentration, an indicator of inflammation, was measured (20 week time-point, n=4-5/treatment group) in duplicate with an enzyme-linked immunosorbent assay (Kamiya Biomedical Company #KT-095), following all manufacturer instructions.

5.3.8 Statistical Analysis

Data were analyzed using SAS (v. 9.1) statistical software. Two-way analysis of variance (ANOVA) test was performed to determine main effects of diet and genotype, followed by Tukey post-hoc tests on main effects when the interaction term was not significant; Least Squares Means was used when interaction values were significant. Statistical significance was set at p ≤ 0.05. Two-way ANOVA was used to determine main effect of diet and genotype on glucose values at each time point during the GTT. Chi-square test was used to determine differences in steatosis and inflammation scores between treatment groups. Statistical significance was set at p < 0.05. Values are expressed as means ± SD.
5.4 Results

5.4.1 Final Weight and Organ Weights

Upon termination at 8 weeks, mean mouse weight (Table 5.3) did not differ between treatment groups. At 20 weeks, with the exception of MD mice, all D6KO treatment groups were significantly (p < 0.05) lighter upon termination than their WT counterparts.

Mean liver weights relative to total body mass (% liver mass) did not significantly differ between treatment groups at 8 weeks (Table 5.3). At 20 weeks a significant (p < 0.05) diet and genotype effect was present; mean liver mass of MD mice was significantly lower than all other diet groups, while D6KO mice had significantly higher liver weights relative to WT mice.

Adipose mass at 8 weeks did not differ between treatment groups. At 20 weeks, the LD, CD and FD KO groups had accumulated significantly (p < 0.05) less adipose tissue relative to WT mice (Table 5.3). Additionally, FD and CD D6KO mice had significantly (p < 0.05) less abdominal adipose tissue compared with MD D6KO mice.
**Table 5.3**: Mouse final body weight and relative organ weight at 8 and 20 weeks

<table>
<thead>
<tr>
<th>Diet</th>
<th>Final Body Weight</th>
<th>% Liver mass</th>
<th>% adipose mass*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lard</td>
<td>WT KO</td>
<td>WT KO</td>
<td>WT KO</td>
</tr>
<tr>
<td></td>
<td>8 Weeks</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>24.2 ± 2.8</td>
<td>5.9 ± 1.5</td>
</tr>
<tr>
<td></td>
<td></td>
<td>26.1 ± 4.4</td>
<td>7.0 ± 1.2</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>33.9 ± 4.0</td>
<td>5.1 ± 1.1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>a 30.1 ± 2.4</td>
<td>6.7 ± 1.0</td>
</tr>
<tr>
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<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Values are means ± SD. 2-way ANOVA for main effect of diet, genotype and interaction. Values with different letters denote significant differences between treatment group means when interaction variable was significant (8 weeks: n= 6-8; 20 weeks n= 8-11). *mass of epididymal fat pads
Table 5.4: Comparison of hepatic phosphatidylcholine fatty acid percent composition across treatment groups at 20 weeks.

