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Title: Long-chain (n-3) PUFA and Non-alcoholic Fatty Liver Disease

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Non-alcoholic fatty liver disease (NAFLD) is defined as excess hepatic lipid accumulation, in the absence of excess alcohol consumption and chronic liver disease. NAFLD can range in severity from simple fatty liver (steatosis) to non-alcoholic steatohepatitis (NASH). NASH is defined as hepatic steatosis with inflammation and hepatic injury and describes the progressive form of NAFLD. NASH promotes development of hepatic fibrosis, cirrhosis, advanced liver disease, and potentially increases risk of hepatocellular carcinoma. The incidence of NAFLD has increased in parallel with that of obesity. It is estimated that by the year 2020 NAFLD will be the leading cause of liver transplants in the United States. The current standard of care for patients diagnosed with NAFLD/NASH is to treat for liver disease and the associated co-morbidities including obesity, Type 2 Diabetes Mellitus (T2D), hyperlipidemia, and the metabolic syndrome. There are no treatments specific to NAFLD or NASH. As such, new treatment modalities and an improved understanding of NAFLD are urgently needed to address this impending public health burden.

20-22 carbon (C20-22) (n-3) polyunsaturated fatty acids (PUFA) have well established anti-inflammatory properties and many known benefits on hepatic lipid metabolism, and thus are attractive agents to potentially combat NAFLD/NASH in humans. As such, my overall goal was to establish the therapeutic efficacy of (n-3) PUFA supplementation as a treatment modality for NAFLD/NASH. First, I assessed several mouse models of NAFLD as follows: high-fat (HF; 60% calories as fat), high fat-high cholesterol (HFHC; 54.4% fat, 0.5% cholesterol), and the western diet (WD; 41% fat, 0.2% cholesterol) feeding to both wild-type (WT) and low density lipoprotein receptor knockout (*Ldlr-/-*) mice. A key finding was that hepatic cholesterol accumulation is a major driver of the progression of NAFLD to NASH. Accordingly, the most severe phenotype resulted from feeding the WD to *Ldlr-/-* mice. To evaluate the ability of C20-22 (n-3) PUFA to combat NAFLD/NASH, mice were fed the experimental diets with and without added C20-22 (n-3) PUFA over the entire feeding period (12 or 16 weeks). Addition of C20-22 (n-3) PUFA found in menhaden oil prevented the development of NAFLD in WT and *Ldlr-/-* mice fed either HF or HFHC diets for 12 weeks. However, when *Ldlr-/-* mice were fed the WD supplemented with C20-22 (n-3) PUFA for 16 weeks, hepatic steatosis developed but hepatic inflammation, oxidative stress, and fibrosis were dramatically attenuated. Furthermore, the capacity of dietary docosahexaenoic acid (DHA; C22:6 (n-3)) was greater than dietary eicosapentaenoic acid (EPA; C20:5 (n-3)) at attenuating WD induced hepatic inflammation, oxidative stress, and fibrosis in *Ldlr-/-* mice.

To further investigate these findings, I used a metabolomics approach to assess the development of NASH and the impact from EPA and DHA supplementation. Development of NASH was wide-reaching and impacted all major pathways of hepatic

metabolism analyzed including lipid, carbohydrate, and amino acid metabolism. There was very strong evidence of membrane lipid remodeling associated with NASH. These changes were most effectively prevented by supplementation with DHA. Additionally, C20-22 (n-3) PUFA supplementation dramatically elevated the accumulation of (n-3) derived oxidized PUFA with anti-inflammatory properties. Since DHA supplementation most potently attenuated NASH progression, I speculate that membrane remodeling and the corresponding changes in oxidized PUFA associated with DHA consumption plays a major role in attenuating inflammation, fibrosis, and oxidative stress in WD-induced NASH in *Ldlr-/-* mice.

In conclusion, I established that the development of NASH has wide-reaching and complex consequences on hepatic metabolism. Ultimately, dietary DHA is more efficacious than dietary EPA at attenuating the progression of NASH induced by a severe model such as WD feeding to *Ldlr-/-* mice. Collectively, my data indicates that dietary DHA at a physiologically relevant dose, either alone or in combination with other therapy is likely to provide significant benefit to obese humans with NAFLD/NASH.

Long-chain (n-3) PUFA and Non-alcoholic Fatty Liver Disease

by

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I understand that my dissertation will become part of the permanent collection of Oregon State University libraries. My signature below authorizes release of my dissertation to any reader upon request.

Christopher Michael Depner, Author

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CONTRIBUTION OF AUTHORS

The author's responsibilities were as follows:

Chapter 1: Christopher Depner wrote.

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Chapter 5: Christopher Depner wrote.

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Dedicated to my family and friends

Chapter 1

Introduction

1.1. Non-alcoholic fatty liver disease

Non-alcoholic fatty liver disease (NAFLD) is defined as excess accumulation of liver fat (steatosis), mainly in the form of neutral lipids consisting of triglyceride (TAG), cholesteryl ester, and diacylglycerol (DAG). NAFLD describes a spectrum ranging from hepatic steatosis to non-alcoholic steatohepatitis (NASH) [1]. NASH is defined as hepatic steatosis with inflammation and hepatic injury [2]. While simple hepatic steatosis is considered a clinically benign condition, NASH is the progressive form of the disease and can advance to fibrosis, cirrhosis, hepatocellular carcinoma, and end stage liver disease [3]. Clinically, liver biopsy is the only true method of diagnosing NASH [2]. Histologically, NASH is indistinguishable from alcoholic liver disease [4]. Thus, significant alcohol consumption and all other potential causes of chronic liver disease must be ruled out prior to NAFLD diagnosis [2].

The incidence of NAFLD has increased in parallel with that of obesity. It is estimated the prevalence of NAFLD in the general population ranges from ~6% to 33% depending on the method of analysis and population studied [5]. Approximately 30% to 40% of individuals with hepatic steatosis progress to NASH [3]. In the general population the prevalence of NASH ranges from 3% to 5% [5]. The prevalence of NAFLD and NASH has been reported to be as high as 87% and 63%, respectively, in patients with type 2 diabetes mellitus (T2D) [6]. It was reported in patients undergoing bariatric surgery that 93% had NAFLD, and of those, 26% had NASH [7]. Mortality in NASH patients > NAFLD patients > general population and it is most often derived from cardiovascular and liver related causes [8,9,10]. It was previously reported that over a 10 year period, 20% of NASH patients developed cirrhosis and 12% died of liver related causes [11]. Given the clinical severity and increasing prevalence of NASH, it is quickly

becoming a significant public health burden. By the year 2020 NASH is projected to be the leading cause of liver transplantation in the United States [12].

Currently, there are no specific treatments for NAFLD or NASH. The standard of care for patients diagnosed with NAFLD or NASH is to treat for liver disease and the associated co-morbidities including obesity, T2D, hyperlipidemia, and the metabolic syndrome (MetS) [2,13,14]. In controlled settings, patients have had success with lifestyle modifications (diet and physical activity), but these approaches have little long-term success outside the laboratory [14,15,16]. To date, no potential therapeutic agents have been effective and without negative side-effects [13]. Given the treatment limitations, disease outcomes, and increasing prevalence, there is a large need for new and improved therapies specific to NAFLD and NASH. One potential treatment warranting further investigation are the long-chain C20-22 (n-3) polyunsaturated fatty acids (PUFA) found in fish oil. The following describes my work investigating the potential therapeutic effectiveness of C20-22 (n-3) PUFA to combat NAFLD/NASH.

1.2. Diagnosis, development, and progression of NAFLD/NASH

Simple hepatic steatosis is considered a clinically benign condition, whereas NASH has severe health consequences and is associated with increased risk of liver failure, cardiovascular disease, T2D, and MetS [11,17]. Whereas imaging methods including magnetic resonance imaging and ultrasound can detect steatosis, they are not capable of determining characteristics of NASH such as inflammation, hepatocellular injury (ballooning), and fibrosis. The gold standard for NASH diagnosis is liver biopsy [18]. In an effort to better classify stage of disease, the NAFLD activity score (NAS) was developed [18]. The NAS is a composite of 14 histological features that can be graded by trained pathologists. While the NAS is beneficial, it still requires a liver biopsy, which

is invasive, expensive, and subject to variability based on location of biopsy. Furthermore, symptoms can vary and some individuals are asymptomatic until their liver begins to fail, at which point conventional treatments are ineffective [19]. Thus, it is often hard to determine when a liver biopsy is appropriate for diagnosis. Currently, there are no easily analyzed plasma biomarkers that consistently predict stage of NAFLD and NASH. Classically, elevated plasma alanine amino transferase (ALT) and/or aspartate amino transferase (AST) are indicative of liver damage. Whereas these enzymes are generally elevated in patients with NAFLD, they are not always increased and do not correlate well with degree of severity [20]. As such, they cannot be reliably used for diagnostic purposes. Given these diagnostic challenges, there is a large need for biomarker discovery and improved understanding of the progression to NASH.

1.2.1) Two-hit hypothesis for the development of NASH

The current model for the development of NASH is a two-hit hypothesis proposed by Day and James [21] **(Fig. 1.1)**. The “first hit” comes from excess lipid accumulation in the liver. This is hypothesized to sensitize the liver to the “second hit” consisting of oxidative stress, inflammation, and insulin resistance. As these abnormalities continue to progress they cause hepatocellular injury, fibrosis, decreased liver function, and eventually cirrhosis. Whereas the two-hit hypothesis is helpful in understanding processes that contribute to development and progression of NASH, it is overly simplistic lacking a true mechanistic explanation. Furthermore, it does not address why the disease progresses in some patients, and yet in others it does not.

Development of hepatic steatosis, the “first hit”, results from a disrupted balance of fatty acid metabolism in the liver. Liver fatty acid sources include circulating non-esterified fatty acids (NEFA) derived from adipose tissue, *de novo* lipogenesis (DNL),

and diet. Fatty acid oxidation (FAO) for fuel and fatty acid export as VLDL are the 2 pathways for removal of fatty acids from the liver. Hepatic steatosis develops when these pathways are chronically unbalanced with fatty acid delivery exceeding export [22]. In steatotic livers, it is estimated about 59% of the excess fatty acid accumulation is derived from circulating NEFA, 26% from DNL, and 15% from diet [23]. Both hepatic and peripheral insulin resistance contribute to the disruption of these pathways leading to hepatic steatosis [22].

Clinically, the most detrimental aspect of NASH is progression to fibrosis [24]. Again, our current understanding does not adequately address or identify the mechanisms and mediators of progressing fibrosis in NASH. Many potential mediators of NASH have been identified such as: dietary cholesterol and fructose, the NEFA profile, excess ceramide accumulation, elevated leptin, and endotoxemia [25,26,27,28]. Likely, a combination of these mediators and others act in parallel and give rise to an environment in the liver that sets the stage for the “second hit” promoting NASH progression. To develop new therapies specific to NASH, it is imperative that we have a more complete understanding of the complex interplay of mechanisms involved and the actual “second hit” mediators driving NASH progression.

1.2.2) Environmental and genetic factors contributing to NAFLD and NASH progression. Several dietary components have been associated with the development and progression of NAFLD and NASH. Unhealthy dietary habits are hypothesized to be the key driver of NAFLD in patients with normal body mass index (BMI) [29]. Given these observations and the challenges associated with long-term weight reduction [30], dietary manipulations that improve NAFLD independent of weight loss are an attractive

therapeutic approach. Thus, a complete understanding of the role various dietary components have on NAFLD and NASH development is imperative.

Both the amount and composition of dietary fat are key contributors to NAFLD and NASH. In mice, it is well established that long-term high fat (45 – 75% kcal) diet (HFD) feeding induces hepatosteatosis with minimal inflammation and liver damage [31]. Whereas there are fewer studies in humans, HFD has been demonstrated to induce hepatosteatosis independent of weight changes [32,33]. Concerning fat composition, NASH patients have a lower ratio of polyunsaturated/saturated fat consumption than the healthy population [34,35]. Specifically, dietary long-chain (n-3) PUFA have been reported to decrease hepatic steatosis and a lower (n-3) to (n-6) PUFA consumption ratio in humans is associated with development of NAFLD [32,36,37].

Trans-fatty acids (TFA) consumption is associated with insulin resistance and cardiovascular disease, however the impact of TFA consumption on NAFLD in humans is less well known [38]. Studies utilizing mice suggest that TFA consumption is associated with a higher degree of both hepatic steatosis and injury [39,40]. Given the well established negative health consequences of TFA consumption, high TFA consumption is likely to promote development and progression of NASH in humans. However, studies specifically investigating human TFA consumption and NASH are still required.

MUFA are known to have a beneficial effect on the plasma lipid profile in humans when they are replacing carbohydrates in the diet [41]. However, similar observations exist for both PUFA and saturated fatty acids (SFA), which questions the role of MUFA in NAFLD. In rats fed a methionine-choline deficient diet (MCDD), MUFAs derived from olive oil decreased liver TAG accumulation by 30% [42]. However, MCDD-induced

hepatosteatosis is not physiologically relevant to development of NAFLD in humans (discussed in detail in section 1.3), questioning the relevance of these results. Patients diagnosed with NAFLD do not have different MUFA consumption compared to healthy controls [43]. Overall, the role of MUFA in human NAFLD has not been well established, however studies to date do not suggest a major role of MUFA in the development and progression of NAFLD.

Studies on the role of dietary cholesterol in development of hepatic steatosis in obese humans have been mixed. Alternatively, in normal weight individuals, elevated dietary cholesterol has been implicated in the development of hepatic steatosis [29]. Whereas there is controversy on the role of dietary cholesterol promoting hepatic steatosis, elevated dietary cholesterol has been consistently implicated in hepatic inflammation and damage [27,35,44]. As such, it is safe to conclude that high dietary cholesterol intake is implicated in NASH progression.

Contrary to lipids, the role of excess sugar consumption in promoting NAFLD and NASH is more clearly defined. Over the last 30 years there has been a dramatic increase in obesity and NAFLD in the United States. Over this time period, fat consumption has remained steady while carbohydrate and total caloric intake dramatically increased [45,46,47,48]. As such, elevated carbohydrate, and specifically fructose consumption has been linked to NAFLD and NASH progression [49,50].

Fructose consumption has more than doubled in the last 30 years and is the monosaccharide most elevated with increased carbohydrate consumption [49]. The liver highly expresses the fructose-specific glucose transporter 5 (GLUT5) and is responsible for metabolizing up to 70% of fructose loads [51,52]. Fructose metabolism is independent of insulin action and drives DNL to a greater extent than glucose. Fructose

is known to promote all the aspects associated with the MetS including hypertension, hepatic steatosis, insulin resistance, dyslipidemia, hyperglycemia, obesity, and endotoxemia, and is thus highly implicated in NAFLD and NASH progression [49]. A detailed discussion of the role of fructose in each of these abnormalities is beyond the scope of this paper. Briefly, since fructose metabolism is insulin independent, the majority of a fructose bolus is converted to pyruvate and then acetyl-CoA which enters the Kreb's cycle in the mitochondria. A large fructose bolus will generate enough acetyl-CoA to overwhelm the Kreb's cycle capacity. Under these conditions, excess acetyl-CoA is exported into the cytosol via the citrate shuttle and is a substrate for DNL, thus promoting steatosis. Malonyl-CoA, an intermediate in DNL, accumulates with elevated DNL and inhibits carnitine palmitoyl transferase-1 (CPT-1), a rate limiting enzyme of fatty acid oxidation (FAO). The elevated DNL and reduced FAO resulting from a large fructose bolus results in rapid development of hepatosteatosis [49]. Glucose, however is tightly regulated by insulin, converted to glycogen, and metabolized by skeletal muscle, and thus does not drive hepatosteatosis as aggressively as excess fructose consumption. Lastly, several mechanisms by which fructose mediates the "second-hit" have been proposed including elevated reactive oxygen species (ROS) and advanced glycation end-product (AGE) formation [53,54,55].

The benefit of physical activity for the array of abnormalities associated with NAFLD is widely accepted including reduction of T2D, insulin resistance, hypertension, dyslipidemia, impaired fasting glucose, and the MetS [38]. Multiple studies have reported an inverse associated between leisure time physical activity and or cardio-respiratory fitness and prevalence of NAFLD [56,57]. Another study reported that lower cardio-respiratory fitness was associated with a higher NAS and progression of NASH [58].

Unfortunately, long-term adherence to elevated physical activity is low with about 20% compliance rates over two year follow-ups [59].

Only a fraction of NAFLD patients progress to NASH. Genetic variation is one potential contribution explaining why some patients develop NASH and others do not. Whereas several gene polymorphisms potentially contributing to NASH have been investigated, variations in patatin-like phospholipase domain containing 3 (PNPLA3) have been most widely studied and associated with NAFLD progression. A polymorphism in PNPLA3 has been associated with degree of NAFLD and fibrosis in several different studies [60]. Among various ancestry groups, Hispanics are most susceptible to NAFLD and most highly express the PNPLA3 (rs738409[G]) allele associated with higher hepatic lipid accumulation and inflammation levels [61]. Furthermore, African Americans have the lowest risk of NAFLD and most commonly express the PNPLA3 (rs6006460[T]) allele that is associated with lower hepatic lipid accumulation [61]. Even though PNPLA3 gene polymorphisms are the best characterized with respect to NAFLD, the specific role of PNPLA3 in development of NAFLD and hepatic lipid metabolism is still not clear. More work is needed to characterize other potential gene polymorphisms that might be contributing to NAFLD and NASH progression.

Overall NAFLD is a very complex disease with a wide range of modifiable risk factors. Generally, studies investigating NAFLD aim to isolate these factors to understand their individual contributions to NAFLD and NASH. However, humans who develop NAFLD are variably exposed to the whole range of risk factors facilitating potential interactions amongst them. These potential complex interactions are much more difficult to investigate, especially in human studies. This has limited the progress in

our understanding of the progression of NAFLD to NASH. Much more work is needed to fully understand the range of risk factors and how they work in tandem throughout the progression of NAFLD. A more complete understanding of the risk factors and mechanisms of progression will hopefully allow for improved treatment options and outcomes for patients with NAFLD.

1.3. Animal models of NAFLD and NASH

NAFLD is now considered the hepatic manifestation of the MetS due to highly correlated risk factors and metabolic abnormalities [62]. Supporting this, development of NASH and fibrosis is most highly associated with diabetes mellitus, insulin resistance, and hypertension, but not BMI [63]. Given these observations, it is critical to study NAFLD in the context of these metabolic abnormalities for a complete understanding. Currently, several genetic and dietary models of NAFLD/NASH have been used, but there is not one specific universal model.

1.3.1) Genetic models of NAFLD and NASH

A wide range of genetic models have been used to investigate NAFLD development and the role specific genes have in the disease. First, mice with defective leptin signaling have been a commonly used model to study NAFLD. In humans, obesity is highly associated with elevated plasma leptin, due to defective leptin signaling [64]. *ob/ob* mice are leptin deficient and *db/db* mice lack the leptin receptor, thus both strains have defective leptin signaling. These mice are characterized by inactivity, hyperphagia, obesity, hyperglycemia, insulin resistance, and hyperinsulinemia [65], resembling the human MetS. Whereas leptin deficient mice develop hepatosteatosis, they do not progress to NASH. This helped identify leptin as essential for the development of hepatic

fibrosis in chronic liver injury [66]. Fibrosis is the most detrimental clinical aspect of NASH, thus the utility of leptin defective mice for the study of NASH is limited.