<table>
<thead>
<tr>
<th>Fatty Acid</th>
<th>Wild-Type</th>
<th>Knock-Out</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>LD</td>
<td>CD</td>
<td>FD</td>
</tr>
<tr>
<td>c16:0</td>
<td>32.7 ± 3.4&lt;sup&gt;b&lt;/sup&gt;</td>
<td>30.3 ± 1.8&lt;sup&gt;c&lt;/sup&gt;</td>
<td>29.8 ± 1.6&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
<tr>
<td>c18:0</td>
<td>2.1 ± 0.3</td>
<td>1.3 ± 0.3</td>
<td>1.4 ± 0.4</td>
</tr>
<tr>
<td>c20:0</td>
<td>0.1 ± 0.1</td>
<td>0.1 ± 0.1</td>
<td>0.2 ± 0.1</td>
</tr>
<tr>
<td>c22:0</td>
<td>nd</td>
<td>nd</td>
<td>trace</td>
</tr>
<tr>
<td>c18:1</td>
<td>2.1 ± 0.3</td>
<td>1.3 ± 0.3</td>
<td>1.4 ± 0.4</td>
</tr>
<tr>
<td>c20:1</td>
<td>13.4 ± 1.1&lt;sup&gt;d&lt;/sup&gt;</td>
<td>17.9 ± 2.8&lt;sup&gt;b&lt;/sup&gt;</td>
<td>10.3 ± 1.0&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>c22:1</td>
<td>0.2 ± 0.4</td>
<td>0.4 ± 0.6</td>
<td>0.2 ± 0.4</td>
</tr>
<tr>
<td>18:2n6 LA</td>
<td>17.1 ± 2.4&lt;sup&gt;c&lt;/sup&gt;</td>
<td>15.7 ± 0.7&lt;sup&gt;f&lt;/sup&gt;</td>
<td>19.3 ± 1.0&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
<tr>
<td>18:3n6</td>
<td>0.2 ± 0.3</td>
<td>0.1 ± 0.2</td>
<td>0.1 ± 0.1</td>
</tr>
<tr>
<td>20:4n6 AA</td>
<td>12.2 ±2.2&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>11.4 ± 1.7&lt;sup&gt;b&lt;/sup&gt;</td>
<td>11.4 ± 1.5&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>22:5n6</td>
<td>0.2 ± 0.2&lt;sup&gt;a&lt;/sup&gt;</td>
<td>nd&lt;sup&gt;b&lt;/sup&gt;</td>
<td>nd&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>20:3 (novel Δ5)</td>
<td>trace&lt;sup&gt;bc&lt;/sup&gt;</td>
<td>nd&lt;sup&gt;c&lt;/sup&gt;</td>
<td>trace&lt;sup&gt;bc&lt;/sup&gt;</td>
</tr>
<tr>
<td>18:3n3 ALA</td>
<td>0.1 ± 0.1&lt;sup&gt;e&lt;/sup&gt;</td>
<td>0.7 ± 0.2&lt;sup&gt;d&lt;/sup&gt;</td>
<td>4.9 ± 0.7&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>20:5n3 EPA</td>
<td>trace&lt;sup&gt;d&lt;/sup&gt;</td>
<td>0.6 ± 0.1&lt;sup&gt;c&lt;/sup&gt;</td>
<td>2.7 ± 0.6&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>22:5n3</td>
<td>0.1 ± 0.2&lt;sup&gt;d&lt;/sup&gt;</td>
<td>0.2 ± 0.2&lt;sup&gt;ed&lt;/sup&gt;</td>
<td>0.7 ± 0.3&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>22:6n3 DHA</td>
<td>4.6 ± 0.8&lt;sup&gt;b&lt;/sup&gt;</td>
<td>5.6 ± 0.9&lt;sup&gt;b&lt;/sup&gt;</td>
<td>3.4 ± 0.5&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>20:4(novel Δ5)</td>
<td>nd&lt;sup&gt;b&lt;/sup&gt;</td>
<td>nd&lt;sup&gt;b&lt;/sup&gt;</td>
<td>nd&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>22:4(novel Δ5)</td>
<td>nd&lt;sup&gt;b&lt;/sup&gt;</td>
<td>nd&lt;sup&gt;b&lt;/sup&gt;</td>
<td>nd&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

Note: Values are presented as mean ± SD (n = 8-11). nd = not detected. Trace = <0.05% of total fatty acids; n= 8-10/treatment group. 2-way ANOVA with significant main effect of diet, genotype and interaction, letters denote significant difference between groups when interaction was significant. SFA, saturated fatty acid; MUFA, monounsaturated fatty acid.
5.4.2 Liver fatty acid composition

As fatty acid composition was similar across the PL fractions, the fatty acid profile of PC, the most abundant PL fraction in the liver, is presented. By 8 weeks, liver PL of LD, FD, and CD D6KO mice were enriched in LA and ALA (Figure 5.1A, B). Hepatic PL of FD D6KO mice had the highest ALA content relative to all other treatment groups (Figure 5.1B). MD D6KO and MD WT mice had equivalent LA and ALA values.

Hepatic fatty acid composition at 20 weeks was comparable to the fatty acid profile at 8 weeks; the hepatic PL of FD D6KO mice continued to contain significantly more ALA than all other treatment groups (Table 5.4). MD WT and MD D6KO groups maintained a significantly lower percentage of AA (Figure 5.1C; p < 0.05) than all other WT and D6KO treatment groups at both time points. Hepatic PL of LD D6KO mice contained significantly less AA relative to all other treatment groups.

By 8 weeks, n-3 HUFA had been nearly eliminated from the liver PLs of LD, FD and CD D6KO mice (Figure 5.1D). In comparison, MD D6KO mice maintained equivalent HUFA composition to MD WT mice.

Together, the three novel Δ-5 desaturase derived fatty acids 20:3n6 (Δ7, 11, 14), 20:4n3 (Δ7, 11, 14, 17) and 22:4n3 (Δ9, 13, 16, 19) comprised <1% of total fatty acids in the PC fraction of each treatment group. A similar distribution of novel Δ-5 fatty acids was seen at 20 weeks (Table 5.4).
By 8 weeks, hepatic PC is enriched in essential fatty acids, and void of very long chain n-3 PUFA in D6KO animals fed lard, canola and flaxseed oil diets. A) % LA, B) % ALA, C) % AA, D) % DHA. Livers of D6KO mice not provided with dietary long chain n-3 and n-6 PUFA are enriched in LA and/or ALA, and void in DHA (n = 6-8/group). Data is presented as mean percentage of total fatty acids in the PC fraction; letters indicate significant differences between groups as determined by 2-way ANOVA with significant main effects of diet, genotype and interaction.
5.4.3 Liver Lipid Weight

After 8 weeks on diet, mouse liver lipid weight differed significantly between KO mice; (Figure 5.2A). Liver lipid weights from lowest to highest were MD < FD < CD, LD.

By 20 weeks, the differences in liver lipid masses seen at 8 weeks had diminished (Figure 5.2B). While differences in liver lipid weight between treatment groups were not significantly different, a diet effect was present. FD mice had significantly lower liver lipid mass than LD mice, while CD mice had a mean liver lipid mass intermediate between LD and FD mice. MD liver lipids were significantly lower than all other treatment groups.
Figure 5.2. Mean liver lipid mass per gram liver tissue is associated with diet. A) At 8 weeks FDKO and MDKO groups have significantly lower mean lipid accumulation relative to the LDKO group. 2-way ANOVA with significant effect of diet, genotype and interaction, letters denote significant difference between groups (n = 6-8), (p < 0.05)  B) At 20 weeks, WT and KO mice did not significantly differ in mean liver lipid accumulation, a diet effect is present (n = 8-11). Letters denote significant difference between diet groups, as analyzed by Tukey’s Post Hoc test (p < 0.05).
5.4.4 Tissue Histology

At 8 weeks, differences in hepatic inflammation were present between genotypes and diet groups. CD and FD D6KO groups had inflammation scores intermediate between LD and MD D6KO scores (Table 5.5). By 20 weeks this trend had disappeared as LD, FD and CD D6KO groups all had significantly higher mean inflammation scores than MD D6KO mice (Table 5.5). At both time-points D6KO inflammation score means were not significantly different from WT scores, with the exception of FD D6KO mean inflammation score, which was significantly lower than the mean FD WT score at 20 weeks.

At 8 weeks, CD and FD D6KO mice had the highest steatosis scores (Table 5.5). In comparison, LD, FD and CD WT mice had equivalent steatosis scores. By 20 weeks there was no statistical difference between mean steatosis scores of LD, CD, and FD mice of both genotypes (Table 5.5; Figure 5.3A-C); all had significantly higher scores relative to MD mice (Figure 5.3D).

Comparing between scores at 8 and 20 weeks, steatosis scores tended to increase over time; however the FD D6KO steatosis score was significantly lower at 20 weeks. Evidence of hepatic fibrosis or necrosis was not observed in any treatment group.
Table 5.5: Liver steatosis and Inflammation scores at 8 and 20 weeks

<table>
<thead>
<tr>
<th>Treatment Group</th>
<th>Inflammation Score</th>
<th>Steatosis Score</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>8 Weeks</td>
<td>20 weeks</td>
</tr>
<tr>
<td>LD WT</td>
<td>1.7&lt;sup&gt;a&lt;/sup&gt;</td>
<td>2.1&lt;sup&gt;ab&lt;/sup&gt;</td>
</tr>
<tr>
<td>LD KO</td>
<td>1.7&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1.9&lt;sup&gt;ab&lt;/sup&gt;</td>
</tr>
<tr>
<td>CD WT</td>
<td>1.1&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>1.7&lt;sup&gt;ab&lt;/sup&gt;</td>
</tr>
<tr>
<td>CD KO</td>
<td>1.1&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>1.8&lt;sup&gt;ab&lt;/sup&gt;</td>
</tr>
<tr>
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<td>1&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>2.4&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>FD KO</td>
<td>0.7&lt;sup&gt;bc&lt;/sup&gt;</td>
<td>1.5&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>MD WT</td>
<td>0.5&lt;sup&gt;bc&lt;/sup&gt;</td>
<td>0&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>MD KO</td>
<td>0.3&lt;sup&gt;c&lt;/sup&gt;</td>
<td>0.2&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

Note: values with different letters indicate significant (p < 0.05) difference between groups within a column, Chi-Square test. (8 weeks, n = 6-8; 20 weeks, n = 8-10).