Second, mice over-expressing sterol regulatory element-binding protein 1c (SREBP-1c) in adipose tissue develop lipodystrophy, insulin resistance, and a hepatic phenotype resembling NASH [67]. However, these mice have reduced adipose tissue mass and circulating leptin, thus their usefulness in studying traditional NAFLD in humans is limited.

Third, mice with hepatocyte specific deletion of the tumor suppressor gene phosphatase and tensin homolog (PTEN) develop hepatosteatosis with liver fibrosis and hepatocellular carcinoma [68]. As such, some studies have used this model to investigate both treatments and development of NAFLD. However, hepatic PTEN-/- mice do not develop obesity and are hypersensitive to insulin. As such, these mice do not develop NAFLD in the same context as humans, limiting their worth as a model of human NAFLD.

Fourth, there are several genetic models of defective cholesterol metabolism in mice. Whole body low density lipoprotein receptor knockout mice (LDLR-/-) develop hypercholesterolemia due to elevated plasma VLDL and LDL half-lives [69]. Historically, LDLR-/- mice have been widely used as a model to study atherosclerosis, however, high-fat high-cholesterol feeding induces hepatosteatosis with hepatic inflammation, injury, and fibrosis [27]. Since LDLR-/- mice develop NAFLD and NASH in a context similar to humans they are a useful model to investigate the contributions of the MetS to the development of NAFLD and NASH. Another model of altered cholesterol metabolism is apolipoprotein E2 knock-in mice (APOE2ki). In these mice, the mouse *apoE* gene is replaced by the human *APOE2* allele [70]. Human *APOE2* has reduced affinity for LDLR

and APOE2ki mice develop a plasma lipoprotein profile very close to that of human type III hyperlipoproteinaemia [70]. Upon HFD feeding, APOE2ki mice develop hepatosteatosis and inflammation [71]. However, with prolonged feeding the inflammation resolves itself and therefore APOE2ki mice do not develop a phenotype as severe as human NASH [72].

There are numerous other genetic models of NAFLD but the ones discussed here are the most commonly used. Each of the models has unique strengths and weaknesses for modeling human NALFD and NASH. Depending on the specific goal of a study these models can be very useful in improving our overall understanding of NAFLD and NASH. When investigating potential treatments for NAFLD one must consider the use of genetic models carefully as there are many aspects of these models that do not match the phenotype or context of human NAFLD. In general, the models of disrupted cholesterol metabolism most closely replicate the human phenotype on the whole and can be a very useful model in assessing both disease progression and treatments. However, these models do not spontaneously progress to the advanced stages of the disease without further environmental stressors such as diet and or reduced physical activity.

1.3.2) Nutritional models of NAFLD and NASH

As with the genetic models, there are multiple nutritional models used to study human NAFLD and NASH. Whereas there are general dietary trends in patients with NAFLD and NASH, there is not one single diet or dietary component responsible for causing NAFLD. Accordingly, multiple methods of inducing NAFLD in animal models have been developed to investigate different aspects of human nutrition potentially contributing to NAFLD and NASH.

First, one of the most common nutritional models of NAFLD and NASH is feeding a methionine choline deficient diet (MCDD). The standard MCDD contains no methionine or choline and consists of 40% sucrose and 10% fat; however it is not uncommon to use a higher fat content. Methionine and choline are essential nutrients for both β-oxidation and hepatic VLDL export. Thus MCDD feeding induces rapid hepatosteatosis with elevated oxidative stress that causes hepatic inflammation and fibrosis [73]. Whereas MCDD feeding induces severe hepatic inflammation and fibrosis, rodents fed the MCDD have reduced body weight, white adipose tissue, and plasma TAG, with elevated systemic insulin sensitivity [74,75]. Since the MCDD induces a very severe hepatic phenotype it is useful to study hepatic specific aspects of human NAFLD and NASH progression. But since MCDD feeding does not induce a phenotype resembling the MetS, the relevancy of this model to the human condition has been questioned.

Second, HFD (45-75% calories as fat) are commonly used to induce hepatosteatosis. HFD feeding in rodents has been demonstrated to induce obesity, insulin resistance, dyslipidemia, hepatosteatosis, oxidative stress, with mild fibrosis, and inflammation [31,76,77]. HFD feeding induces a less severe hepatic phenotype compared to MCDD and requires a much longer feeding period (~12 weeks). But, the pathophysiology associated with HFD feeding much more closely resembles human NAFLD versus MCDD feeding. As such, HFD models are very commonly used to study NAFLD and often times combined with either genetic or other dietary modifications to induce a phenotype with severe hepatic inflammation and fibrosis.

Classic HFD manipulations used in atherosclerosis studies such as increased cholesterol (2-5% wt/wt), cholic acid, fructose, and sucrose are increasingly being used

to study NAFLD too. These dietary manipulations induce a more severe phenotype resembling human NASH with fibrosis and inflammation [27,78,79,80]. Overall, these manipulations support the idea that not one single dietary component is responsible for NAFLD or NASH and a holistic dietary approach is needed for modeling NAFLD and NASH.

Lastly, models of over-nutrition in rodents have been used to induce insulin resistance and NAFLD. In humans, the most common cause of these metabolic ailments is over-nutrition, or chronic energy intake exceeding energy expenditure [19]. HFD feeding can result in over-nutrition in rodents; however it is not uncommon for rodents to metabolically adapt to a HFD and fail to develop metabolic abnormalities associated with NAFLD [81]. This can be avoided through forced feeding by gavage or gastrosomy tube. Forced over-nutrition in rodents results in obesity, hyperglycemia, hyperinsulinemia, hyperleptinemia, glucose intolerance, hepatic inflammation, damage, and fibrosis [82]. These types of over-nutrition models develop a similar pathophysiology as human NAFLD and NASH. The technical procedures associated with these models are complex and very time intensive. As such, these over-feeding models are less frequently used compared to other approaches that resemble the human pathophysiology of NAFLD.

In all, there is a wide range of animal models at our disposal for the study of NAFLD and NASH. Unfortunately, no one model replicates all aspects of the human condition. With the combination of models available, all aspects of the human condition can be probed with thoughtful study designs. One must very carefully consider their specific aims and hypotheses to properly choose the correct model system for a given study. The lack of a specific model that replicates all aspects of the human disease is one of the limiting factors in properly assessing potential treatments for NAFLD.

Continued work on model development will hopefully provide a more complete understanding of NAFLD and NASH and the effectiveness of potential treatments.

1.4. Current treatment options for patients with NAFLD/NASH

To date, there are no pharmacologic treatments specific to NAFLD and NASH. Patients with either suspected or diagnosed NAFLD or NASH are recommended to gradually lose weight through lifestyle modifications (diet and physical activity) and treat risk factors of the MetS [83]. Unfortunately, long-term success of lifestyle modifications is low and inadequate to address the rapidly increasing prevalence of NAFLD and NASH. As such, there are currently 198 registered clinical trials (www.clinicaltrials.gov) investigating NAFLD or NASH and 130 of these are focused on potential treatments. A very wide range of treatment modalities are being assessed including but not limited to vitamin E, probiotics, pioglitazone, exercise versus diet, (n-3) fatty acids, Hoodia, iron depletion via phlebotomy, statins, and bovine colostrum. This wide range of potential treatments stems from the complexity and range of abnormalities associated with development of NAFLD and NASH. Most trials completed to date are either observational or do not have histological end points. Thus effective treatments have yet to be identified.

Both calorie restriction and specific diets such as fat restriction, carbohydrate restriction, ketogenic diets, and high-protein diets have been evaluated as therapies for NAFLD and NASH [83]. To date, no one specific diet has been identified as a superior treatment option versus the others. It is well accepted that weight loss should be gradual and avoiding specific foods rather than calorie restriction will likely be more successful in the long run [83,84]. Some diets or dietary components are hypothesized to have potential benefit for NAFLD and NASH patients such as the Mediterranean diet and (n-3)

PUFA. Reduced liver fat accumulation in NAFLD patients consuming a Mediterranean style diet has been reported [85]. Liver histology was not included in this trial so impact on fibrosis was not determined. NAFLD patients have lower dietary (n-3)/ (n-6) ratios and long-chain (n-3) PUFA have known benefits on hepatic lipid metabolism [83]. Accordingly, elevated long-chain (n-3) PUFA consumption may significantly benefit NAFLD and NASH patients. Studies investigating the benefit of (n-3) PUFA for NAFLD are limited and further research is required. In all, the complex interplay between diet, NAFLD, and NASH progression is incompletely understood. Studies identifying cause and affect mechanisms associated with specific dietary components implicated in both progression and treatment of NAFLD/NASH are urgently needed.

1.5. Long-chain (n-3) PUFA potential benefits for NAFLD/NASH patients

The most abundant dietary (n-3) PUFA consist of α-linolenic acid (ALA, C18:3 (n-3)), stearidonic acid (SDA, C18:4, (n-3)), eicosapentaenoic acid (EPA, C20:5, (n-3)), docosapentaenoic acid (22:5, (n-3)), and docosahexaenoic acid (DHA, C22:6, (n-3)) [86]. ALA is an essential nutrient required for life and cannot be synthesized by humans, is converted to longer more desatured PUFA, and therefore is considered the essential (n-3) PUFA [87] **(Fig.1.2)**. The potential health benefits of high (n-3) PUFA consumption stem from epidemiological studies in the 1970s indicating reduced rates of myocardial infarction after high dietary consumption of C20-22 (n-3) PUFA in Greenland Inuits compared to individuals in westernized countries [88,89,90]. Since these early studies, there has been an abundance of research investigating the potential benefits of high (n-3) PUFA consumption with respect to cardiovascular disease [86]. Additionally, the impact of C20-22 (n-3) PUFA on hepatic lipid metabolism has been well established [91,92]. However, as NAFLD is only recently becoming a major public health burden, the

body of research investigating the impact of high C20-22 (n-3) PUFA intake on NAFLD is limited and further research is needed.

1.5.1) C20-22 (n-3) PUFA potential mechanisms of action against NAFLD/NASH

EPA and DHA are well studied pleiotropic molecules known to regulate DNL, MUFA and PUFA synthesis, membrane architecture, promote FAO, and attenuate inflammation [91]. These characteristics make EPA and DHA attractive for the potential treatment and or prevention of NAFLD and NASH. Currently there are 11 clinical trials (www.clinicaltrials.gov) investigating the use of EPA and or DHA to combat NAFLD/NASH. However, data is limited and while some specific mechanisms of C20-22 (n-3) PUFA are defined, their overall efficacy as a therapeutic agent to combat NAFLD/NASH is unknown.

The transcription factors sterol regulatory element binding protein-1c (SREBP1c) and carbohydrate response element binding protein (ChREBP) are equivalently responsible for the regulation of hepatic DNL [93]. It is estimated ~26% of excess liver fat is derived from DNL in patients with NAFLD, thus regulation of SREBP1c and ChREBP are attractive therapeutic targets of NAFLD [23]. Whereas glucose metabolism is required to induce nuclear translocation of ChREBP, both EPA and DHA are known to suppress nuclear ChREBP [94]. Nuclear SREBP1c regulation is complex involving both transcriptional and post-transcriptional mechanisms. Precursor SREBP1c is synthesized and tethered to the endoplasmic reticulum (ER), escorted to the Golgi where it is cleaved to the mature form, and finally transported to the nucleus where it binds sterol regulatory elements in promoters of target genes [91]. Blood insulin is the predominant regulator of nuclear SREBP1c. In fasted conditions when insulin is low, nuclear SREBP1c is low; in the postprandial period when blood insulin is elevated, there is a parallel increase in

nuclear SREBP1c [91]. Both the phosphoinositol-3-kinase-Akt2 and mammalian target of rapamycin complex-1 and 2 pathways have been implicated in the control of insulin induced nuclear SREBP1c [95,96,97,98]. Whereas all C20-22 (n-3) and (n-6) PUFA depress SREBP1c at the mRNA level, DHA uniquely stimulates SREBP1c proteasomal degradation [91,99]. As such, DHA is the most potent PUFA suppressor of nuclear SREBP1c and, thus, DNL. SREBP1c also regulates desaturases and elongases involved in MUFA and PUFA synthesis [91]. Thus, EPA and DHA regulate MUFA and PUFA synthesis through changes in nuclear SREBP1c content. Hepatic DNL, MUFA, and PUFA synthesis are elevated with NAFLD and are therapeutic targets of EPA and DHA through regulation of nuclear SREBP1c and ChREBP [100].

Another target of C20-22 (n-3) PUFA is modification of membrane characteristics. DHA especially, is known to accumulate in plasma membrane phospholipids thereby altering fluidity, lipid rafts, and fatty acid substrate availability for synthesis of oxidized PUFA [101]. PUFA double bonds are extremely flexible and allow disordered conformations relative to the more ordered less flexible conformations of SFA with no double bonds [102]. DHA supplementation enriches DHA in phospholipids of the plasma membrane by 2 – 10 fold in most tissues [103]. DHA is the PUFA with the highest number of double bonds that accumulates in membranes and, therefore, most dramatically alters membrane architecture [104]. Membrane architecture is critical for proper functioning of membrane associated proteins. Some receptors known to be impacted by elevated membrane DHA content are the toll-like receptors (TLR) and Src-family kinases [105,106]. Disruption of these receptor systems in the membranes impedes their downstream signaling including nuclear factor kappa light chain enhancer of activated B cells (NF-κB), a major regulator of inflammation [107]. Altered membrane

architecture is thought to be one of the major mechanisms by which C20-22 (n-3) PUFA exert their wide-ranging beneficial effects.

Another component of membranes influenced by (n-3) PUFA enrichment is phospholipase excision of fatty acids for oxidation by cyclooxygenases (COX), lipoxygenases (LOX), and cytochrome P450 enzymes (CYP2C and CYP4A). (n-3) PUFA are competitive inhibitors of (n-6) PUFA for these enzymes and in general (n-3) derived oxidized PUFA promote resolution of inflammation [108]. Thus, enrichment and reorganization of membranes with (n-3) PUFA can shift the balance of oxidized PUFA towards promoting resolution of inflammation. Whereas this is another attractive feature of the (n-3) PUFA for NALFD treatment, the physiological function of many of the (n-3) derived oxidized PUFA in the liver are unknown.

Lastly, C20-22 (n-3) PUFA have been implicated in regulating FAO for fuel. Peroxisome proliferator-activated receptors (PPAR) regulate genes involved in fatty acid elongation, desaturation, oxidation, and transport [91,92]. PPAR are fatty acid regulated nuclear receptors that heterodimerize with retinoid X receptors and bind *cis*-regulatory elements of target genes [109]. Both EPA and DHA have been demonstrated to bind and induce PPARα, and thereby elevate FAO [92,110]. The key feature of NAFLD is excess hepatic lipid accumulation and, thus, a potential increase in FAO through C20-22 (n-3) PUFA treatment is a potential treatment option. However, the ability of either EPA or DHA to elevate FAO in NAFLD/NASH patients is not defined. Furthermore, simple hepatosteatosis without inflammation is considered benign and it is the “second-hit” that is responsible for inducing the pathologies associated with NASH. Accordingly, simply elevating FAO in patients with progressing NASH may be of little benefit on overall outcome.

In all, C20-22 (n-3) PUFA, especially EPA and DHA have a wide-range of known physiological functions that could potentially contribute to prevention and treatment of NAFLD/NASH. Additionally, EPA and DHA consumption at levels currently used to treat hypertriglyceridemia (~2% total calories) is well tolerated with minimal side-effects, further supporting their potential therapeutic use for NAFLD. However, the ability of EPA and DHA to combat NAFLD in humans is largely unknown. The time is ripe for investigations into the effectiveness of EPA and DHA to combat the rapidly increasing prevalence of NAFLD/NASH.

1.6. Summary

As the prevalence of NAFLD increases in parallel with obesity, and because NASH is anticipated to be the leading cause of liver transplants in the United States by 2020 [12], NAFLD/NASH is rapidly becoming a major public health burden. NAFLD is associated with an array of metabolic abnormalities including hyperglycemia, hyperlipidemia, insulin resistance, obesity, elevated risk of cardiovascular disease and T2D. As such NAFLD is now considered the hepatic manifestation of the MetS [62]. Development of NASH can progress to severe fibrosis, cirrhosis, end-stage liver disease, and hepatocellular carcinoma in some cases. Currently the only treatment option for patients with NAFLD/NASH is lifestyle modifications (diet and physical activity) and while this approach can be successful in the short-term, in the long-term this is not a viable treatment option for most people. Thus, new treatments and prevention techniques are urgently needed. Unfortunately, our incomplete understanding of the development and progression of NAFLD/NASH has dramatically hampered the development of effective prevention and treatment methods.

EPA and DHA are well studied PUFA with several known impacts on hepatic lipid metabolism and inflammation. A lower (n-3) to (n-6) PUFA consumption ratio is associated with development of NAFLD [32]. Thus, the potential use of EPA and DHA as therapeutic agents to combat NAFLD/NASH is of high interest. Preliminary studies from our lab suggested that C20-22 (n-3) PUFA supplementation has the capacity to prevent development of NAFLD in mice fed HFD. The overall goal of my dissertation is to establish the therapeutic efficacy of (n-3) PUFA supplementation as a treatment modality for NAFLD/NASH at doses relevant to human consumption. The following aims were completed to accomplish this goal.

Aim 1: Develop a mouse model of diet induced NASH closely resembling human pathophysiology. The hypothesis was that *Ldlr*$^{-/-}$ mice fed a WD for 16 weeks would develop a phenotype resembling human NASH and provide a model for future studies investigating NASH.

Aim 2: Determine if EPA and DHA are equally effective at preventing diet-induced NASH. My preliminary data indicated there may be differences in the effectiveness of EPA versus DHA. Accordingly, I hypothesized that supplementation with DHA would prevent the progression of NASH in mice fed the WD for 16 weeks more robustly than supplementation with EPA.

Aim 3: Identify metabolic pathways perturbed by diet-induced NASH and the impact from EPA and DHA supplementation on those pathways. This was a hypothesis generating aim to identify mechanisms of NASH progression and targets of dietary EPA and DHA.

The outcome of these aims provided novel information on dietary components that promote development of NAFLD/NASH and also the efficacy of EPA and DHA supplementation to prevent NAFLD/NASH.