Figure 5.3. Representative H&E stained sections of D6KO mouse livers following 20 weeks of one of four dietary treatments: A) Lard, B) Canola, C) Flax, D) Menhaden diet. D6KO mice fed menhaden oil (MD) had significantly lower inflammation and steatosis scores than all other treatment groups.
5.4.5 Serological Profile

At 20 weeks mean serum CRP concentrations did not significantly differ between treatment groups (Figure 5.4).

5.4.6 Whole Body Glucose Tolerance Test

At 8 weeks, glucose tolerance test (GTT) curves and area under the curve (AUC) values (Figure 5.5) between genotypes and diets were not significantly different.

A diet effect was present for differences in blood glucose and AUC values at 20 weeks (Figure 5.6). FD and MD-fed animals had significantly lower fasting blood glucose in comparison to lard and canola fed animals (Figure 5.7). Additionally, FD and CD groups had AUC values intermediate between LD and MD values (Figure 5.6).

Figure 5.4. Serum CRP levels at 20 weeks. CRP levels were not significantly different between treatment groups. (n=4-5/treatment group).
Figure 5.5. Diet and genotype are not associated with blood glucose clearance at 8 weeks ($n = 6-8$).

![Graph showing glucose clearance at 8 weeks](image)

Figure 5.6. Glucose tolerance is modified by diet by 20 weeks. Two-way ANOVA was significant ($p < 0.05$) for main effect of diet; different letters denote significant differences between diet groups ($n = 8-11$).

![Graph showing glucose tolerance at 20 weeks](image)
Figure 5.7. Mean GTT curve of the FD group is intermediate between LD and MD treatment groups at 20 weeks. Blood glucose of CD animals did not significantly differ from LD blood glucose values for the duration of the GTT. 2-way ANOVA was significant (p < 0.05) for main effect of diet; different letters represent significant differences between diet groups at each time.
5.5 Discussion

Long chain n-3 PUFA have long been acknowledged as bioactive metabolites,\textsuperscript{20,39} and various mechanisms for their direct and indirect effects in combatting MetS and its associated pathologies have been described.\textsuperscript{5} In contrast, while the metabolic effects of ALA-rich oils have been investigated,\textsuperscript{12,122,173} such studies cannot firmly attribute findings exclusively to ALA, given that some ALA is converted to EPA and DHA. To our knowledge, this is the first study to provide evidence that ALA can act independently to prevent the development of hepatic steatosis and inflammation associated with NAFLD. In this study, D6KO mouse was effectively used to evaluate the independent effects of ALA in disease prevention, as the tissues of the D6KO mouse are void of n-3 HUFA if these fatty acids are not provided in the diet.

In the present study, the metabolic outcomes of D6KO mice supplemented with canola oil+ ARASCO or flaxseed oil+ ARASCO, which contain ~8% ALA or ~55% ALA respectively, were compared to D6KO control animals supplemented with either lard, which contains negligible n-3 PUFA, or menhaden oil which is rich in EPA and DHA. Three potential outcomes were predicted. First, resulting similarities between D6KO animals fed canola or flaxseed oil, and lard-fed animals would suggest that the beneficial effects of ALA arose entirely through conversion to EPA and DHA. Secondly, if D6KO mice fed diets containing ALA and menhaden oil performed similarly, this would indicate that ALA could act independently, as well as through conversion, to modify disease progression. Thirdly, if outcome measures of D6KO mice fed ALA were intermediate between lard-fed and menhaden-fed D6KO groups, this would suggest that ALA had biological benefits which approached those conferred by EPA/DHA. Through contrasting the effects of these four dietary treatments on several metabolic outcomes, this study
presented evidence that ALA-rich oils have effects which approach those of fish oil in the prevention of hepatic steatosis and inflammation.

5.5.1 Final weight and organ weights are influenced by dietary fat quality

At 20 weeks, the three D6KO groups which consumed diets deficient in EPA and DHA (i.e LD, CD, and FD) weighted significantly less upon termination than associated WT mice. Additionally, while FD and CD D6KO mice had lower final weights and less visceral adiposity relative to MD D6KO mice by 20 weeks, CD and FD groups had the heaviest livers. While blood lipids were not measured, these findings suggest that in FD and CD D6KO mice, lipids accumulate in the liver with reduced transport to peripheral fat stores.

In general, increased adiposity is strongly associated with the development of hepatic steatosis; however, visceral adiposity is not a prerequisite for the development of fatty liver. In the methionine-choline deficient model of NAFLD, the livers of choline-deficient animals are unable to produce VLDL to export lipids, resulting in the development of fatty liver along with a reduction in adiposity. Similarly, overexpression of nuclear SREBP-1c in adipose tissue of mice resulted in lipodystrophy, characterized by marked reduction in adipose tissue, along with severe fatty liver. This phenotype is similar to the phenotype exhibited by FD and CD D6KO mice in this study. As n-3 PUFA are potent inhibitors of SREBP-1c, uninhibited SREBP-1c activity in LD, CD and FD D6KO mice may have contributed to adipose lipolysis and increased liver lipid accumulation over time, similar to changes observed in nSERBP-1c transgenic mice. Additionally, increased SREBP-1c activity in the liver in the absence of DHA would have activated enzymes associated with hepatic fatty acid synthesis, further contributing to the
development of fatty liver. Future studies with the D6KO model may aim to determine how the rate of adipogenesis/adipose lipolysis and SREBP-1c activity contribute to the development of fatty liver.

5.5.2 Liver PL composition is altered by diet

Eight weeks after supplementation began, it was confirmed that the livers of D6KO mice fed canola and flaxseed oil contained significantly less very long chain n-3 PUFA relative to WT animals. The liver PLs of flaxseed and canola fed D6KO animals also contained higher levels of ALA compared to WT mice. In addition, the production of novel Δ5 desaturase fatty acids was greatly diminished relative to studies in which fatty acids downstream of D6D were not provided.207 Together, these findings validated the use of the D6KO mouse model in investigating the role of ALA in preventing hepatic steatosis, independent of the effects of EPA and DHA. Not surprisingly, a significant increase in LA was also observed in D6KO animals which consumed HUFA-deficient diets. MD D6KO animals had liver lipid profiles that mirrored those of their WT counterparts; supporting our previous finding that menhaden oil supplementation can indeed provide the requisite long chain PUFA to rescue the D6KO fatty acid profile.

Of interest, after 24 weeks of consuming diets completely void of n-3 HUFA, many D6KO animals maintained trace levels of EPA and DHA in liver PL. One dietary study evaluating n-3 deficiency in rodents reported the presence of trace levels of DHA in the brains and retinas of 7 week old rat pups maintained on the same n-3 deficient diets as their mothers.176 As in this study, trace levels of DHA continue to be detected in the 2nd generation of absolute n-3
restriction; thus, maternal transfer of DHA must have occurred. Similarly, in this study, persistence of DHA in liver PL of D6KO mice after prolonged n-3 HUFA restriction suggested that at least some DHA obtained through maternal transfer via the milk from heterozygous D6KO dams was maintained into adulthood. This finding, in line with the findings of previous n-3 restriction studies,\textsuperscript{176,214} supports the importance of DHA.

5.5.3 Dietary ALA results in a lower liver lipid weight

At 8 weeks, liver lipid accumulation was significantly greater in LD and CD D6KO mice relative to WT groups, while smaller differences in liver lipid weight relative to WT animals were observed in FD and MD D6KO groups (Figure 5.2). This finding supports the initial hypothesis that ALA is associated with lower liver lipid accumulation, although EPA and DHA have much more potent effects in preventing steatosis. Differences in liver lipid weight between genotypes disappeared at 20 weeks, but a significant diet effect remained, suggesting that ALA-rich oils were also associated with lower liver lipid accumulation over longer durations. Given that both D6KO and WT mice fed CD and FD were similar in response at 20 weeks, this further supports the notion that the effects observed are specific to ALA, as D6KO mice had no EPA/DHA.

Trends in liver lipid weight data at both 8 and 20 weeks supported a beneficial effect of ALA exclusively at high doses, as liver lipid weights of CD mice and LD mice did not significantly differ. However, as MD D6KO mice had several-fold lower liver lipid mass relative to FD D6KO mice, preformed HUFA were still shown to provide the greatest inhibition of liver lipid accumulation.
5.5.4 ALA does not lead to lower steatosis scores but improves hepatic inflammation

In contrast to trends in liver lipid weight data at 8 weeks, D6KO mice fed oils containing ALA had the highest steatosis scores. Methodological aspects could account for differences in these two methods of evaluating liver lipid accumulation, as histological scores provide a qualitative measure of steatosis on a 2-dimensional plane, while lipid extraction and weighing provides a direct quantitative measure of liver lipid accumulation. At 20 weeks, trends in steatosis scores more closely reflected liver lipid weight data. At this later time-point, ALA-rich oils did not seem to protect against steatosis in either WT or KO animals.