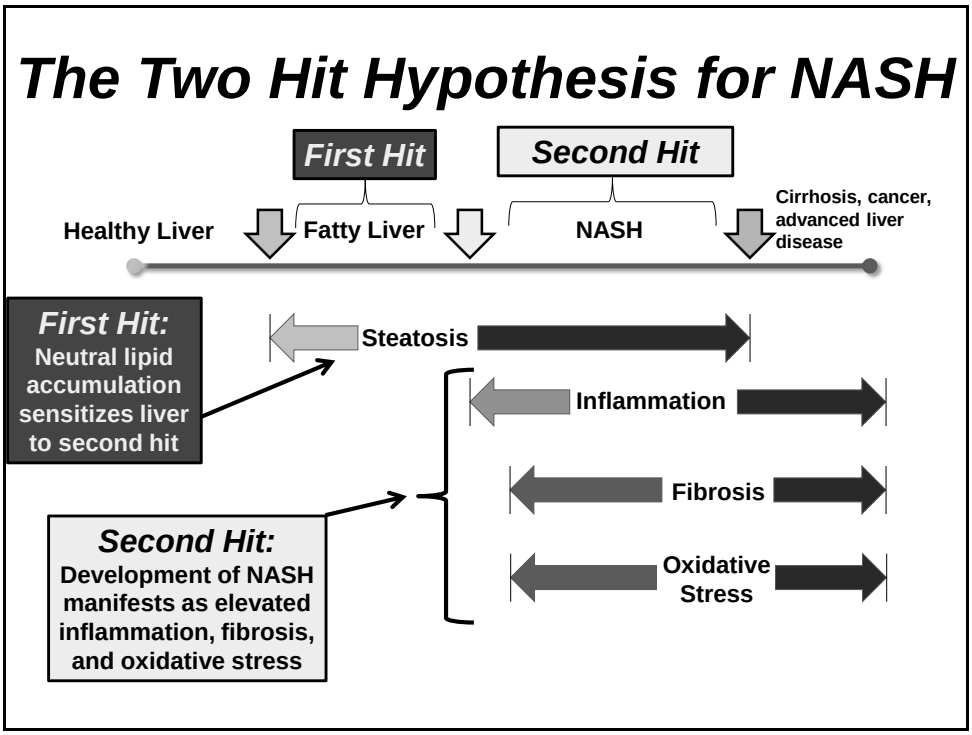

Figure 1.1. Model representing the two-hit hypothesis for the development of NASH. The "first hit" comes from excess lipid accumulation in the liver. This is hypothesized to sensitize the liver to the "second hit" consisting of oxidative stress, inflammation, and insulin resistance. If these metabolic abnormalities continue to progress they can cause hepatocellular injury, fibrosis, decreased liver function, cirrhosis, and end-stage liver disease. The shading indicates degree of severity and represents the spectrum of symptoms derived from NAFLD.

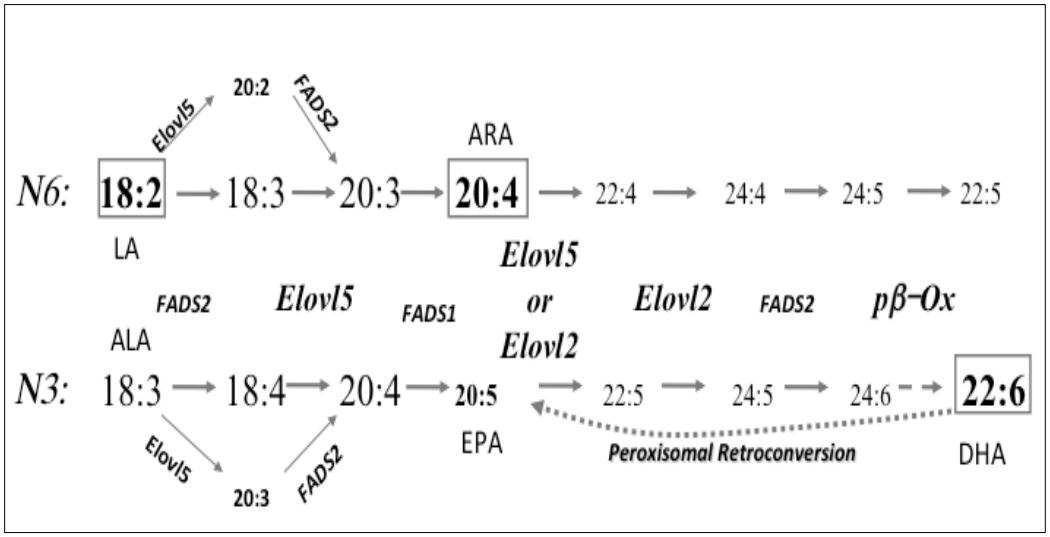

Figure 1.2. Synthesis of long-chain PUFA derived from essential fatty acids. The (n-6) essential fatty acid linoleic acid (18:2,(n-6); LA) and the (n-3) essential fatty acid is α-linolenic acid (18:3,(n-3); ALA). These essential fatty acids are obtained through the diet and converted to longer more desaturated PUFA through desaturation (Δ^5D[FADS1] and Δ^6D[FADS2]), elongation (Elovl2 and Elovl5), and peroxisomal β-oxidation steps. The boxed fatty acids accumulate in the highest abundance in hepatic membranes.

Chapter 2

Menhaden oil decreases high-fat diet-induced markers of hepatic damage, steatosis, inflammation, and fibrosis in obese *Ldlr*$^{-/-}$ mice

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2.1. Abstract

The frequency of non-alcoholic fatty liver disease (NAFLD) and non-alcoholic steatohepatitis (NASH) has increased in parallel with obesity in the US. NASH is progressive and characterized by hepatic damage, inflammation, fibrosis and oxidative stress. Since C20-22 (n-3) PUFA are established regulators of lipid metabolism and inflammation, we tested the hypothesis that C20-22 (n-3) PUFA in menhaden oil (MO) prevents high fat diet-induced fatty liver disease in mice. Wild type (WT) and *Ldlr*$^{-/-}$ C57BL/6J mice were fed the following diets for 12 wk: non-purified (NP), high fat (HF; 60% fat energy), high fat-high cholesterol with olive oil (HFHC-OO; 54.4% fat energy, 0.5% cholesterol), or HFHC-OO supplemented with MO (HFHC-MO). When compared to the NP diet, the HF and HFHC-OO diets significantly induced (P<0.05) hepatosteatosis and hepatic damage (elevated plasma alanine aminotransferase (ALT) and aspartate aminotransferase (AST) and elevated hepatic expression of markers of inflammation [monocyte chemo-attractant protein-1], fibrosis [pro-collagen1α1] and oxidative stress [heme oxygenase-1]. Hepatic damage, as reflected by plasma ALT, correlated (r = 0.74, P<0.05) with quantitatively higher (244%, P<0.05) hepatic cholesterol in *Ldlr*$^{-/-}$ mice fed the HFHC-OO diet compared to WT mice fed the HF or HFHC-OO diets. Plasma and hepatic markers of liver damage, steatosis, inflammation and fibrosis, but not oxidative stress, were significantly (P<0.05) lower in WT and *Ldlr*$^{-/-}$ mice fed HFHC-MO versus HFHC-OO. In conclusion, MO [C20-22 (n-3) PUFA at 2% energy] decreases many, but not all, high fat diet-induced markers of fatty liver disease in mice.

Key words: non-alcoholic fatty liver disease, (n-3) PUFA, inflammation, fibrosis, oxidative stress, triglycerides, cholesterol.

2.2. Introduction

Non-alcoholic fatty liver disease (NAFLD) has increased in parallel with central obesity and its prevalence is anticipated to continue to increase [111,112]. NAFLD is now the most common cause of liver disease in developed countries [113] and is defined as excessive lipid accumulation in the liver, i.e., hepatosteatosis [114,115]. The American Liver Foundation estimates that ~25% of the United States population has NAFLD and 75% of obese and 100% of morbidly obese individuals have NAFLD. NAFLD is the hepatic manifestation of metabolic syndrome (MetS) [62]; MetS risk factors include obesity, elevated plasma triglycerides and LDL cholesterol, reduced HDL cholesterol, high blood pressure and fasting hyperglycemia [116].

NAFLD ranges in severity from simple fatty liver (steatosis) to non-alcoholic steatohepatitis (NASH) [1]. Simple steatosis is relatively benign until it progresses to NASH, which is characterized by hepatic injury (hepatocyte ballooning and cell death), increased blood levels of hepatic enzymes (alanine aminotransferase [ALT]), hepatic inflammation, oxidative stress and fibrosis [111,112,117]. Approximately 30-40% of individuals with simple steatosis progress to NASH [3] and NASH can progress to cirrhosis [3], a major risk factor for hepatocellular carcinoma [112]. In the “2 hit hypothesis” for NASH [21], the first hit involves chronic hepatosteatosis as triglyceride and cholesterol (free cholesterol and cholesterol esters) accumulation. Excessive hepatic lipid sensitizes hepatocytes to the second hit that is manifested by increased inflammation derived from resident (Kupffer cells) and recruited macrophages, induction of oxidative stress, activation of stellate cells and fibrosis [112,118].

While management of life style (diet and exercise) is one approach to control the onset and progression of NAFLD, the best strategy for managing NAFLD has yet to be

defined [15]. Based on the well-established effects of C20-22 (n-3) PUFA to regulate hepatic lipid metabolism, dyslipidemia and inflammation [101,119,120], we tested the hypothesis that dietary (n-3) PUFA prevents high fat diet-induced fatty liver disease in mice. Recent clinical studies have indicated that dietary (n-3) PUFA have the potential to reduce hepatic lipid content in children and adults [121,122,123,124,125]. Our studies, however, go beyond the analysis of hepatic lipids and examine the capacity of (n-3) PUFA to regulate markers of NASH, such as hepatic damage, inflammation, oxidative stress and fibrosis. We used wild type (WT) and *Ldlr*$^{-/-}$ mice and two high fat diets: a high fat lard (HF, 60% fat energy) diet that induces obesity and insulin resistance [126,127] and a high fat-high cholesterol (HFHC, 54.4% fat energy; 0.5% cholesterol) diet that induces fatty liver and oxidative stress [128]. Menhaden oil (MO), a rich source of eicosapentaenoic acid [EPA, 20:5(n-3)] and docosahexaenoic acid [DHA, 22:6(n-3)], was used as a supplement to the HFHC-OO diet. EPA and DHA in the HFHC-MO diet are at 2% total energy, a level comparable to that used to treat dyslipidemia [129]. Our studies establish that dietary C20-22 (n-3) PUFA have the capacity to regulate some, but not all, high fat diet-induced markers of NASH.

2.3. Materials and methods

2.3.1) Animals and diets

All procedures for the use and care of animals for laboratory research were approved by the Institutional Animal Care and Use Committee at Oregon State University. Male WT and *Ldlr*$^{-/-}$ mice [on the C57BL/6J background](Jackson Laboratories) at 2 months of age were fed one of the following diets *ad libitum* for 12 wk: 1) Purina chow 5001 (NP, non-purified) [13.5% energy as fat and 58.0% energy as carbohydrates]; 2) high fat (HF) [60% energy as fat, D12492, Research Diets]; 3) high

fat-high cholesterol (HFHC) supplemented with olive oil (HFHC-OO) [54.4% energy as fat, 0.5% cholesterol (weight%); D08010702, Research Diets] or 4) high fat-high cholesterol supplemented with olive oil plus MO (HFHC-MO) [54.4% energy as fat, 0.5% cholesterol (weight%); D08010703, Research Diets] **(Table 2.1)**. The HF, HFHC-OO and HFHC-MO diets were described previously [127,128]. Fat energy density in the HFHC-OO and HFHC-MO diets was identical, 54.4% energy as fat. C20-22 (n-3) PUFA in the HFHC-MO diet was at ~2% of total energy **(Table 2.1)**. All diets were stored frozen (-20^{o}C) until used to feed the mice; diets were replenished every other day in an effort to reduce the formation of oxidation products.

The study was carried out twice with 8 mice/diet group in each study. Energy intake was monitored every other day and body weight was monitored weekly. At the end of the 12 wk feeding period, all mice were feed-deprived overnight (18:00 to 08:00 the next day); then half of the mice were refed their diets for 4 h (08:00 to 12:00). Feed-deprived and refed mice were euthanized (isoflurane anesthesia and exsanguination) at 08:00 and 12:00, respectively, for the collection of blood and liver. Blood was collected in tubes containing EDTA; plasma was collected by centrifugation. Livers were removed, weighed and rapidly frozen in liquid nitrogen. Plasma, blood cells and liver was stored frozen (-80^{o}C) until used for specific assays.

Our studies used WT and *Ldlr*$^{-/-}$ mice fed the NP diet as controls. Since there was no statistical difference (Student's t-test) in any variable measured between WT and *Ldlr*$^{-/-}$ mice fed the NP diet, NP fed *Ldlr*$^{-/-}$ mice served as the control for all studies described below. The following 6 group designations are used to describe the study: *Ldlr*$^{-/-}$ fed NP (NP), WT fed HF (WT-HF), WT fed HFHC-OO (WT-HFHC-OO), WT fed HFHC-MO (WT-HFHC-MO), *Ldlr*$^{-/-}$ fed HFHC-OO (*Ldlr*$^{-/-}$HFHC-OO), and *Ldlr*$^{-/-}$ fed HFHC-MO (*Ldlr*$^{-/-}$

HFHC-MO). During the course of our studies, we found that markers of NASH were induced to higher levels in *Ldlr*$^{-/-}$ mice, when compared to WT mice. As such, much of the data presented below describes the capacity of MO to regulate NASH markers in *Ldlr*$^{-/-}$ mice. A comparison of the effects of the 4 diets on inflammation and fibrosis markers in WT and *Ldlr*$^{-/-}$ mice is in **Table 2.2.**

2.3.2) Measurement of plasma markers

Plasma glucose (Autokit Glucose), plasma triglycerides (L-type TG H triglyceride), non-esterified fatty acids (NEFA-C), and cholesterol (Cholesterol E) were measured using kits from Wako. Plasma β-hydroxybutyrate was measured using a kit (β-hydroxybutyrate Liquicolor) from Stanbio. Plasma ApoB (ApoB K-Assay) and ApoCIII (ApoCIII K-assay) were measured by immunoturbidimetric assay from Kamiya Biomedical. Alanine aminotransferase (ALT) and aspartate aminotransferase (AST) were measured using kits from Thermo Electron.

2.3.3) Measurement of urinary isoprostanes

After 11 wk on the NP or HFHC diets, *Ldlr*$^{-/-}$ mice were placed in metabolic cages for 24 hour urine collection. Collected urine was stored at -80^{o}C until extracted for F2- and F3- isoprostanes and F4-neuroprostanes analysis. Results are normalized to urinary creatinine as previously described [128,130].

2.3.4) Lipid extraction and analysis

Total lipids were extracted from liver and plasma; fatty acid methyl esters were prepared and quantified by gas chromatography [126]. To measure hepatic total triglyceride and cholesterol, extracted hepatic lipids were dried, dissolved in 10% Triton X-100 and assayed for triglyceride and cholesterol content using the L-type TG H triglyceride and total cholesterol assay kits from Wako described above [126]. Hepatic

protein was measured using the Quick Start Bradford Reagent (Bio-Rad) and bovine serum albumin (Sigma) as standard [126].

2.3.5) RNA extraction and quantitative real time-polymerase chain reaction (qRT-PCR)

Total RNA was extracted from liver and specific mRNA were quantified by qRT-PCR as done previously [126,131]. Specific primers for each gene were described previously [126,131] or are listed in **Table 2.3.** Cyclophilin was used as the internal control for all genes.

2.3.6) Immunoblotting

Hepatic nuclear protein extracts were prepared using both protease (Roche Diagnostics) and phosphatase inhibitors (1 mmol/L β-glycerol phosphate, 2.5 mmol/L Na-pyrophosphate, 1 mmol/L Na_3VO_4) as described [126]. Proteins (25-100 µg) were separated electrophoretically and transferred to nitrocellulose for immunoblotting. TATA-binding protein (TBP) served as a loading control for all immunoblot studies. Antibodies used in these studies were described previously [126]; antibodies for NFκB-p50 and NFκB-p65 were purchased from Santa Cruz Biotechnology and Cell Signaling, respectively.

2.3.7) Statistical analysis

The statistical analysis of data used ANOVA (one- and two-way) and Tukey's HSD post hoc analysis to establish significant differences. One-way ANOVA was used to detect dietary effects when only *Ldlr*$^{-/-}$ mice were included in the analysis. Two-way ANOVA was used to detect diet-gene interactions when both WT and *Ldlr*$^{-/-}$ mice were included in the analysis and for the analysis of refeeding effects. Data was analyzed for homogeneous variances using the Levene test. If unequal variances were detected, data were log-transformed. ANOVA was performed on both transformed and untransformed

data. Untransformed data are presented because it is more meaningful for interpretation. If only 2 groups were being compared, then Student's t-test was used. *P<0.05* was considered statistically different. The correlation analysis was a linear regression analysis. Statistical analysis was done with VassarStats and Statgraphics. All values reported are mean ± SD.

2.4. Results

2.4.1) Body weight and plasma markers

After 12 wk of being fed the NP and test diets, body weight of WT mice fed the HF or HFHC diets and *Ldlr*$^{-/-}$ mice fed the HFHC diets was 45-63% higher (*P*<0.05) than the NP group **(Table 2.4)**. Body weight of WT and *Ldlr*$^{-/-}$ mice was not different amongst the groups fed the HF, HFHC-OO or HFHC-MO diets. Plasma markers were measured in feed-deprived mice. Diet and genotype did not significantly affect plasma glucose. Plasma triglycerides were elevated 290% (*P*<0.05) in the *Ldlr*$^{-/-}$ HFHC-OO group compared to the NP group. Plasma triglycerides in the *Ldlr*$^{-/-}$ HFHC-MO group were ~50% lower (*P*<0.05) than the *Ldlr*$^{-/-}$ HFHC-OO group and not different from NP. For plasma cholesterol there was a dietary effect (*P*<0.05) where all HFHC-OO fed mice were elevated ~180% compared to all HFHC-MO fed. Additionally, there was a genotype effect (*P*<0.05) with all *Ldlr*$^{-/-}$ fed mice elevated ~650% compared to all WT fed mice. There was not a significant diet by genotype interaction for plasma cholesterol. Plasma ApoB and ApoCIII paralleled diet-induced changes in plasma triglyceride in the *Ldlr*$^{-/-}$ HFHC-OO and *Ldlr*$^{-/-}$ HFHC-MO groups. When compared to the NP group, plasma NEFA were elevated 188% (P<0.05) in the *Ldlr*$^{-/-}$ HFHC-OO group. Finally, plasma β-hydroxybutyrate was ~200% (P<0.05) higher in the *Ldlr*$^{-/-}$ HFHC-OO and *Ldlr*$^{-/-}$ HFHC-MO groups when compared to the NP group.

2.4.2) Hepatic damage

Plasma ALT and AST are markers of hepatic damage; these markers were measured in feed-deprived mice. When compared to the NP group, ALT was elevated ≥200% ($p<0.05$) in the WT and *Ldlr*$^{-/-}$ mice fed the HF, HFHC-OO or HFHC-MO diets, while AST was elevated ≥260% ($p<0.05$) in the WT-HFHC-OO, *Ldlr*$^{-/-}$ HFHC-OO and *Ldlr*$^{-/-}$ HFHC-MO groups **(Table 2.4)**. Plasma ALT and AST in the *Ldlr*$^{-/-}$ HFHC-OO group was ≥195% ($p<0.05$) higher than the WT-HFHC-OO group. Plasma ALT (P=0.12) and AST (P<0.05) in the *Ldlr*$^{-/-}$ HFHC-MO group were ~50% lower than the *Ldlr*$^{-/-}$ HFHC-OO group, however, only the change in AST was statistically significant.

2.4.3) Hepatic lipids

Hepatic triglycerides in the WT-HF, WT-HFHC-OO, and *Ldlr*$^{-/-}$ HFHC-OO groups were ≥276% (P<0.05) higher than the NP group **(Table 2.4)**. This level of triglyceride accumulation is comparable to mice fed a HF diet [126,132,133]. Hepatic triglycerides in the WT-HFHC-MO and *Ldlr*$^{-/-}$ HFHC-MO groups were not different from the NP group. Hepatic cholesterol was increased 244% ($P<0.05$) only in the *Ldlr*$^{-/-}$HFHC-OO group when compared to the NP group **(Table 2.4)**. Thus, supplementing the HFHC-OO diet with C20-22 (n-3) PUFA, i.e., the HFHC-MO diet, prevented the HFHC-OO diet induced accumulation of hepatic triglycerides and cholesterol in *Ldlr*$^{-/-}$ mice.

The level of plasma ALT correlated with hepatic cholesterol ($r = 0.74$, P<0.05), but not hepatic triglyceride, in WT and *Ldlr*$^{-/-}$ mice. This outcome agrees with recent studies linking hepatic cholesterol to NASH [132,133,134]. Since hepatic damage, as reflected by plasma ALT, was quantitatively greater (195%, P<0.05) in the *Ldlr*$^{-/-}$ HFHC-OO group, compared to WT-HFHC-OO group **(Table 2.4)**, studies described below

examined the effects of the HFHC-OO and HFHC-MO diets on hepatic fatty acids and markers of NASH in *Ldlr*$^{-/-}$ mice, i.e., oxidative stress, inflammation and fibrosis.

2.4.4) Hepatic fatty acids

Hepatic fatty acid composition is significantly affected by diet. Since the HFHC-OO and HFHC-MO diets had similar affects on hepatic fatty acid profiles in WT and *Ldlr*$^{-/-}$ mice, only the *Ldlr*$^{-/-}$ data is shown **(Table 2.5)**. Oleic acid [18:1(n-9)] is the predominant fatty acid in the HFHC-OO diet **(Table 2.1)**; the mole% of hepatic oleic acid was 360% and 247% higher (*P<0.05*) in the *Ldlr*$^{-/-}$-HFHC-OO and *Ldlr*$^{-/-}$-HFHC-MO groups, respectively, when compared to the NP group. Hepatic oleic acid in the *Ldlr*$^{-/-}$ HFHC-MO group was ~32% lower (*P<0.05*) than the *Ldlr*$^{-/-}$HFHC-OO group. Hepatic 20:4(n-6) was 48% and 68% lower (P<0.05) in the *Ldlr*$^{-/-}$ HFHC-OO and *Ldlr*$^{-/-}$ HFHC-MO groups, respectively, when compared to NP. Hepatic C20-22 (n-3) PUFA were ~70% lower (*P<0.05*) in *Ldlr*$^{-/-}$ HFHC-OO group compared to NP, but 309% higher (*P<0.05*) in the *Ldlr*$^{-/-}$ HFHC-MO group. The ratio of 20:4(n-6) to 18:2(n-6) or 20:3(n-6) to 18:2(n-6) was the same in both the NP and HFHC-OO groups, but $\geq$50% lower (P<0.05) in the *Ldlr*$^{-/-}$ HFHC-MO group **(Table 2.5)**. Similar effects of diet were seen in plasma C20-22 (n-3) PUFA and the ratio of 20:4(n-6) to 18:2(n-6) **(Fig. 2.1)** as well as in liver and plasma of WT mice (not shown).

2.4.5) Whole body and hepatic oxidative stress

F2-, F3-isoprostanes and F4-neuroprostanes are non-enzymatic oxidation products of 20:4(n-6), 20:5(n-3) and 22:6(n-3), respectively [128,130]. Since urinary isoprostanes are a measure of whole body oxidative stress [135], we analyzed urinary isoprostanes (F2 and F3) and neuroprostanes (F4) in the NP, *Ldlr*$^{-/-}$ HFHC-OO and

Ldlr$^{-/-}$ HFHC-MO groups [128,130] **(Table 2.6)**. F2 and F3 isoprostanes and F4-neuroprostanes in urine of NP were comparable to previous reports [136]. F2 isoprostanes were not elevated in urine of *Ldlr*$^{-/-}$HFHC-OO, but increased 213% (P<0.05) in *Ldlr*$^{-/-}$ HFHC-MO compared to NP. While urinary F3-isoprostanes and F4-neuroprostanes were not significantly affected by the HFHC-OO diet, these oxidized lipids were respectively increased 595% and 155% (P<0.05) in the urine of the *Ldlr*$^{-/-}$ HFHC-MO group when compared to the NP and *Ldlr*$^{-/-}$ HFHC-OO groups. Supplementing the HFHC-OO diet with C20-22 (n-3) PUFA, i.e., the HFHC-MO diet, increases lipid peroxidation as measured by urinary F2- and F3-isoprostanes and F4-neuroprostanes.

To assess hepatic oxidative stress, we measured hemeoxygenase-1 (*Hmox1*) expression **(Table 2.7)**. *Hmox1* is induced in NAFLD in response to oxidative stress [137,138,139,140,141,142]; it is also cytoprotective [137,138,139,143]. Hepatic *Hmox1* mRNA was induced 420% and 320% (P<0.05) in the *Ldlr*$^{-/-}$ HFHC-OO and *Ldlr*$^{-/-}$ HFHC-MO groups, respectively, when compared to the NP group. Supplementing the HFHC-OO diet with MO did not lower HFHC-OO induced hepatic oxidative stress, i.e., *Hmox1* expression, in *Ldlr*$^{-/-}$ mice.

2.4.6) Lipid-regulated hepatic transcription factors and gene expression

Transcription factors controlling *de novo* lipogenesis (DNL), MUFA and triglyceride synthesis include SREBP1, ChREBP, MLX, LXR, PPARα and HNF4α [119]. We examined the effect of feed-deprivation and refeeding on the nuclear abundance of these transcription factors in *Ldlr*$^{-/-}$ mice maintained on the HFHC-OO or HFHC-MO diets **(Fig. 2.2)**. The nuclear abundance of SREBP1 and ChREBP, but not MLX, was increased $\geq$300% (P<0.05) in *Ldlr*$^{-/-}$ mice 4 hr after refeeding the HFHC-OO group. The

induction of nuclear SREBP1 was reduced by ~50% (P<0.05) in the *Ldlr*$^{-/-}$-HFHC-MO group when compared to the *Ldlr*$^{-/-}$ HFHC-OO group **(Fig. 2.2A)**. Since the induction of *Srebp-1* mRNA abundance was not different in the *Ldlr*$^{-/-}$ HFHC-OO and *Ldlr*$^{-/-}$ HFHC-MO groups **(Fig. 2.3)**, MO suppression of hepatic SREBP1 nuclear abundance involved a post-translational mechanism [99]. The refeeding response of ChREBP was not affected by MO. The nuclear abundance of other transcription factors controlling hepatic metabolism, i.e., LXRα, PPARα, HNF4α and FoxO1, remained unaffected by diet (not shown).

We next measured the abundance of *Acc1, Fasn, Scd1* and *Elovl6* mRNA in livers of the re-fed mice described above. Of these transcripts, hepatic abundance of *Acc1* and *Fasn* was suppressed by 50% (P<0.05) in the *Ldlr*$^{-/-}$ HFHC-MO group compared to the *Ldlr*$^{-/-}$ HFHC-OO group **(Fig. 2.2B)**. PPARα regulates the expression of multiple genes involved in fatty acid oxidation [119], including acyl CoA oxidase (*Aox*), CYP450-4A10 (*Cyp4A10*), acyl-CoA thioesterase-1 (*Acot1*), carnitine palmitoyl transferase 1 (*Cpt1*) and *Cpt2.* None of these transcripts were affected by the HFHC-OO or HFHC-MO diets when compared to the NP diet (not shown).

Enzymes involved in converting essential fatty acids [18:2(n-6) and 18:3(n-3)] to C20-22 (n-6) and (n-3) PUFAs include fatty acid elongases (*Elovl2* and *Elovl5*) and desaturases (*fads1* and *fads2*). Expression of these enzymes is controlled, at least in part, by SREBP1 and PPARα [119,144,145,146]. Hepatic E*lovl2* was induced 220% (P<0.05) by in the *Ldlr*$^{-/-}$ HFHC-OO and *Ldlr*$^{-/-}$ HFHC-MO groups when compared to the NP group **(Table 2.7)**. Hepatic E*lovl5* and *Fads1* mRNAs were reduced by 70-80% (P<0.05) in the *Ldlr*$^{-/-}$ HFHC-MO group compared to the NP or *Ldlr*$^{-/-}$ HFHC-OO groups. Hepatic *Fads2* was not affected by diet. Changes in *Elovl5* and *Fads1* expression

parallel the decline in the hepatic 20:4/18:2 ratio in *Ldlr*$^{-/-}$ fed the HFHC-MO diet **(Table 2.5)**.

Since the combination of the HFHC-OO diet and the *Ldlr*$^{-/-}$ genotype significantly increased hepatic cholesterol **(Table 2.4)**, we examined pathways controlling hepatic cholesterol content **(Table 2.7)**. The mRNA abundance of SREBP2 target genes (*Hmg CoA red* and *Hmg CoA syn1*) was not affected. Of the LXR target genes examined, only the ABC transporters (*Abca1*, *Abcg5*) were induced ≥320% (P<0.05) in livers of *Ldlr*$^{-/-}$ fed the HFHC-OO and HFHC-MO diets when compared to the NP group. *Acat1* is expressed in Kupffer cells [147], while *Acat2* is expressed in hepatocytes [148]. The expression of hepatic *Acat1* and *Acat2* mRNA was induced 480% and 200%, respectively, by the HFHC-OO when compared to the NP diet. Hepatic *Acat1* mRNA, however, was 38% lower (P<0.05) in the *Ldlr*$^{-/-}$ HFHC-MO group when compared to the *Ldlr*$^{-/-}$ HFHC-OO group **(Table 2.7)**.

2.4.7) Hepatic inflammation

We next examined markers of inflammation. Monocyte chemo-attractant protein-1 (*Mcp1*) mRNA, an early marker of inflammation [149], was induced ≥720% (*P*<0.05) in the WT-HF, WT-HFHC-OO groups **(Table 2.2)**, and 2280% (*P*<0.05) in the *Ldlr*$^{-/-}$ HFHC-OO group when compared to NP **(Table 2.7)**. Furthermore, *Mcp1* mRNA was 272% higher (*P*<0.05) in *Ldlr*$^{-/-}$ HFHC-OO compared to WT-HFHC-OO. In contrast to the HFHC-OO, the HFHC-MO diet did not induce hepatic *Mcp1* mRNA in either genotype **(Tables 2.2 and 2.7)**.

Cd68 and *Clec4f* are cell surface markers of monocytes and resident macrophages (Kupffer cells), respectively. Hepatic *Cd68* mRNA was induced 270%

(*P*<0.05) in the WT-HFHC-OO group, 770% (*P*<0.05) in the *Ldlr*$^{-/-}$HFHC-OO group and 410% (*P*<0.05) in the *Ldlr*$^{-/-}$ HFHC-MO group when compared to the NP group **(Tables 2.2 and 2.7)**. Comparing the *Ldlr*$^{-/-}$ HFHC-OO and the *Ldlr*$^{-/-}$ HFHC-MO groups shows a ~50% (*P*<0.05) reduction in *Cd68* mRNA **(Table 2.7)**. *Clec4f* mRNA was elevated 630% and 470%, respectively (*P*<0.05) in the *Ldlr*$^{-/-}$ HFHC-OO and *Ldlr*$^{-/-}$HFHC-MO groups compared to the NP group. Hepatic *Clec4f* was not induced by either HF or HFHC-OO diet in WT mice **(Table 2.2)**.

NFκB-p50 and NFκB-p65 are transcription factors controlling the expression of multiple genes involved in inflammation [107]. We examined the hepatic nuclear abundance of NFκB-p50 and NFκB-p65 in *Ldlr*$^{-/-}$ mice fed the NP, HFHC-OO and HFHC-MO diets. Nuclear content of NFκB-p50 and NFκB-p65 was induced ≥200% (*P*<0.05) in mice fed the HFHC-OO diet versus the NP diet. While the level of nuclear NFκB-p50 in mice fed the HFHC-MO diet was not different from mice fed the NP diet, NFκB-p65 nuclear content in mice fed the HFHC-OO and HFHC-MO diets was not different **(Fig. 2.4)**.

2.4.8) Hepatic fibrosis

Markers of fibrosis examined in this study include transforming growth factor-β1 (*Tgfβ1*) and pro-collagen 1α1 (*Procol1α1*) [150,151,152,153]. These proteins are expressed in Kupffer and stellate cells, but not in hepatic parenchymal cells. *Tgfβ1* mRNA was not induced in the WT-HF or WT-HFHC-OO groups when compared to NP **(Table 2.2)**, but was induced 300% and 210% (*P*<0.05) in *Ldlr*$^{-/-}$mice fed the HFHC-OO and HFHC-MO diets, respectively **(Table 2.7)**. *Procol1α1* mRNA was induced 150%, 430% and 200% (P<0.05) in the WT-HFHC-OO, *Ldlr*$^{-/-}$ HFHC-OO and *Ldlr*$^{-/-}$ HFHC-MO

groups, respectively **(Tables 2.2 and 2.7)**. While *Procol1α1* mRNA abundance in the WT-HFHC-MO and NP groups was not different, *Procol1α1* mRNA abundance in the *Ldlr*$^{-/-}$ HFHC-MO group was ~50% lower (P<0.05) when compared to the *Ldlr*$^{-/-}$ HFHC-OO group.

2.5. Discussion

We have examined the effect of two high fat diets on the development of fatty liver disease in WT and *Ldlr*$^{-/-}$ mice. Feeding WT mice the HF and HFHC-OO diets and feeding *Ldlr*$^{-/-}$ mice the HFHC-OO diet for 12 wk increased body weight by 41-63% and multiple markers of dyslipidemia and NASH; including hepatic damage (plasma ALT and AST), hepatosteatosis, oxidative stress (urinary isoprostanes and hepatic *Hmox1* [*Ldlr*$^{-/-}$ only]), inflammation (mRNAs encoding *Mcp1*, *Cd68*, *Clec4f*) and fibrosis (*Tgfβ1* and *Procol1α1)* **(Tables 2.4, 2.6, and 2.7)**. While hepatic triglyceride content increased equally in the WT-HF, WT-HFHC-OO and *Ldlr*$^{-/-}$ HFHC-OO groups, the accumulation of hepatic cholesterol in *Ldlr*$^{-/-}$ correlated with hepatic damage [plasma ALT versus hepatic cholesterol, r = 0.74, P<0.05] and the induction of markers of inflammation and fibrosis. Thus, the combination of diet lipid composition and the genotype contributed to the severity of fatty liver disease in mice.

Because fatty liver disease was more robust in *Ldlr*$^{-/-}$ fed the HFHC-OO diet when compared to WT mice fed the same diet, most of our studies focused on determining if MO supplementation of the HFHC-OO diet, i.e., the HFHC-MO diet, could prevent diet-induced fatty liver disease in *Ldlr*$^{-/-}$ mice. As expected, feeding *Ldlr*$^{-/-}$ the HFHC-MO diet significantly elevated hepatic and plasma C20-22 (n-3) PUFA **(Fig. 2.1 and Table 2.5)** and reduced the expression of many NASH markers when compared to

Ldlr$^{-/-}$ mice fed the HFHC-OO diet **(Tables 2.4 and 2.7)**. The exception was oxidative stress.

Both *Ldlr*$^{-/-}$ HFHC-OO and *Ldlr*$^{-/-}$ HFHC-MO groups expressed the hepatic oxidative stress marker, *Hmox1,* at levels 420% above that seen in the NP group **(Table 2.7)**. Moreover, *Ldlr*$^{-/-}$ mice fed the HFHC-MO diet increased urinary isoprostanes **(Table 2.6)**. *Hmox1* expression is controlled by nuclear factor E2-related factor-2 (NRF2), a transcription factor regulated by reactive oxygen species (ROS) [141]. NRF2 binds anti-oxidant response elements in the *Hmox1* promoter as well as other promoters responsive to ROS, e.g., *Gstα1.* Induction of hepatic *Hmox1* by the HFHC-OO and HFHC-MO diets is an indirect marker of elevated hepatic ROS.

NAFLD patients have elevated oxidative stress [137,138,139,140,141,142] and dietary supplement approaches which counter oxidative stress have been tested as a potential NAFLD therapy [154,155]. Finding that dietary C20-22 (n-3) PUFA increased urinary isoprostanes may raise concerns for their use in NAFLD therapy **(Table 2.6)** [156]. Interestingly, urinary F2-isoprostane levels were not different in the NP and HFHC-OO groups, but increased 213% in the HFHC-MO group. One explanation for this outcome is the impact of dietary fat composition on tissue (n-6) and (n-3) PUFA content. In contrast to the NP diet, the HFHC-OO diet contains high levels of saturated and MUFA, relative to (n-6) PUFA **(Table 2.1)**. Hepatic 20:4(n-6), the substrate for F2-isoprostanes, was lower in the HFHC-OO group than the NP group **(Table 2.5)**. Thus, low tissue 20:4(n-6) content may not favor increased F2 isoprostane formation, even when hepatic ROS is elevated. Moreover, the HFHC-OO diet did not suppress expression of hepatic enzymes involved in PUFA synthesis **(Table 2.7)**. The hepatic and plasma ratio of 20:4(n-6) to 18:2(n-6) also indicated that n-6 PUFA synthesis was not

suppressed by the HFHC-OO diet **(Fig. 2.1 and Table 2.5)**. One explanation for the decline in hepatic 20:4(n-6) in the *Ldlr*$^{-/-}$ HFHC-OO group is that synthesized 20:4(n-4) is exported via VLDL and stored in extra-hepatic tissues. Additional studies are required to define the impact of high fat diets on hepatic and whole body 20:4(n-6) metabolism.

While the HFHC-OO and HFHC-MO diets have the same SFA, MUFA, (n-6) PUFA content, they differ in (n-3) PUFA content; the HFHC-MO diet increased urinary isoprostanes **(Table 2.6)**. In contrast to the HFHC-OO group, low hepatic 20:4(n-6) in the HFHC-MO group **(Table 2.5)** can be explained by low expression of enzymes involved in PUFA synthesis and a 50% reduction in the 20:4(n-6) to 18:2(n-6) ratio **(Tables 2.5 and 2.7 and Fig. 2.4)**. Moreover, the HFHC-OO and MO diets induced hepatic oxidative stress, i.e., *Hmox1*, and livers enriched with C20-22 (n-3) PUFA paralleled increased F3-isoprostane and F4 neuroprostane formation [128]. Increased production of isoprostanes from C20-22 (n-3) PUFA may stimulate F2 isoprostane formation [128,156]. F2-isoprostanes activate thromboxane and prostaglandin F2α receptors; they activate platelets and promote smooth muscle cell-mediated vasoconstriction [156,157], whereas isoprostanes generated from (n-3) PUFA do not [156]. By analogy to the pro-inflammatory (series 2) and anti-inflammatory (series 3) cyclooxygenase-derived eicosanoids [158], F3 isoprostanes may be anti-inflammatory. If so, some effects of C20-22 (n-3) PUFA on inflammatory markers reported in **Table 2.7 and Fig. 2.4** may be attributed to the action of F3-isoprostanes and F4-neuroprostanes. Clearly, more study is required to establish whether isoprostanes generated from (n-3) PUFA are beneficial.