Of note, the steatosis scores decreased over time in CD and FD D6KO mice; this reduction was significant in FD D6KO mice. A similar trend was observed in liver lipid mass data. There are several interpretations for this observation. First, decreased steatosis scores are associated with increased fibrosis;\textsuperscript{215} however, this could not be possible as fibrosis and signs of necrosis were not observed in any treatment groups. Alternately, a build-up of ALA in FD D6KO livers over time could trigger increased fat oxidation and decreased lipogenesis, causing a reduction in lipid accumulation. A similar situation has been described in the fatty acyl-CoA (AOX) knockout mouse,\textsuperscript{216} which cannot produce a critical enzyme required for β-oxidation. The AOX knockout mouse model develops steatohepatitis, but, by 6-8 months the hepatic accumulation of long chain fatty acids results in the over-activation of PPAR-α, causing a reversal of steatosis. Thus, it is feasible that accumulating ALA in FD and CD D6KO mice could trigger similar PPAR-α dependent mechanisms as those triggered by ALA’s long chain metabolites in the AOX knockout mouse in order to initiate a similar reversal of steatosis after several months. However, evaluation of hepatic gene expression would be required to determine if PPAR-α was significantly up-regulated by 20 weeks of experimental treatment.
At 8 weeks, liver inflammation scores of FD and CD D6KO mice were intermediate between LD and MD D6KO mice. The trend in liver inflammation scores at 8 weeks supported an independent and dose dependent role of ALA in preventing early inflammatory cell infiltration of the liver. At 20 weeks, this trend in inflammation scores disappeared, as LD, CD and FD D6KO mice all had statistically similar inflammation scores. However, WT LD, FD and CD groups also had statistically similar inflammation scores, suggesting that the anti-inflammatory benefits conferred by ALA attenuated over time.

Overall, ALA-rich oils were associated with reduced hepatic inflammation at an early stage of NAFLD. Additionally, steatosis scores did not provide evidence to support direct or indirect protective effects of ALA, but 8 week steatosis scores did suggest that the inhibition of ALA’s conversion to EPA and DHA could exacerbate liver lipid accumulation. Additionally, it is possible that ALA may act independently over time to reverse steatosis in the absence of long chain n-3 PUFA.

5.5.5 Serum CRP levels are not effected by diet

Serum CRP values did not significantly differ between treatment groups. CRP is primarily synthesized in the liver,217 and is commonly used as a marker of systemic inflammation in humans and rodent models. While it is an acute response protein, CRP levels are also raised during chronic inflammation associated with several metabolic diseases218 including fatty liver disease.219,220 ALA deficiency leads to elevated levels of CRP in mice,221 while supplementation with ALA has been shown to reduce circulating CRP in rodent and clinical studies.85,221,222 This then provided the rationale for examining CRP values across treatment groups. However, CRP is one of several inflammatory markers which may be elevated
in mouse models of NAFLD.\textsuperscript{223} While there were no differences in serum CRP observed in this study, the presence of systemic inflammation cannot be ruled out, as histological analysis of the livers provided clear evidence of moderate to severe inflammatory cell infiltration in several treatment groups by 20 weeks.

5.5.6 ALA is associated with improved glucose tolerance

Glucose tolerance did not differ across treatment groups at 8 weeks; however, a diet effect was present at 20 weeks. Raised fasting blood glucose concentrations signal the potential development of insulin resistance, and are associated with the development of NAFLD in rodent studies.\textsuperscript{223} Differences in body weight could not solely account for differences in glucose tolerance between diet groups, as mean body weights of MD and LD groups were similar, yet MD animals had significantly lower AUC values relative to LD mice. The ability of ALA-rich oils to modify blood glucose clearance is controversial, as some\textsuperscript{224} but not all\textsuperscript{225,226} studies have found flaxseed oil to have a beneficial effect. In this study, animals which consumed flaxseed oil had significantly lower fasting blood glucose relative to lard-fed animals, supporting an independent protective effect of ALA on the prevention of glucose intolerance. As canola oil-fed animals did not have blood glucose values that significantly differed from the lard treatment group, ALA seems to have an effect on glucose tolerance strictly at high doses. However, the mechanisms by which ALA-rich flaxseed oil improved glucose tolerance were not explored in this study.