Since (n-3) PUFA inhibit hepatic fatty acid synthesis and induce fatty acid oxidation [119,159], we hypothesized that increasing hepatic (n-3) PUFA would

suppress hepatic accumulation of triglyceride and prevent the induction of all markers linked to fatty liver disease. Major regulators of DNL include SREBP1 and the ChREBP/MLX heterodimer [93,160], both transcription factors play a role in hepatosteatosis [99,112,119,161,162]. The nuclear abundance of SREBP1, but not the ChREBP/MLX heterodimer, was vulnerable to C20-22 (n-3) PUFA regulation in obese *Ldlr*$^{-/-}$ mice **(Fig. 2.2)**. The suppression of mRNAs encoding *Acc1* and *Fasn* parallels the suppression of hepatic nuclear abundance of SREBP1 **(Fig. 2.2B)**. While C20-22 (n-3) PUFA suppression of nuclear SREBP1 accounts for some reduction of hepatic triglycerides, other mechanisms are likely involved. In humans with NAFLD, fatty acids entering hepatic triglycerides are derived from multiple sources, including DNL, portal circulation, and NEFA mobilized from adipose tissue [23,118,163]. In feed-deprived mice, plasma NEFA were reduced by 35% in the *Ldlr*$^{-/-}$ HFHC-MO group compared to the *Ldlr*$^{-/-}$ HFHC-OO group **(Table 2.4)**. Thus, (n-3) PUFA control of lipid mobilization from adipose tissue and delivery of NEFA to the liver may contribute to the low hepatic triglyceride in the *Ldlr*$^{-/-}$ HFHC-MO group.

The combination of HFHC-OO and the *Ldlr*$^{-/-}$ genotype was required to increase hepatic cholesterol content 244% ($P<0.05$) **(Table 2.4)** and induced high levels of expression of inflammation and fibrosis markers **(Table 2.7)**. Several recent reports have linked elevated hepatic cholesterol to the induction of NASH markers [132,133,134,164]. In those studies, increased hepatic cholesterol activated SREBP2, up-regulated the LDL-receptor, reduced biotransformation of bile acids, and suppressed canalicular pathways for cholesterol and bile acid export [134,164]. In our study, however, the LDL-receptor is absent and SREBP2 target genes and the expression of CYP7A1, the rate limiting step in bile acid synthesis, were not affected by the HFHC-OO or HFHC-MO

diets **(Table 2.7)**. As such, alternative mechanisms are needed to explain our observation. Since both HFHC-OO and HFHC-MO diets induced *Abca1* and *Abcg5*, cholesterol efflux may not have been affected. The very high plasma triglycerides and cholesterol in feed-deprived *Ldlr*$^{-/-}$ mice **(Table 2.4)** likely promotes LDL uptake via hepatic scavenger receptors. Using the same diet and mice as reported herein, Saraswathi, et al [128] found that dietary C20-22 (n-3) PUFA decreased hepatic cholesterol, but increased cholesterol storage in adipose tissue. *Ldlr*$^{-/-}$ mice fed the HFHC-MO diet had significantly lower ApoB, ApoCIII (a lipoprotein lipase inhibitor), as well as plasma triglycerides and cholesterol when compared to the HFHC-OO group **(Table 2.4)**. Therefore, part of the control of hepatic triglycerides and cholesterol may involve VLDL assembly and secretion as well as VLDL/LDL clearance by non-hepatic tissues. More studies are required to verify this mechanism.

A key finding of our study was the effect of the HFHC-OO and HFHC-MO diets on markers of NASH in non-parenchymal hepatic cells. *Cd68* and *Clec4f* are expressed in macrophage and Kupffer cells [133], while *Tgfβ1* is expressed predominantly in Kupffer cells and stellate cells, but not at all in parenchymal cells. *Procol1α1* is expressed in stellate cells [150]. Feeding WT and *Ldlr*$^{-/-}$ mice the HFHC-OO diet induced expression of mRNA and nuclear proteins linked to inflammation (*Mcp1*, *Cd68*, *Clec4f* and nuclear NFκB p50 and p65) and fibrosis (*Tgfβ1*, *Procol1α1*) **(Fig. 2.4 and Tables 2.2 and 2.7)**. More importantly, supplementing the diet with MO (HFHC-MO) lowered the levels of most of these markers (except *Clec4f* and *Tgfβ1*) when compared to HFHC-OO-fed mice.

While we have a good understanding of C20-22 (n-3) PUFA control of hepatic parenchymal cell gene expression [119], much less is known about (n-3) PUFA

regulation of Kupffer and stellate cells. *Mcp1* is an early marker of inflammation and is regulated by NFκB [149,165]. In the *Ldlr*$^{-/-}$ HFHC-OO group, the nuclear abundance of NFκB p50 and NFκB-p65 was significantly induced when compared to the NP group **(Fig. 2.4)**. The induction of nuclear abundance of NFκB-p50, but not NFκB-p65, was prevented in mice fed the HFHC-MO diet **(Fig. 2.4)**. Changes in *Mcp1* mRNA paralleled changes in hepatic nuclear NFκB-p50 content. This outcome suggests that genes requiring the classical p50/p65 heterodimer or the p50/50 homodimer for transcriptional activation will be attenuated by C20-22 (n-3) PUFA, while inflammatory genes that are regulated by p65 heterodimerization with other partners, like c/EBPβ, will be less sensitive to C20-22 (n-3) PUFA control. In addition to NFκB, other regulatory factors control the expression of hepatic inflammatory and fibrotic markers, including TGFβ, cJun, TNFα and the Toll-like receptors-2 and 4 [132,166]. *Procol1α1* expression in stellate cells is induced by TGFβ1 through a H_2O_2-c/EBPβ-dependent mechanism [152,153]. The HFHC-MO diet had a modest effect on *Tgfβ1* expression in *Ldlr*$^{-/-}$ *mice*. In contrast, *Procol1α1* expression was significantly attenuated in the WT- and *Ldlr*$^{-/-}$ HFHC-MO groups when compared to the WT and *Ldlr*$^{-/-}$ HFHC-OO groups **(Tables 2.2 and 2.7)**. A systematic analysis of C20-22 (n-3) PUFA effects on hepatic target genes controlled by these regulatory factors will clarify the breadth of C20-22 (n-3) PUFA effects on hepatic inflammation and fibrosis markers.

In summary, we used an established model of high-fat diet induced fatty liver disease in mice [128] and assessed the capacity of dietary C20-22 (n-3) PUFA in MO to prevent fatty liver disease in WT and *Ldlr*$^{-/-}$ mice. *Ldlr*$^{-/-}$ mice developed more severe fatty liver disease than WT mice when fed the HFHC-OO diet. Mice fed the HFHC-MO diet,

which was supplemented with a physiologically relevant level (2% of total energy) of C20-22 (n-3) PUFA, had lower plasma and hepatic lipids (triglycerides and cholesterol), hepatic damage, inflammation and fibrosis, than mice fed the HFHC-OO diet. The HFHC-MO diet, however, did not prevent the induction of other high fat diet-induced markers of fatty liver disease or MetS; including obesity, hyperglycemia, and hepatic or whole body oxidative stress. Thus, the C20-22 (n-3) PUFA in MO reduced many, but not all, metabolic abnormalities associated with high fat diet-induced fatty liver disease in mice.

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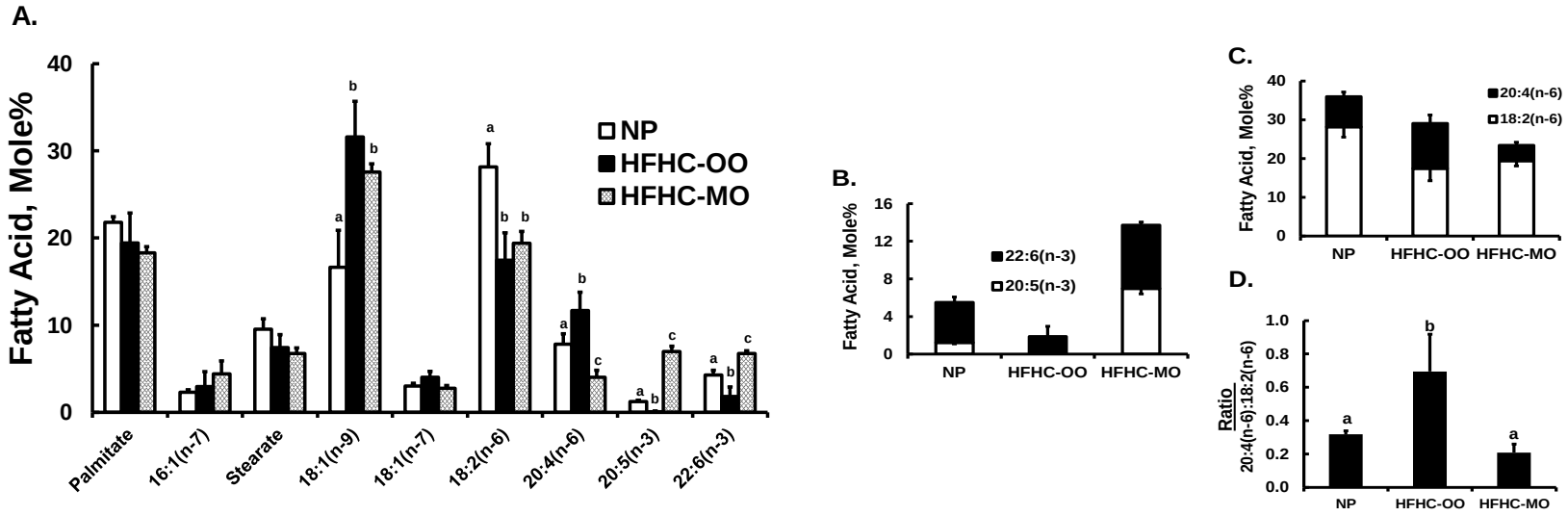

Figure 2.1. Effect of diet on plasma fatty acid composition in *Ldlr*$^{-/-}$ mice. [A]: *Ldlr*$^{-/-}$ mice fed NP (white bars), HFHC-OO (black bars) or HFHC-MO (cross-hatched bars) for 12 wk were fasted overnight, euthanized the next day for the collection of blood and plasma. Plasma lipids were extracted and fatty acid methyl esters were prepared and quantified (Methods). Results are reported as Fatty Acid, Mole%, mean ± SD, n=8. Labeled means without a common letter differ, *P<0.05*. [B, C & D] used data from [A] to illustrate the cumulative abundance of hepatic PUFA: [B]: (n-3) PUFA: 20:5(n-3) and 22:6(n-3) and [C] (n-6) PUFA, 18:2(n-6) and 20:4(n-6). [D] used data from [A] to calculate the molar ratio of 20:4(n-6) to 18:2(n-6). All results are the mean ± SD, *n*=4. Labeled means without a common letter differ, *P<0.05*.

Table 2.1. Diet Composition

	NP	HF	HFHC-OO	HFHC-MO
Composition, *energy%*				
Protein	28.5	20.0	18.6	18.6
Carbohydrate	58.0	20.0	27.0	27.0
Fat	13.5	60.0	54.4	54.4
% Energy as:				
Saturated fat	3.5	22.7	21.0	21.0
MUFA	2.8	22.6	24.4	22.0
(n-3) PUFA	0.2			2.0
(n-6) PUFA	7.0	14.7	9.4	9.4
Cholesterol, weight%	0.02	0.03	0.5	0.5
Fatty Acid Composition, *Mole%*				
16:0	16.0	23.5	14.2	16.7
18:0	4.0	11.5	7.4	8.1
18:1,n-9	19.5	35.8	54.9	40.4
18:2,n-6	50.1	23.2	14.1	14.4
18:3,n-3	0.4	0.5	0.6	0.5
20:5,n-3				3.5
22:6,n-3	1.1			1.8

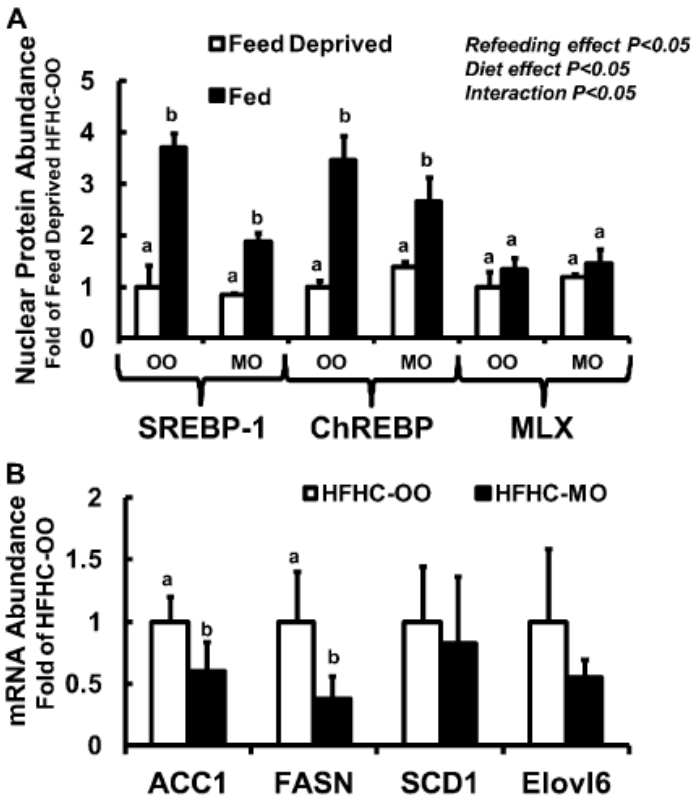

Figure 2.2. Effect of HFHC-OO and HFHC-MO diets on the hepatic SREBP1, ChREBP and MLX nuclear abundance (**A**) and hepatic lipogenic gene expression (**B**). [**A**]: *Ldlr*$^{-/-}$ mice maintained on the HFHC-OO or HFHC-MO diets for 12 wk; mice were feed deprived overnight. The next day half the animals were fed their diets; 4 hr later, feed-deprived and refed mice were euthanized for the isolation of liver, extraction and quantitation of hepatic nuclear protein abundance (Methods). Hepatic nuclear abundance of SREBP1 (white bars), ChREBP (black bars) and MLX (gray bars) was measured in as described in Methods. TATA-binding protein (TBP) was the loading control and all results are normalized to TBP abundance. Results are expressed as nuclear protein abundance (Fold Change), relative to feed-deprived *Ldlr*$^{-/-}$ mice maintained on the HFHC-OO diet; mean ± SD, n = 8. Labeled means without a common letter differ, $P < 0.05$.
[**B**]: Hepatic RNA from the refed mice described in [A] (above) was used to quantify *Acc1*, *Fasn*, *Scd1* and *Elovl6* mRNA abundance by qRT-PCR. Results are expressed as mRNA abundance (Fold Change) relative to *Ldlr*$^{-/-}$ mice fed the HFHC-OO diet; mean ± SD, n = 8. Labeled means without a common letter differ, $P < 0.05$.

Table 2.2. mRNA abundance of markers of inflammation and fibrosis in wild type (WT) and *Ldlr* $^{-/-}$ mice fed NP, HF, HFHC-OO or HFHC-MO diets for 12 wks[1].

		Wild Type			*Ldlr*$^{-/-}$	
	NP	**HF**	**HFHC-OO**	**HFHC-MO**	**HFHC-OO**	**HFHC-MO**
Inflammation mRNA		*Fold of NP*			*Fold of NP*	
Mcp1[2-4]	1.0 ± 0.5[a]	7.2 ± 1.8[b]	8.3 ± 3.6[b]	3.3 ± 0.9[a]	22.8 ± 10.4[c]	4.6 ± 2.8[a]
Cd68[2-4]	1.0 ± 0.5[a]	2.2 ± 0.7[a]	2.7 ± 0.9[b]	2.0 ± 1.1[a]	7.7 ± 2.5[c]	4.1± 2.5[b]
Clec4F[3]	1.0 ± 0.5[a]	1.4 ± 0.2[a]	1.7 ± 0.3[a]	1.5 ± 0.3[a]	6.3 ± 1.2[b]	4.7 ± 1.3[b]
Fibrosis mRNA						
Tgfβ1[3]	1.0 ± 0.2[a]	1.1 ± 0.3[a]	1.2 ± 0.4[a]	1.6 ± 0.2[a]	3.0 ± 0.9[b]	2.1 ± 0.4[b]
ProCol1α1[2-4]	1.0 ± 0.7[a]	7.4 ± 4.1[a]	15.0 ± 12.6[b]	2.5 ± 0.9[a]	43.0 ± 24.0[c]	20.1 ± 14.0[b]

1Data are mean ± SD, *n* = 8 per treatment group and are represented as fold of NP. Labeled means in a row with superscripts without a common letter differ, $P < 0.05$. [2]2-way ANOVA diet effect $P<0.05$; [3]2-way ANOVA genotype effect $P<0.05$; [4]2-way ANOVA diet by gene interaction effect $P<0.05$.

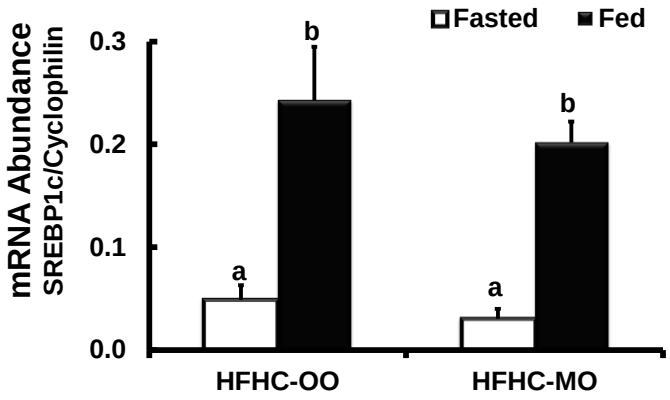

Figure 2.3. Effect of diet on the fasting-feeding response of hepatic SREBP1 mRNA in *Ldlr*$^{-/-}$ mice. Hepatic RNA from fasted and refed *Ldlr*$^{-/-}$ mice was extracted and assayed for the abundance of SREBP1 by qRTPCR; cyclophilin was the internal control. Results are expressed as mRNA Abundance: SREBP1/Cyclophilin; Mean $\pm$ SD, n =8.
Labeled means without a common letter differ, $P<0.05$. Fold induction of SREBP1c mRNA following refeeding food-deprived mice was 4.9 $\pm$ 1.1 for the HFHC-OO group and 6.6 $\pm$ 1.1 for the HFHC-MO group; these values were not significantly different.

Table 2.3. Mouse Primer Pairs for qRT-PCR

	Accession#	Forward	Reverse
Cyclophilin	NM_008907	CTTCTTGCTGGTCTTGCCATTCCT	GGATGGCAAGCATGTGGTCTTTG
Sterol Metabolism			
ABC-transporter A1 (*Abca*1)	NM_013454.3	GCTTTCAATGAGACCAACCA	CAGAACTTCCTCTCGTCCAG
ABC-transporter G5 (*Abcg5*)	NM_031884.1	CCAGAACAACACGCTAAAGG	AGCCCAGAGTCCAATAACAC
ABC-transporter G8 (*Abcg8*)	NM_026180.2	TGACATCTTCAGGCTATTTGAC	TCAAGTCCACGTAGAAGTCC
Acyl cholesterol-acyl transferase 1 (*Acat1*)	NM_009230.3	GAGAAATCAAGCAAAGATCCAC	GGAACAGTATCAAGACACCT
Acyl cholesterol-acyl transferase 2 (*Acat2*)	NM_146064.1	CTACAAGCAAGACCCAAGAG	TCAAACTCCAGCATCAACCT
Cytochrome P450-7A (*Cyp7a*)	NM_007824.2	TGGAATAAGGAGAAGGAAAGTAGG	AGGGAGTTTGTGATGAAGTG
HMG CoA reductase (*HMG-CoA red*)	NM_008255.2	TCTTTCCGTGCTGTGTTCTG	TTTTAACCCACGGAGAGGTG
HMG CoA synthase 1 (HMG CoA syn*1)*	NM_145942.3	GGCTGCCACGCTGTACTCCC	ATTTGTGAAGGGGCGCCGGG
Fatty acid metabolism			
Acyl CoA oxidase (*Aox*)	NM_015729.2	GGTGGCTGTTAAGAAGAGTG	AAGATGAGTTCCATGACCCA
Acyl CoA thioesterase 1 (*Acot1*)	NM_012006	CGCAGCCACCCCGAGGTAAA	TCTCAGGATGGTCACAGGGGGT
Acetyl CoA carboxylase-1 (*Acc1*)	NM_133360.2	TACTGAACTACATCTTCTCCC	AAGCAATAAGAACCTGACGA
Carnitine palmitoyl transferase 1 (*Cpt1*)	NM_013495	TTAACAGCAACTACTACGCC	CCAGAAGACGAATAGGTTTGAG
Cytochrome P450-4A10 (*Cyp4a*)	NM_010011	TGTTTGACCCTTCCAGGTTT	CAATCACCTTCAGCTCACTCA
Δ^5-desaturase (*Fads1*)	NM_146094	TGTGTGGGTGACACAGATGA	GTTGAAGGCTGATTGGTGAA
Δ^6-desaturase (*Fads2*)	NM_019699	CCACCGACATTTCCAACAC	GGGCAGGTATTTCAGCTTCTT
Fatty acid elongase-2 (*Elovl2*)	NM_019423	ACGCTGGTCATCCTGTTCTT	GCCACAATTAAGTGGGCTTT
Fatty acid elongase-5 (*Elovl5*)	NM_134255.2	CGGGAGAATCCGATATGAAG	CATGGTAGCGTGGTGGTAGA
Fatty acid elongase-6 (*Elovl6*)	NM_130450.2	ACAATGGACCTGTCAGCAAA	GTACCAGTGCAGGAAGATCAGT
Fatty acid synthase (*Fasn*)	NM_007988	CAAATACAATGGCACCCTGA	TGGCGAAGCCGTAGTTAGTT
HMG CoA synthase-2 (*Hs2*)	NM_008256	CCTTGAACGAGTGGATGAGA	CAGATGCTGTTTGGGTAGCA
Stearoyl CoA desaturase-1 (*Scd1*)	NM_009127	TCAACTTCACCACGTTCTTCA	CTCCCGTCTCCAGTTCTCTT
Oxidant Stress			
Hemoxygenase-1 (*Hmox1*)	NM_010442.1	GCCACCAAGGAGGTACACAT	GCTTGTTGCGCTCTATCTCC
Inflammation			
Cell Determination-68 (*Cd68*)	NM_009853.1	TCAAATCCGAATCCTATACCCA	ATGTAGGTCCTGTTTGAATCCA
C-type lectin domain family 4f (*Clec4f*)	NM_016751.3	CTGCGGCACGTCCAGGTCAT	CCAAGGCTGTGAAGCCACCACA
Monocyte chemoattractant protein-1 (*Mcp1*)	NM_011333.3	TCCCAATGAGTAGGCTGGAG	TCTGGACCCATTCCTTCTTG
Tumor necrosis factor-α (*Tnfα*)	NM_013693.2	AGCCCCCAGTCTGTATCCTT	ACAGTCCAGGTCACTGTCCC
Fibrosis			
Procollagen 1α1 (*Procol1α1*)	NM_007742.2	GACATCCCTGAAGTCAGCTGC	TCCCTTGGGTCCCTCGAC
Transforming growth factor 1β (*Tgfβ1*)	NM_011577.2	TTGCCCTCTACAACCAACACAA	GCTTGCGACCCACGTAGTA

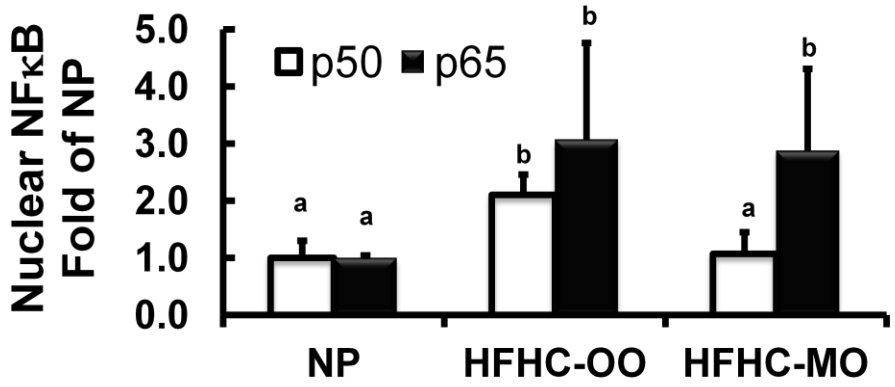

Figure 2.4. Effect of the NP, HFHC-OO and HFHC-MO diets on hepatic nuclear abundance of NFκB-p50 and NFκB-p65 in *Ldlr*$^{-/-}$ mice. *Ldlr*$^{-/-}$ mice were fed the NP, HFHC-OO or HFHC-MO diets for 12 wk; hepatic proteins were extracted from food-deprived mice (Table 1). The nuclear abundance of NFκB p50 and NFκB p65 was measured by immunoblotting, quantified and normalized to TBP. Results are expressed as nuclear NFκB-Fold Change, relative to NP control mice; mean ± SD, n = 4; Labeled means without a common letter differ, $P < 0.05$.

Table 2.4. Body weight, plasma, and liver parameters in feed-deprived WT and *Ldlr*$^{-/-}$ mice after 12 wk feeding NP, HF, HFHC-OO, or HFHC-MO diets[1]

	Wild Type				*Ldlr*$^{-/-}$	
	NP	HF	HFHC-OO	HFHC-MO	HFHC-OO	HFHC-MO
Body Weight[4], *g*	27.3 ± 1.1^{a}	45.0 ± 3.7^{b}	42.7 ± 1.2^{b}	39.0 ± 2.2^{b}	40.3 ± 4.6^{b}	39.9 ± 5.2^{b}
Plasma Parameters[5]						
Glucose, *mmol/L*	6.4 ± 1.0	9.3 ± 2.7	8.3 ± 2.8	5.6 ± 0.7	11.9 ± 4.6	11.0 ± 4.6
Triglycerides[5,6], *mg/dL*	88.3 ± 31^{a}	90.0 ± 16^{a}	66.0 ± 27^{a}	106 ± 25^{a}	352.0 ± 141^{b}	185.0 ± 83^{a}
Cholesterol[4,5], *mg/dL*	51.6 ± 20.9	107.9 ± 45.7	138.0 ± 65.8	81.0 ± 6.4	956.6 ± 251.9	598.7 ± 145.0
ApoB[5,6], *mg/dL*	11.0 ± 1.7^{a}	14.1 ± 2.2^{a}	15.0 ± 4.3^{a}	13.6 ± 2.0^{a}	159.9 ± 70.1^{b}	50.3 ± 21.0^{a}
ApoC3[5,6], *mg/dL*	7.1 ± 4.0^{a}	12.7 ± 7.2^{a}	9.9 ± 3.6^{a}	8.0 ± 1.5^{a}	21.3 ± 6.0^{b}	10.5 ± 6.2^{a}
NEFA[5,6], *mEq/mL*	0.8 ± 0.1^{a}	0.7 ± 0.2^{a}	0.6 ± 0.1^{a}	0.7 ± 0.3^{a}	1.5 ± 0.6^{b}	0.9 ± 0.4^{a}
β-hydroxybutyrate[5], *mmol/L*	1.4 ± 0.3^{a}	1.3 ± 0.4^{a}	1.5 ± 0.3^{a}	1.7 ± 0.2^{a}	2.9 ± 0.8^{b}	2.6 ± 0.5^{b}
ALT[4,5,6], *U/L*	5.1 ± 0.4^{a}	18.7 ± 10.1^{b}	19.6 ± 11.0^{b}	8.2 ± 2.1^{b}	38.3 ± 8.7^{c}	20.5 ± 16.3^{b,c}
AST[4,5,6], *U/L*	10.5 ± 1.4^{a}	17.6 ± 2.1^{a}	29.7 ± 11.8^{b}	12.8 ± 2.2^{a}	55.5 ± 1.2^{c}	27.3 ± 11.6^{b}
Liver Parameters						
Weight, g	1.0 ± 0.1	1.6 ± 0.5	1.9 ± 0.5	1.2 ± 0.1	1.0 ± 0.6	1.6 ± 0.4
% Body weight[4]	3.7 ± 0.1^{a}	3.4 ± 1.0^{a}	4.5 ± 1.2^{b}	3.0 ± 0.2^{a}	4.8 ± 0.9^{b}	3.9 ± 0.7^{a}
Triglycerides[2,4], μg/mg	56.0 ± 39^{a}	157.0 ± 88^{b}	141.0 ± 63^{b}	72.0 ± 15.3^{a}	129.0 ± 13^{b}	64.0 ± 26^{a}
Cholesterol[3,4,5,6], μg/mg	6.9 ± 1.1^{a}	6.4 ± 1.5^{a}	8.0 ± 1.4^{a}	8.3 ± 1.0^{a}	17.1 ± 3.2^{b}	10.4 ± 4.8^{a}

[1]Data are mean ± SD, n = 8 per treatment group unless otherwise noted. Labeled means in a row with superscripts without a common letter differ, $P < 0.05$. Stats by 2-way ANOVA include L*dlr*$^{-/-}$NP, but are not shown as WT and L*dlr*$^{-/-}$ fed NP are not statistically different in any parameter (t-test). [2]Data are expressed as μg triglyceride per mg protein (liver). [3]Data are expressed as μg cholesterol per mg protein (liver). [4]2-way ANOVA diet effect $P<0.05$. [5]2-way ANOVA genotype effect $P<0.05$. [6]2-way ANOVA diet by gene interaction effect $P<0.05$. [7]To convert from mg/dL to mmol/L, multiply by: 0.0113 for triglycerides; 0.02586 for cholesterol. To convert from mg/dL to g/L, multiply by: 0.01 for ApoB and ApoC3. To convert from mEq/mL to mEq/L multiply by 1000 for NEFA.

Table 2.5. Hepatic fatty acid composition in feed-deprived *Ldlr*$^{-/-}$ mice after 12 wk feeding NP, HFHC-OO, or HFHC-MO diets[1].

	Ldlr$^{-/-}$		
	NP	HFHC-OO	HFHC-MO
Fatty acids, *mol%*			
C16:0	26.9 ± 1.0^{a}	18.0 ± 1.9^{b}	14.0 ± 4.7^{b}
C16:1(n-7)	2.2 ± 0.7	3.4 ± 0.9	2.2 ± 1.9
C18:0	11.7 ± 2.7^{a}	2.4 ± 0.5^{b}	4.0 ± 2.3^{b}
C18:1(n-9)	15.2 ± 1.8^{a}	55.0 ± 4.3^{b}	37.6 ± 3.7^{b,c}
C18:1(n-7)	3.1 ± 0.3^{a}	7.3 ± 1.2^{b}	5.1 ± 1.1^{c}
C18:2(n-6)	24.7 ± 2.8^{a}	14.2 ± 1.6^{b}	16.4 ± 3.0^{b}
C18:3(n-3)	0.8 ± 0.2	0.3 ± 0.1	0.3 ± 0.1
C20:0	0.4 ± 0.1	0.3 ± 0.1	0.6 ± 0.1
C20:2(n-6)	0.2 ± 0.1^{a}	1.8 ± 0.1^{b}	0.8 ± 0.2^{a}
C20:3(n-6)	0.7 ± 0.2	0.6 ± 0.1	0.4 ± 0.1
C20:4(n-6)	6.2 ± 0.1^{a}	3.2 ± 0.7^{b}	2.0 ± 1.1^{b}
C20:5(n-3)	0.6 ± 0.1^{a}	0.2 ± 0.1^{a}	7.9 ± 2.3^{b}
C22:5(n-6)	0.2 ± 0.1	0.8 ± 0.2	0.6 ± 0.2
C22:5(n-3)	0.6 ± 0.1^{a}	0.2 ± 0.1^{a}	2.0 ± 0.3^{b}
C22:6(n-3)	5.1 ± 0.9^{a}	1.4 ± 0.3^{b}	9.6 ± 3.8^{c}
Sum C_{20-22} PUFA			
C_{20-22}(n-3)	6.3 ± 0.6^{a}	1.8 ± 0.3^{b}	19.5 ± 3.3^{c}
C_{20-22}(n-6)	7.3 ± 1.3^{a}	6.4 ± 0.8^{a}	3.7 ± 1.1^{b}
Fatty acid ratios			
C20:4(n-6)/C18:2(n-6)	0.26 ± 0.07^{a}	0.27 ± 0.08^{a}	0.14 ± 0.09^{b}
C20:3(n-6)/C18:2(n-6)	0.04 ± 0.02^{a}	0.05 ± 0.01^{a}	0.02 ± 0.01^{b}

[1]Data are mean ± SD, n = 4 per treatment group and are representative of two separate studies. Labeled means in a row with superscripts without a common letter differ, $P < 0.05$.

Table 2.6. Urinary isoprostanes (F2-IsoP, F3-IsoP, F4-NP) in *Ldlr*$^{-/-}$ mice after 12 wk feeding NP, HFHC-OO, or HFHC-MO diets[1].

	Ldlr$^{-/-}$		
	NP	HFHC-OO	HFHC-MO
Isoprostane	*ng/mg creatinine*		
F2-Isoprostane	2.2 ± 0.1[a]	2.6 ± 1.1[a]	4.7 ± 1.2[b]
F3-Isoprostane	2.1 ± 0.1[a]	0.9 ± 0.6[a]	12.5 ± 8.2[b]
F4-Neuroprostane	70.7 ± 5.7[a]	45.5 ± 12.8[a]	110.3 ± 57.6[b]

[1]Data are the mean ± SD of 4 pooled samples/diet group; samples are derived from 2 separate studies. Labeled means in a row with superscripts without a common letter differ, $P < 0.05$.

Table 2.7. Fold change in mRNA abundance in feed-deprived *Ldlr*$^{-/-}$ mice after 12 wk feeding NP, HFHC-OO, or HFHC-MO diets[1].

	Ldlr$^{-/-}$		
	NP	HFHC-OO	HFHC-MO
Oxidative Stress		*Fold of NP*	
Hmox1	1.0 ± 0.3 [a]	4.2 ± 1.0 [b]	3.2 ± 1.1 [b]
PUFA synthesis			
Elovl2	1.0 ± 0.4[a]	2.2 ± 0.9[b]	2.4 ± 1.1[b]
Elovl5	1.0 ± 0.2 [a]	0.8 ± 0.1[a]	0.3 ± 0.1[b]
Fads1	1.0 ± 0.1 [a]	0.8 ± 0.3[a]	0.2 ± 0.1[b]
Fads2	1.0 ± 0.1	1.1 ± 0.2	0.7 ± 0.3
Cholesterol metabolism			
HMG CoA red	1.0 ± 0.3	1.0 ± 0.2	1.2 ± 0.4
HMG CoA syn 1	1.0 ± 0.2	1.0 ± 0.1	0.9 ± 0.2
Cyp7A1	1.0 ± 0.3	1.2 ± 0.2	2.1 ± 1.3
Abca1	1.0 ± 0.2 [a]	4.7 ± 0.3[b]	4.0 ± 0.4[b]
Abcg5	1.0 ± 0.1[a]	3.2 ± 0.3[b]	4.0 ± 0.5[b]
Acat1	1.0 ± 0.4[a]	4.8 ± 1.5[b]	3.0 ± 1.1[c]
Acat2	1.0 ± 0.1[a]	2.0 ± 0.5[b]	2.3 ± 0.7[b]
Inflammation			
Mcp1	1.0 ± 0.5[a]	22.8 ± 10.4[b]	4.6 ± 2.8[a]
Cd68	1.0 ± 0.5[a]	7.7 ± 2.5[b]	4.1 ± 2.5[c]
Clec4f	1.0 ± 0.5[a]	6.3 ± 1.2[b]	4.7 ± 1.3[b]
Fibrosis			
Tgfβ1	1.0 ± 0.2[a]	3.0 ± 0.9[b]	2.1 ± 0.4[b]
Procol1α1	1.0 ± 0.7[a]	43.0 ± 24.0[b]	20.1 ± 14.0[c]

[1]Data are mean ± SD, n = 8 per treatment group and are represented as fold change from NP-fed mice. Labeled means in a row with superscripts without a common letter differ, $P < 0.05$.

Chapter 3

Docosahexaenoic Acid Attenuates Hepatic Inflammation, Oxidative Stress, and Fibrosis without Decreasing Hepatosteatosis in a *Ldlr*$^{-/-}$ Mouse Model of Western Diet-Induced Non-Alcoholic Steatohepatitis

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3.1. Abstract

The incidence of non-alcoholic fatty liver disease (NAFLD) and non-alcoholic steatohepatitis (NASH) has increased in parallel with the incidence of obesity. While both NAFLD and NASH are characterized by hepatosteatosis, NASH is characterized by hepatic damage, inflammation, oxidative stress and fibrosis. We previously reported that feeding *Ldlr*$^{-/-}$ mice a high fat-high cholesterol diet containing menhaden oil attenuated several markers of NASH, including, hepatosteatosis, inflammation and fibrosis. Herein, we test the hypothesis that docosahexaenoic acid [22:6(n-3); DHA], is more effective than eicosapentaenoic acid [20:5(n-3); EPA] at preventing western diet (WD)-induced NASH in *Ldlr*$^{-/-}$ mice. Mice were fed the WD supplemented with either olive oil (OO), EPA, DHA, or EPA + DHA for 16 wk. WD + OO feeding induced a severe NASH phenotype, characterized by robust hepatosteatosis, inflammation, oxidative stress, and fibrosis. While none of the C20-22 (n-3) fatty acid treatments prevented WD-induced hepatosteatosis, all three (n-3) PUFA containing diets significantly attenuated WD-induced inflammation, fibrosis and hepatic damage. The capacity of dietary DHA to suppress hepatic markers of inflammation (*Clec4F, F4/80, Tlr4, Tlr9, CD14, Myd88*), fibrosis (*Procol1*α*1, Tgf*β*1) and* oxidative stress (NADPH oxidase subunits *Nox2, p22phox, p40phox, p47phox, p67phox*) was significantly greater than dietary EPA. The effects of DHA on these markers paralleled DHA mediated suppression of hepatic *Fads1* mRNA abundance and hepatic arachidonic acid content. Since DHA suppression of NASH markers does not require a reduction in hepatosteatosis, dietary DHA may be useful in combating NASH in obese humans.