While insulin resistance and steatosis commonly occur together, a causal relationship between these conditions has not been firmly established.\textsuperscript{227} In this study insulin resistance was not measured directly; however, steatosis developed before changes in glucose tolerance were
observed. Other dietary or genetic rodent studies have found that the development of fatty liver preceded the initiation of glucose intolerance and insulin resistance.\textsuperscript{228,229} In particular, one study\textsuperscript{229} reported that hepatic lipid accumulation was associated with increased gluconeogenesis and impaired intracellular insulin signalling resulting in reduced glycogen synthesis. Thus, the early development of hepatic steatosis may lead to perturbations in glucose homeostasis over a longer time-frame.

Interestingly, a genotype effect was also observed; when grouped together, KO mice had significantly lower AUC values relative to WT mice at 20 weeks. One explanation for this observation is that the deletion of D6D may have conferred protection against glucose intolerance. In the ApoE null mouse, another mouse model of MetS, animals develop hypercholesterolemia, hypertriglyceridemia, atherosclerosis and fatty livers; however, they have significantly lower plasma glucose levels relative to WT mice.\textsuperscript{230} In this model, while most features of the MetS were present, ApoE null mice are protected from glucose intolerance through an undetermined mechanism. Future work with the D6KO model may aim to determine how inhibition of HUFA synthesis provides protection against glucose intolerance.
5.6 Summary and Conclusions

Of note, this study has provided evidence that variation in the quantity and type of dietary PUFA strongly influences metabolic outcomes. Various protective effects were observed in animals provided with high dietary ALA, as FD D6KO mice consistently had lower liver lipid masses, were slower to develop hepatic inflammation, and had improved glucose tolerance at 20 weeks. However, animals provided with the low-ALA canola diet performed similarly to animals fed the n-3 depleted lard diet. This suggests that the protective effects of ALA are conferred at high levels of intake through the consumption of ALA-rich oils such as flaxseed oil, but also possibly through raised consumption of oils with moderate ALA contents such as canola oil. Other studies have also suggested that several-fold greater quantities of ALA must be consumed to provide the similar benefits to EPA and DHA.\textsuperscript{231}

A diet rich in EPA and DHA provided the strongest protection against steatosis, hepatic inflammation and glucose intolerance despite the fact that menhaden fed D6KO mice were significantly heavier and had significantly more abdominal adiposity than all other D6KO treatment groups at 20 weeks. Indeed, while central adiposity is a major risk factor for the development of MetS and fatty liver disease,\textsuperscript{25} this study found that increased liver lipid accumulation seemed to be more strongly associated with the development of adverse metabolic events. Furthermore, it was the quality of fat ingested that most affected liver lipid accumulation and inflammation, as all animals consumed equivalent quantities of fat. The risk of developing NAFLD is therefore modified by the type of fat consumed, as well as the relative proportion of healthy fats to unhealthy fats ingested, rather than the total caloric intake from fat.
Chapter Six

GENERAL DISCUSSION AND FUTURE DIRECTIONS
6.1 General Discussion

Together, the two studies presented in this thesis validated the use of the D6KO mouse in metabolic investigations, and utilized the D6KO mouse to provide evidence that ALA, the precursor n-3 PUFA, can independently improve risk factors associated with NAFLD. At the time of writing, this is the first study to provide evidence that ALA can improve metabolic abnormalities, independent of the effects of EPA and DHA. While ALA was found to confer some protection against several risk factors associated with NAFLD, this study also confirmed that the consumption of preformed n-3 HUFA provided the best protection against metabolic abnormalities in both WT and D6KO mice. Recent studies have identified several FADS2 SNPs that result in decreased D6D activity;\textsuperscript{232} thus, the study finding that menhaden oil can restore the fatty acid balance in D6KO mice provides evidence that individuals with genetic abnormalities in PUFA metabolism can effectively protect themselves from increased risk of metabolic disorders through regular consumption of HUFA-rich foods. Overall, the findings of this study suggest that the consumption of ALA-rich oils such as flaxseed oil should be encouraged, especially in place of other vegetable oils with low or negligible n-3 PUFAs, for individuals at risk of developing metabolic disease. However, fish oil consumption should also be heavily encouraged in this population as the primary mode of dietary prevention of NAFLD and CVD.
6.2 Strengths and Limitations

The studies presented in this thesis offer several strengths. The first key strength was that the changes in PUFA metabolism which occurred as a result of genetic manipulation were explored and accounted for in the D6KO mouse model. The first study confirmed the presence of three novel Δ5 fatty acids, and as their metabolic actions were unknown, an effective and natural dietary method to prevent their production was determined. Furthermore, this finding was applied when formulating the diets for the second study. In the second study, hepatic production of EPA, DHA and novel Δ5 fatty acids was negligible in D6KO mice fed diets containing lard, canola or flaxseed oil. In the confirmed absence of ALA’s long chain metabolites, any resulting metabolic benefits of flaxseed oil or canola oil consumption in D6KO mice could be attributed to ALA with greater confidence. Previous studies which have attempted to inhibit D6D could not fully eliminate EPA and DHA from liver tissues. Another overall strength was that all animals were bred by the study authors, insuring that harems and experimental animals were regularly monitored throughout their lifespan, and that the offspring of each dam were evenly distributed across dietary treatment to prevent unwanted hereditary effects.