3.2. Introduction

The incidence of non-alcoholic fatty liver disease (NAFLD) parallels the incidence of obesity in western societies [19,112]. NAFLD spans a spectrum of hepatic disorders ranging from simple fatty liver (hepatosteatosis) to non-alcoholic steatohepatitis (NASH) [1]. While simple hepatosteatosis is generally considered a benign condition, NASH is the progressive form of the disease that can lead to fibrosis, cirrhosis, and hepatocellular carcinoma [1,3]. Progression of NAFLD to NASH has been modeled by a "two-hit" hypothesis [21]. The "first hit" involves excess hepatic triglyceride (TG) and cholesterol accumulation that is attributed to: 1) elevated *de novo* lipogenesis, 2) accumulation and re-esterification of non-esterified fatty acids (NEFA), 3) decreased fatty acid oxidation and 4) reduced VLDL secretion [13]. The "first hit" sensitizes the liver to the "second hit", which consists of elevated hepatic oxidative stress, insulin resistance, and inflammation. Inflammation promotes hepatocellular damage and death. Furthermore, hepatic fibrosis is activated in stellate cells, which leads to excessive collagen deposition [167]. While management of lifestyle (diet and exercise) is one approach to control the onset and progression of NAFLD, the best strategy for managing NAFLD has yet to be defined [15].

Over the last 30 years the overall consumption of fat in the United States has remained steady and even declined with respect to trans and saturated fatty acids (SFA) [45,46]. Total energy consumed, however, has increased due to elevated carbohydrate consumption, mainly in the form of simple sugars [47,48]. Thus elevated carbohydrate and specifically fructose consumption has been linked to NAFLD and its progression to NASH [49,50].

We recently reported that menhaden oil has the capacity to attenuate several markers linked to NAFLD, including hepatosteatosis, inflammation, hepatic damage and fibrosis [168]. Menhaden oil is enriched in C20-22 (n-3) polyunsaturated fatty acids (PUFA). In that study we used a high fat-high cholesterol diet supplemented with olive oil (HFHC + OO) to induce NAFLD/NASH [168]. The HFHC + OO diet contained starch as the main source of carbohydrate. In an effort to further define the strengths and limitations of C20-22 (n-3) PUFA in controlling NAFLD and NASH, we have used a western diet (WD) to induce NASH in *Ldlr*$^{-/-}$ mice in this study. The WD consists of 41% total energy from fat and 43% total energy from carbohydrate; sucrose comprises 26.6% of total energy. The WD has been used extensively in atherosclerosis studies to model human disease [169]. In this report, we test the hypothesis that docosahexaenoic acid [22:6(n-3); DHA] is more effective than eicosapentaenoic acid [20:5(n-3); EPA] at preventing the onset and progression of NASH.

Twelve clinical trials [170] are investigating the use of (n-3) PUFA in NAFLD and NASH therapy. While some trials use either EPA or DHA as their source of dietary (n-3) PUFA, none are comparing the efficacy of EPA versus DHA to manage fatty liver disease. Some animal studies have examined specific (n-3) PUFA for the management of NAFLD and liver metabolism [171,172,173,174,175]. One study focusing on insulin resistance directly compared EPA versus DHA; both fatty acids lowered hepatic lipid content equally [175]. The JELIS trial, a randomized clinical trial assessing the impact of dietary EPA on cardiovascular events, reported improvement in cardiovascular outcomes when patients received dietary EPA versus placebo. Since these improvements correlated with increased blood levels of EPA, but not DHA[176], EPA alone may be sufficient to prevent diet induced NASH. Our goal is to examine the

capacity of EPA versus DHA to prevent WD-induced NASH. The outcome of our study reveals a clear benefit in the use of DHA in attenuating the progression of NAFLD to NASH.

3.3. Materials and methods

3.3.1) Animals and diets

All procedures for the use and care of animals for laboratory research were approved by the Institutional Animal Care and Use Committee at Oregon State University. Male *Ldlr*$^{-/-}$ mice [on the C57BL/6J background](Jackson Laboratories) at 2 months of age were fed one of the following five diets *ad libitum* for 16 wk; each group consisted of 8 male mice. The control diet was Purina chow 5001 (NP, non-purified) consisting of 13.5% energy as fat and 58.0% energy as carbohydrates **(Table 3.1)**. The western diet (WD) (D12709B, Research Diets] was used to induce NAFLD/NASH; it consists of 17% energy as protein, 43% energy as carbohydrate and 41% energy as fat; cholesterol was at 0.2% w/w. The WD was supplemented with olive oil (WD + OO), EPA (WD + EPA), DHA (WD + DHA) or EPA plus DHA (WD + EPA + DHA). Supplementation of the WD with OO, EPA, DHA or EPA + DHA increased total fat energy to 44.7% and reduced protein and carbohydrate energy to 15.8 and 39.5%, respectively. Olive oil was added to the WD to keep all WD diets isocaloric with a uniform level of fat energy. Preliminary studies established that the addition of olive oil had no effect on diet-induced fatty liver disease in *Ldlr*$^{-/-}$ mice.

Dietary EPA was purchased from Futurebiotics (Hauppauge, NY) as Newharvest EPA. This product, developed by DuPont, is a mix of triglycerides; 61 mol% of the fatty acyl chains consists of EPA. Other fatty acids in this product include 16:0 (2.3%), 18:0 (2.3%), 18:1(n-9) (4.7%), 18:2(n-6) (19.5%), 20:2(n-6) (2.1%), 20:3(n-3) (2.4%), 22:5(n-

3) (2.8%). This product contains no arachidonic acid [20:4(n-6); ARA], adrenic acid [22:4(n-6)] or DHA. Dietary DHA was obtained as DHASCO and was a generous gift from Martek Bioscience (Columbia, MD). This product is a mix of triglycerides; 39% of the fatty acyl chains are DHA. Other fatty acids found in this product include: 12:0 (5.7%), 14:0 (15%), 16:0 (8.9%), 16:1(n-7) (3.5%), 18:1(n-9) (27%) and 18:2(n-6) (1.2%). DHA is the only C20-22 (n-3) or (n-6) PUFA in this product. The fatty acid composition of both products was verified in our laboratory by gas chromatographic analysis of fatty acid methyl esters (FAME). Once the diets were prepared, the fatty acid composition was again examined by gas chromatography of FAME **(Table 3.1)**. Total fat content in all the WD diets was 44.7% total energy as fat. C20-22 (n-3) PUFA content in the WD + EPA, WD + DHA and WD + EPA + DHA diets was 2% of total energy. Diets were stored frozen (-20^{o}C) until used and replenished every other day to reduce the formation of oxidation products. Food consumption was monitored every other day and body weight was monitored weekly. At the end of the 16 wk feeding period, all mice were feed-deprived overnight (18:00 to 08:00 the next day) then euthanized (isoflurane anesthesia and exsanguination) at 08:00 for blood and liver harvesting as described [168].

3.3.2) Liver histology

Liver (~100 mg) was fixed in formalin overnight then transferred to methanol. Livers were embedded, sliced and stained with either hematoxylin-eosin or trichrome at the Veterinary Diagnostic Lab at Oregon State University.

3.3.3) Analysis of body composition

One week prior to the termination the study, body mass, lean and fat mass were quantified using a Piximus-dual energy x-ray absorptiometer (PIXImus2, Lunar).

Animals were anesthetized by isoflurane and scanned for body composition. Isoflurane was administered using a precision vaporizer (Summit Anesthesia Solutions). A mixture of 2% isoflurane, 98% O_2 was delivered. The head was excluded from analysis.

3.3.4) Measurement of plasma and hepatic parameters

Plasma markers, hepatic lipids, RNA and proteins were quantified as described previously [168]. The quantitation of specific mRNA and proteins used quantitative real time-polymerase chain reaction (qRT-PCR) and immunoblotting, respectively, as described previously. Primers used for qRT-PCR are listed in **Table 3.2**. Extracted hepatic lipids were also analyzed by thin layer chromatography (TLC) [silica gel 60A, Whatman]; the chromatogram was developed in hexane:diethyl ether:acetic acid (60:40:0.8), dried, stained with iodine and photographed. Lipid standards, e.g., cholesterol esters, triglycerides, non-esterified fatty acids (NEFA); diacylglycerols (DAG) and polar lipids, were used to verify type.

3.3.5) Statistical analysis

One-way ANOVA was used to detect dietary effects when more than 2 groups were included in the analysis [168]. Data were analyzed for homogeneous variances by the Levene test. If unequal variances were detected, the data were log-transformed. ANOVA was performed on both transformed and untransformed data. Untransformed data are presented for interpretation purposes. When only 2 groups were compared, Student's *t* test was used for analysis. $P < 0.05$ was considered significantly different. All values are reported as mean ± SD.

3.4. Results

3.4.1) Body weight, body composition, plasma, and liver parameters

The WD + OO was used to induce obesity and fatty liver. **Table 3.3** compares body weight, plasma, and liver parameters in *Ldlr*$^{-/-}$ mice fed the HFHC + OO diet reported previously [168] and the WD + OO used here. Of all the parameters measured, hepatic TG (154%, $P < 0.05$) and cholesterol (100%, $P < 0.05$) were significantly elevated in *Ldlr*$^{-/-}$ mice fed the WD + OO versus mice fed the HFHC + OO. Of the gene expression markers examined; C-type lectin domain family 4f (*Clec4f*) a Kupffer cell marker [27] and procollagen-1α1 mRNA (*Procol1α1*), derived from stellate cells [150] and *Tgfβ1* were elevated 56% ($P < 0.05$), 106%, ($P < 0.05$) and 55% ($P < 0.05$), respectively, after feeding *Ldlr*$^{-/-}$ mice the WD + OO versus the HFHC + OO diet **(Table 3.4)**. These results indicate that 16 wk of feeding *Ldlr*$^{-/-}$ mice the WD induces a more severe form of NASH than the 12 wk of feeding the HFHC + OO diet [168].

Feeding *Ldlr*$^{-/-}$ mice the WD + OO for 16 wk induced significant ($P < 0.05$) weight gain (≥30%) when compared to age-matched NP-fed *Ldlr*$^{-/-}$ mice **(Table 3.5)**. Body composition analysis revealed a significant increase (≥100%, $P < 0.05$) in fat mass in the WD + OO group. None of the C20-22 (n-3) PUFA-containing diets reduced body weight or fat mass below levels seen in WD + OO fed mice. None of the diets significantly affected blood glucose in feed-deprived mice. Plasma TG levels in feed-deprived mice were elevated 167% ($P < 0.05$) in the WD + OO group compared to the NP group. While plasma TG in feed-deprived mice fed the WD + DHA were not different from NP, feed-deprived mice in the WD + EPA and WD + EPA + DHA groups had plasma TG above (≥95% $P < 0.05$) the NP group **(Table 3.5)**. Plasma total cholesterol in feed-deprived mice was elevated ~340% ($P < 0.05$) in the WD + OO group relative to NP. While WD + DHA and WD + EPA + DHA lowered total cholesterol ($P < 0.05$), neither fully prevented WD-induced increases in blood cholesterol **(Table 3.5)**. Plasma ApoB and ApoC3 were

also induced ($P < 0.05$), 400% and 96%, respectively, in the WD + OO group when compared to the NP group. ApoB was attenuated (≥48%, $P < 0.05$) by addition of C20-22 (n-3) PUFA to the WD. In contrast, WD + DHA was the only (n-3) PUFA that attenuated (~40%, $P < 0.05$) WD + OO induction of ApoC3 **(Table 3.5)**, a result consistent with a previous human study [177]. Plasma NEFA were elevated (~57%, $P < 0.05$) by WD + OO feeding relative to NP. This response was attenuated ($P < 0.05$) by WD + EPA and WD + EPA + DHA, but not by WD + DHA **(Table 3.5)**. Plasma β-hydroxybutyrate was induced (≥80%, $P < 0.05$) in all mice fed the WD compared to NP. Liver weight as percent body weight was increased in WD fed mice; this was prevented ($P < 0.05$) only in the WD + DHA group **(Table 3.5)**.

3.4.2) Hepatic histology and lipid content

Histology of livers from the 5 groups is shown in **Figure 3.1**. Evidence of hepatosteatosis is shown by the appearance of highly vacuolated liver; both micro- and macro-hepatosteatosis was observed. Chemical analysis of each group showed that mice fed the WD + OO had a ≥320% ($P < 0.05$) increase in hepatic TG content and ≥160% ($P < 0.05$) increase in hepatic total cholesterol relative to NP fed mice **(Table 3.5)**. Further analysis of liver lipids is shown in **Figure 3.2** where hepatic lipid extracts were fractionated by thin layer chromatography. Hepatic TG and cholesterol esters were higher in mice fed the WD, supporting the chemical analysis in **Table 3.5**. In contrast to our previous studies [168], no EPA or DHA containing diet significantly lowered WD-induced accumulation of hepatic TG or total cholesterol **(Table 3.5 and Figs. 3.1 & 3.2)**.

3.4.3) Hepatic fatty acid composition

Oleic acid [18:1(n-9)] is the most abundant fatty acid in the WD **(Table 3.1)**; hepatic 18:1(n-9) was elevated >2000% ($P < 0.05$) in the WD + OO group when

compared to the NP group. Addition of EPA or DHA to the WD diet was equally effective in reducing hepatic 18:1(n-9) by >50% ($P < 0.05$) when compared to the WD + OO group. Hepatic ARA is formed by desaturation and elongation of the essential fatty acid linoleic acid [18:2(n-6)]. Feeding mice the WD + OO increased 20:4(n-6) by 87% ($P < 0.05$) relative to NP. Of the EPA or DHA containing diets tested, DHA containing diets were most effective at reducing (>75%, $P < 0.05$) WD-induced hepatic ARA content **(Table 3.6)**.

Addition of EPA or DHA to the WD elevated hepatic EPA and DHA, when compared to either the NP or WD + OO groups. Feeding mice the WD + EPA increased hepatic EPA >1300% and >5000% ($P < 0.05$) when compared to the NP and WD + OO groups, respectively. However, feeding mice the WD + EPA only increased hepatic DHA content ~100% when compared to the NP or WD + OO groups. Feeding mice the WD + DHA, however, increased hepatic DHA >570% and >670% ($P < 0.05$) compared to the NP and WD + OO groups, respectively. Dietary DHA was clearly more effective than dietary EPA at increasing hepatic DHA content. Dietary DHA, however, significantly increased hepatic EPA (≥720%, $P < 0.05$) and 22:5 (n-3) (≥580%, $P < 0.05$) when compared to the NP and WD + OO groups **(Table 3.6)**. These diets have similar effects on plasma fatty acid profiles (not shown).

The ratio of hepatic 20:4(n-6)/18:2(n-6) and 20:3(n-6)/18:2(n-6) is an indicator of hepatic PUFA synthesis **(Table 3.6)**. The 20:4(n-6)/18:2(n-6) and 20:3(n-6)/18:2(n-6) ratios were increased 54% ($P < 0.05$) and 100% ($P < 0.05$), respectively in the WD + OO group compared to the NP group. The WD + DHA diet was the most effective (n-3) PUFA diet at lowering these ratios.

3.4.4) Hepatic fatty acid synthesis

Fatty acid synthase (FASN), stearoyl CoA desaturase-1 (SCD1) and fatty acid elongases (ELOVL)-1, 3, 6 and 7 play a major role in de novo fatty acid synthesis, MUFA and sphingolipid synthesis [119,178]. The WD + OO group suppressed *Elovl3* expression by ~80% ($P < 0.05$) compared to the NP group. In contrast, this diet induced *Elovl1* (100%, $P < 0.05$), *Elovl6* (50%, $P < 0.05$), *Elovl7* (450%, $P < 0.05$) and *Scd1* (500%, $P < 0.05$), respectively **(Table 3.7)**. *Elovl3* mRNA abundance in mice fed the EPA or DHA containing diets was not different from mice in the WD + OO group. Hepatic abundance of *Fasn* (66%, $P < 0.05$), *Scd1* (60%, $P < 0.05$), *Elovl1* (45%, $P < 0.05$), *Elovl6* (34%, $P < 0.05$) and *Elovl7* (62%, $P < 0.05$) were significantly lower in the WD + DHA group relative to the WD + OO group. These results indicate that the WD + DHA is more effective than WD + EPA at reversing the effects of the WD + OO on pathways controlling hepatic SFA, MUFA and sphingolipid production.

Enzymes involved in PUFA synthesis include Elovl2 and Elovl5 and fatty acid desaturases 1 (Fads1) and 2 (Fads2) **(Table 3.7)**. These enzymes are responsible for the conversion of 18:2(n-6) and 18:3(n-3) to C20-22 (n-6) and (n-3) PUFAs, respectively. Hepatic abundance of *Elovl2*, *Elovl5*, and *Fads2* mRNA was significantly (≥50%, $P < 0.05$) elevated by feeding mice the WD + OO, when compared to the NP group. These changes in expression paralleled the induction of both hepatic 20:4(n-6) and the ratio of 20:4(n-6)/18:2(n-6). Unlike the WD + EPA or the WD + EPA + DHA diets, the WD + DHA completely blocked ($P < 0.05$) the WD + OO-mediated induction of all three enzymes. The mRNA encoding *Fads1*, in contrast, was not induced in livers of mice fed the WD + OO. This mRNA, however, was suppressed (>60%, $P < 0.05$) in livers of mice in the WD + DHA and WD + EPA + DHA groups versus mice in the NP or WD + OO

groups. The changes in mRNA abundance for the fatty acid elongases and desaturases paralleled WD + DHA and WD + EPA + DHA induced changes in hepatic 20:4(n-6) **(Table 3.6)**.

3.4.5) Hepatic triglyceride hydrolysis and fatty acid oxidation

Impairment of TG hydrolysis and fatty acid oxidation contributes to hepatosteatosis [179]. We analyzed mRNA abundance of several hepatic TG lipases, key enzymes involved in VLDL assembly and fatty acid oxidation **(Table 3.7)**. Of these, only adiponutrin (*Adpn*) was induced (110%, $P < 0.05$) by the WD + OO when compared to mice fed the NP diet. This induction was blocked in the C20-22 (n-3) supplemented groups. We also quantified mRNAs for several PPARα target genes, including ones involved in fatty acid oxidation, i.e., carnitine palmitoyl transferase 1 and 2 and acyl CoA oxidase. Hepatic abundance of these transcripts was not affected by diet (not shown).

3.4.6) Hepatic damage

Increased levels of plasma alanine aminotransferase (ALT) and aspartate aminotransferase (AST) reflect hepatic damage. Plasma levels of both enzymes were induced ≥270% ($P < 0.05$) by WD + OO feeding when compared to the NP diet **(Table 3.5)**. All EPA and DHA containing WD diets significantly attenuated ($P < 0.05$) the WD + OO induction for ALT and AST.

3.4.7) Hepatic inflammation

Hepatic damage is a result of increased hepatic inflammation and cell death. Histological evidence of inflammation is seen by leukocyte (monocytes and macrophage) infiltration in liver; this phenomenon was only detected in livers of mice fed the WD + OO **(Fig. 3.1)**. In agreement with this observation, monocyte chemo-attractant protein-1 (*Mcp1*) mRNA, an early marker of inflammation [149], was induced 3100% ($P <$

0.01) in the WD + OO group compared to the NP group. Moreover, the cell surface markers for Kupffer cells and macrophages (*Clec4f*, *Clec10a,* cell determination-68 [*Cd68]* and *F4/80)* were also induced ≥180% ($P < 0.05$). In every case, expression levels of these transcripts was lower in livers of mice in the WD + DHA group than mice in the WD + EPA or the WD + EPA + DHA groups **(Table 3.8)**.