Despite careful consideration, there are several limitations to the present studies. Thorough comparisons to LD D6KO animals were limited in the second study due to the unexpectedly low AA content of lard. AA is naturally found in lard and menhaden, but is not found in flaxseed oil and canola oil; therefore, 4g/kg diet ARASCO (~40% AA) was added to these two diets to ensure all diets contained equivalent amounts of AA to prevent n-6 PUFA deficiency, and also to inhibit the production of novel Δ5 fatty acids. When the hepatic PL of LD D6KO animals were found to contain substantially less AA than predicted after eight weeks of feeding, it was discovered that this was due to a lower than expected AA content in lard, rather than endogenous
changes in PUFA metabolism. While the PL fractions of D6KO mice did contain AA, the lower abundance of this fatty acid may limit the comparison of LD D6KO outcome measures to the outcomes of other D6KO treatment groups at both time-points.

Another limitation was the lack of clearly defined criteria to diagnose Mets and NAFLD in mice. Most scoring systems and diagnostic criteria used in animal studies have been adapted from systems established for human diagnosis; however, the degree of similarity between rodent and human metabolic disorders and their pathogenesis has not been clearly established. In this study, a commonly used NAFLD scoring system adapted for rodents was used to evaluate steatosis and inflammation.

As mice are social animals, they were caged in groups of 2-4 for the study second study, as this study was of a much longer duration than the first study. However, this meant that individual food intake of each mouse could not be quantified in the second study, so it is unknown if mice in each diet group were consuming equivalent quantities of diet. As mouse weight at 20 weeks differed between groups, there is a possibility that some groups consumed more food than others. However, final weights in all four WT treatment groups and MD D6KO mice were statistically similar, suggesting that dietary intake was not affected by type of fat or genotype. Thus, any weight differences observed across D6KO groups, and between WT and D6KO mice was due to EPA/DHA restriction in the D6KO mouse.

Finally, every rodent diet contained natural fats and oils, with each oil having a unique fatty acid composition. This could be argued to limit the ability to attribute results specifically to variations in n-3 content in the second study. However, the pattern of results described in this study were
most closely associated with variations in n-3 PUFA content, this finding made it reasonable to attribute changes observed between treatment groups to the effects of ALA or EPA and DHA.

6.3 Future Directions

Based on the present findings, future studies could aim to determine the mechanisms by which ALA could improve metabolic outcomes, and if these mechanisms are similar to those of EPA and DHA. Specifically, the activity of transcription factors associated with hepatic fatty acid metabolism such as PPARα and SREBP-1c could be examined to determine if ALA directly regulates lipid metabolism. In addition, quantifying serum lipoprotein and TG content would clarify if D6KO mice accumulate liver lipids through impaired fatty acid export or increased adipose lipolysis, and how ALA might affect this process.

Future studies could also aim to better characterize the metabolic pathologies associated with NAFLD. In particular, while glucose tolerance was assessed in this study, future investigations could quantify fasting insulin and insulin resistance. In addition, as chronic inflammation is closely related to the development of MetS and its associated disorders, further evaluation of systemic inflammation might provide a better characterization of the whole body changes associated with the depletion of n-3 HUFA and provision of ALA. Overall, quantification of these variables would provide a better understanding of how the development and progression of NAFLD is associated with other metabolic disturbances, and how diet affects this relationship.

Finally, D6KO is the first enzyme in the conversion of both ALA and LA. As such, while the current study focused exclusively on the effects of ALA, future studies can readily use the D6KO mouse model to investigate the independent effects of LA in the prevention of metabolic diseases.
Chapter Seven

REFERENCES
REFERENCES


   Ref Type: Generic


    Ref Type: Generic


48. Statistics Canada. Table 105-2014 - Percentage of total energy intake from fat, by dietary age-sex reference intake group, household population aged 4 and over, Canadian Community Health Survey (CCHS 2.2), Canada and provinces, occasional, CANSIM (database). 2010.