Cytokines produced by Kupffer cells and macrophages play a major role in promoting cell damage. Hepatic abundance of mRNA encoding *Il-1*β and *Tnf-*α was induced 290% ($P < 0.01$) and 760% ($P < 0.01$), respectively, in livers of mice fed the WD + OO when compared to NP group. The WD + EPA and WD + DHA diets attenuated ($P < 0.01$) the induction of *Il-1*β and *Tnf-*α relative to the WD + OO group. The WD-mediated induction of *Clec4* and *F4/*80 was significantly lower in mice fed the WD + DHA when compared to mice fed the WD + EPA or WD + EPA + DHA diets **(Table 3.8)**.

Toll-like receptors (TLR) -2, -4 and -9 play a role in the progression of NASH [180]. Stimulation of these pattern recognition receptors augments NF-κB nuclear content and the induction of cytokines. *Tlr9* mRNA was the most responsive to WD + OO; *Tlr9* was induced nearly 700% ($P < 0.01$) relative to the NP group. The induction of *Tlr4* and *Tlr9* was attenuated ($P < 0.01$) in the WD + DHA group, but not in the WD + EPA group. All C20-22 (n-3) containing diets attenuated the WD + OO induction of *Tlr2*. Cluster of differentiation-14 (CD14), myeloid differentiation factor-2 (MD-2) and myeloid differentiation primary response gene-88 (MyD88) are also involved in TLR signaling. Of these, *Cd14* and *Myd88* mRNA were induced in the WD + OO group; only the WD + DHA group blocked induction of both these genes ($P < 0.05$) **(Table 3.8)**.

NF-κB, a downstream target of TLR-signaling, is a transcription factor that controls a wide range of genes involved in inflammation, including cytokines and chemo-attractant proteins [107]. We quantified the hepatic nuclear abundance of NF-κB-p50 and NF-κB-p65 subunits **(Fig. 3.3)**. Feeding mice the WD + OO or WD + EPA increased hepatic nuclear content of NF-κB-p50 (~100%, $P < 0.01$) relative to NP. Diets containing DHA blocked the WD-induced accumulation of NF-κB-p50 in hepatic nuclei. We also analyzed *Nf-κb-p105* mRNA, the precursor to NF-κB-p50. *Nf-κb-p105* mRNA abundance changed in parallel with NF-κB-p50 nuclear protein abundance. The hepatic nuclear content of NF-κB-p65, a NF-κB-p50 heterodimer partner, was significantly elevated in all WD groups.

3.4.8) Hepatic oxidative stress

Oxidative stress is implicated in the progression of fatty liver to NASH [181]. Nuclear factor-E2-related factor-2 (NRF2) is a transcription factor that responds to oxidative stress; it accumulates in nuclei under conditions of oxidative stress and induces expression of multiple genes involved in the anti-oxidative response, including glutathione-S transferase (GST1α) and hemeoxygenase-1 (HMOX1) [182,183]. Hepatic mRNA expression of *Nrf2* was induced in the WD + OO group; this induction was attenuated ($P < 0.05$) only in the WD + DHA group **(Table 3.8)**. The nuclear abundance of hepatic NRF2 protein was not significantly ($P = 0.09$) elevated in all WD groups regardless of C20-22 (n-3) PUFA (not shown). HMOX1 is induced under conditions of oxidative stress [184,185]. *Hmox1* mRNA was induced (400%, $P < 0.05$) in the WD + OO group; this induction was moderately attenuated ($P < 0.05$) by the WD + EPA and WD + DHA diets **(Table 3.8)**. Hepatic HMOX1 protein abundance, however, was elevated in all WD groups **(Fig. 3.4)**.

NADPH oxidase is an important pathway for the generation of reactive oxygen species, and its role in NASH progression, especially fibrosis, has been well-established [186,187,188]. We measured hepatic mRNA abundance of several NADPH oxidase subunits (NOX), including *Nox1*, *Nox2* (*gp91*), *Nox4*, *Noxa1*, *Noxo1*, RAS-related C3 botulinum substrate 1 (*Rac1*), cytochrome b-245 light chain (*P22phox*), neutrophil cytosolic factor 4 (*P40phox*), neutrophil cytosolic factor 1 (*P47phox*), and neutrophil cytosolic factor 2 (*P67phox*) **(Table 3.8)**. Of these *Nox2*, *P22phox*, *P40phox*, *P47phox*, and *P67phox* were induced 400-600% ($P < 0.05$) in mice fed the WD + OO compared to NP. Of the C20-22 (n-3) PUFA diets examined, the WD + DHA was most effective at attenuating the WD + OO induction of these transcripts.

3.4.9) Hepatic fibrosis

Histological evidence of hepatic fibrosis was detected in mice fed the WD + OO and WD + EPA diets only. Fibrosis is represented by the blue staining of collagen **(Fig. 3.1)**. The mRNA encoding *Procol1α1* was elevated 1700% ($P<0.01$) by WD + OO and 1500% in mice fed the WD + EPA when compared to mice fed the NP diet. In mice fed the DHA- and EPA+DHA containing diets, *Procol1α1* was induced 500 and 870% respectively. This result indicates that DHA was more effective than EPA at reducing *Procol1α1* expression in WD-fed mice **(Table 3.8)**. TGFβ-1 is a cytokine involved in the activation of hepatic stellate cells and the production of *Procol1α1 [189,190]*. Hepatic *Tgfβ-1* mRNA was induced 150% ($P < 0.01$) by WD + OO feeding compared to NP and this induction was attenuated ($P < 0.01$) by the WD + DHA and WD + EPA + DHA groups. As such, inclusion of DHA in the WD attenuated *Tgfβ-1* similar to *Procol1α1* **(Table 3.8)**. BMP and activin membrane-bound inhibitor homolog (BAMBI) is a TGFβ1 pseudo-receptor and has been linked to development of fibrosis [180]. However there

were no changes in *Bambi* expression in any of the WD groups. We quantified the mRNA encoding tissue inhibitor of metalloproteinase-1 (TIMP-1) and plasminogen activator inhibitor-1 (PAI-1). Both are involved in inhibiting extracellular matrix degradation and their over expression is associated with progression of fibrosis [191,192]. *Timp-1* and *Pai-1* hepatic mRNA was elevated 3780% ($P < 0.01$) and 640% ($P < 0.01$), respectively, by WD + OO feeding relative to NP. This WD induced response was attenuated ($P < 0.01$) by all C20-22 (n-3) PUFA containing groups **(Table 3.8)**.

3.5. Discussion

The goal of this study was to examine the capacity of the major C20-22 (n-3) PUFA in menhaden oil, i.e., EPA and DHA, to attenuate the WD-mediated induction of hepatic markers associated with NASH in the *Ldlr*$^{-/-}$ mouse model. Our previous report established the requirement of using the HFHC + OO diet and the *Ldlr*$^{-/-}$ mouse to induce hepatosteatosis, inflammation, fibrosis, oxidative stress and hepatic damage [168]. In this report, we used the WD + OO diet and a longer feeding time in an effort to increase the severity of fatty liver disease in *Ldlr*$^{-/-}$ mice. Clearly, feeding *Ldlr*$^{-/-}$ mice the WD + OO for 16 weeks significantly increased hepatosteatosis (triglyceride and cholesterol), inflammation (*Clec4f*), fibrosis (*proCol1*α*1* and *Tgf*β*1*) and hepatic damage (ALT) when compared to *Ldlr*$^{-/-}$ mice fed the HFHC + OO diet for 12 wk **(Tables 3.3 and 3.4)**. Others using a WD of similar composition and *Ldlr*$^{-/-}$ mice have reported similar effects on the development of NASH markers [193]. In addition to the longer feeding time, the WD + OO diet has higher sucrose content (26.6% total energy) than the HFHC + OO diet (6.8% total energy). High fructose consumption promotes NASH and significantly alters liver metabolism [49,194].

Using the WD + OO diet and the *Ldlr*$^{-/-}$ mouse model, we tested the hypothesis that DHA was more effective than EPA at preventing the induction of markers associated with NASH. We were surprised to find that no EPA or DHA containing diet attenuated WD + OO-induced hepatosteatosis **(Table 3.5 and Figs. 3.1 and 3.2)**. While details on the mechanism for this effect remain to be established, we speculate that the high fructose content of the WD overrides the effects of EPA and DHA on lipogenesis and triglyceride synthesis and storage. Despite this outcome, both EPA and DHA attenuated multiple WD + OO-induced markers of inflammation, oxidative stress and fibrosis. This outcome makes clear that a reduction of hepatosteatosis is not required to lower hepatic inflammation, oxidative stress, fibrosis, and hepatic damage.

3.5.1) Inflammation

Hepatic histology **(Fig. 3.1)** and gene expression analysis indicated that EPA and DHA attenuated WD + OO-induced expression of *Mcp1*, cell surface markers for macrophages (*Cd68*, *Clec4f*, *Clec10a*, *F4/80*), cytokines (*Il1β, Tnfα*) and TLR signaling components (*Tlr2*, *Tlr4*, *Tlr9*, *Cd14*, *Myd88* but not *Md-2*) **(Table 3.8)**. No monocyte infiltration was observed in mice fed any of the WD + EPA or WD + DHA diets **(Fig. 3.1)**. In no case was the WD + EPA diet more effective than the WD + DHA diet at suppressing expression of markers of inflammation **(Table 3.8)**. Notable amongst the transcripts examined are the TLR signaling components. *Tlr4, Tlr9* and *Cd14* are major targets for DHA. TLR2 and TLR4 are plasma membrane receptors, while TLR9 is associated with endosomes in stellate cells [180]. CD14 plays a role in antigen presentation to plasma membrane-associated TLR. Antigens for TLR activation are derived from hepatic necrosis, fatty acids, or bacteria, i.e., endotoxin. We are currently assessing these components as regulators of TLR signaling. A key downstream target of

TLR is NFκB, a major transcription factor controlling expression of multiple genes involved in inflammation. As shown previously [168], and in **Fig. 3.3**, the WD + OO induced nuclear abundance of both NFκB-p50 and NFκB-p65. TLR regulates NFκB nuclear abundance, at least in part, by controlling cytosolic sequestration of NFκB subunits by regulating phosphorylation of NFκB inhibitor β. While regulation at this level was not observed, results in **Fig. 3.3** suggest that DHA, but not EPA, controls NFκB-p50, by regulating its mRNA abundance.

3.5.2) Oxidative Stress

Our previous report established that NASH was associated with the induction of hepatic expression of *Hmox1*, a marker of oxidative stress [168]. This report expands the analysis of oxidative stress markers. The WD + OO group had increased hepatic expression of multiple markers of oxidative stress, including transcripts encoding *Nrf2*, *Gsta1*, *Hmox1* and several NADPH oxidase components (*Nox2*, *Noxa1, Noxo1, P22phox*, *P40phox*, *P47phox*, *P67phox*). Since all WD fed groups had similar elevations in NRF2 and HMOX1 protein abundance it is unlikely these proteins are involved in the protective effects observed with dietary EPA or DHA. In contrast to the other markers, expression of the WD + OO-mediated induction of NADPH oxidase subunits was attenuated most effectively by the WD + DHA group **(Table 3.8)**. NADPH oxidase plays a major role in NASH, particularly in fibrosis [186]. NADPH oxidase generates superoxide from molecular oxygen by using NADPH as an electron donor. The superoxide is converted to hydrogen peroxide by superoxide dismutase [195]. Stellate cells from mice with ablated NOX1 or NOX2 have decreased generation of reactive oxidant species and do not show increased collagen expression [187]. As seen

previously, and in **Table 3.8 and Fig. 3.1**, collagen is a major target for induction by the HFHC + OO [168] or WD + OO diets; this response is suppressed by DHA. In our previous report [168], we suggested that C20-22 (n-3) PUFA in menhaden oil did not decrease expression of genes linked to oxidative stress, i.e., *Hmox1*. Those studies provided a limited view of n-3 PUFA effects on hepatic oxidative stress. The results presented in this report clearly show dietary C20-22 (n-3) PUFA selectively suppressed expression of several NADPH oxidase subunits. Effects of EPA and DHA on NADPH oxidase subunits may be one mechanism for the attenuation of *Procol1α1* expression.

3.5.3) Fibrosis

Hepatic histological and gene expression evidence establish that the WD + OO feeding induced fibrosis. Trichrome staining of collagen in liver slices paralleled *Procol1α1* expression. TGFβ1 is a major regulator of fibrosis. Similar to *Procol1α1*, *Tgfβ1* was regulated most potently by inclusion of DHA in the WD. BAMBI is a TGFβ1 pseudo-receptor and a target of NFκB signaling [180]. Despite the effect of the WD and dietary DHA on NFκB nuclear abundance, significant changes in *Bambi* expression are not linked to WD or (n-3) PUFA regulation of fibrosis. *Timp1* induction is essential for the generation of fibrosis [196]; TIMP1 essentially blocks degradation of collagen. While the WD + OO significantly induced *Timp1* nearly 40-fold, EPA and DHA are equally effective at repressing this response **(Table 3.8)**. One explanation is that *Timp1* is not regulated by NFκB, but by the RUNX1 and RUNX2 transcription factors [196]. Effects of EPA and DHA on RUNX1 and 2 are unknown. Like TIMP1, PAI1 also inhibits collagen breakdown [192]. Both *Pai1* and *Timp1* are induced by the WD + OO diet and repressed when the WD contains EPA or DHA.

When taken together, the outcome of our analysis indicates that EPA and DHA are not equivalent in controlling multiple markers associated with NASH. In no case was DHA less effective than EPA in regulating markers of inflammation, fibrosis or oxidative stress. In contrast, we identified several cases where EPA was less effective than DHA in regulating NASH markers. These effects can be attributed to different mechanisms of EPA and DHA regulation of specific genes. From a prevention perspective, however, DHA is quantitatively more effective at attenuating WD + OO-induced markers of NASH. While dietary EPA significantly elevated hepatic EPA, it had only modest effects on hepatic DHA accumulation. In contrast, dietary DHA significantly induced both hepatic EPA and DHA **(Table 3.6)**. Moreover, dietary DHA more than EPA, lowered hepatic ARA content, at least in part by down regulating expression of enzymes involved in its synthesis, i.e., *Fads1*, *Fads2* and *Elovl5* **(Table 3.7)**. Our finding of differential impacts on hepatic fatty acid profiles between dietary EPA and DHA are consistent with human studies, i.e., the JELIS trial [176].

In conclusion, the WD + OO diet was used to induce markers associated with NASH in *Ldlr*$^{-/-}$ mice. We tested the hypothesis that dietary DHA was more effective than dietary EPA at preventing the induction of these markers. This is the first study that examines the capacity of EPA versus DHA to regulate a wide range of markers linked to NASH. More importantly, EPA and DHA supplementation was used at a physiologically relevant dose (2% total energy) to examine their effects on fatty liver disease. Unlike our previous study [168], none of the C20-22 (n-3) PUFA containing diets prevented diet-induced hepatosteatosis. This is likely due to high dietary fructose and the severity of hepatosteatosis induced following 16 wk of feeding the WD + OO diet. Thus, a reduction in hepatosteatosis is not required for EPA and DHA to attenuate WD + OO induced

hepatic damage, inflammation, oxidative stress, and fibrosis. DHA was more effective than EPA at attenuating inflammation, oxidative stress (NOX subunits), fibrosis and hepatic damage. Based on these results, DHA may be a more attractive dietary supplement than EPA for the prevention and potential treatment of NASH in obese humans.

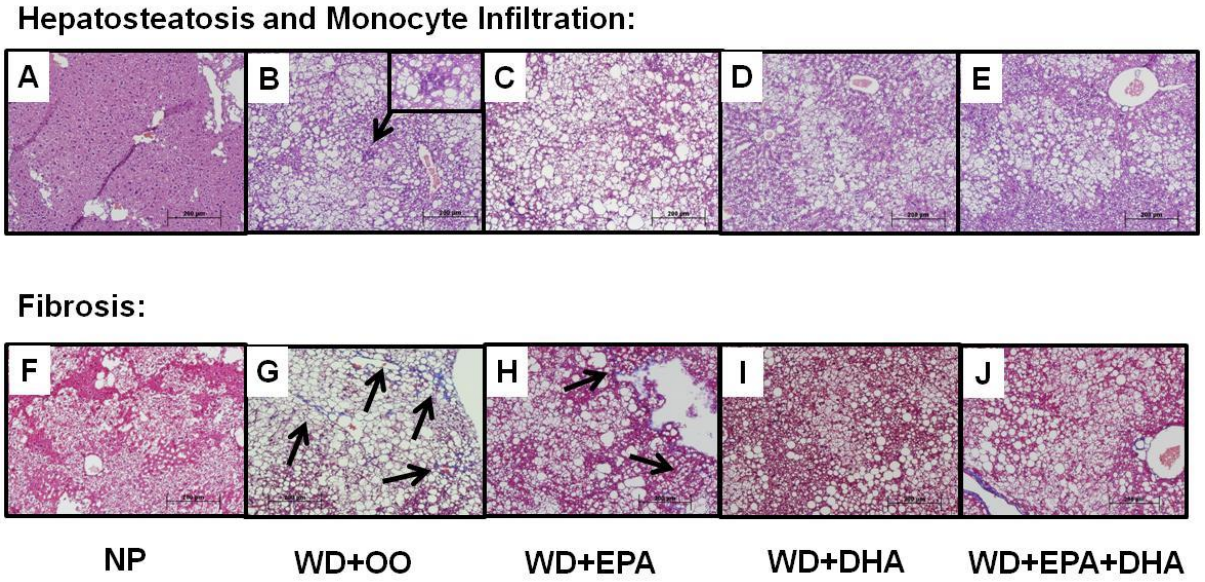

FIGURE 3.1. Histological analysis of liver from *Ldlr*$^{-/-}$ mice fed the NP, WD + OO, WD + EPA, WD + DHA, and WD + EPA + DHA diets for 16 wk. Panels A-E: Hematoxylin and eosin (H&E) staining of liver sections. The enlarged insert in Panel B illustrates a cluster of leukocytes, i.e., inflammation. Panels (F-J): Trichrome stain of liver sections. The blue stained regions (marked by arrows) designates collagen staining, i.e., fibrosis. All images are representative of mice fed their respective diets. NP, non-purified diet; WD + OO, western diet supplemented with olive oil; WD + EPA, western diet supplemented with eicosapentaenoic acid; WD + DHA, western diet supplemented with docosahexaenoic acid; WD + EPA + DHA, western diet supplemented with eicosapentaenoic acid and docosahexaenoic acid.

TABLE 3.1. Composition of NP, WD + OO, WD + EPA, WD + DHA, and WD + EPA + DHA diets[1]

	NP	WD + OO	WD + EPA	WD + DHA	WD + EPA + DHA
Composition, *energy%*					
Protein	28.5	15.8	15.8	15.8	15.8
Carbohydrate	58	39.5	39.5	39.5	39.5
Fat	13.5	44.7	44.7	44.7	44.7
% Energy as:					
Sucrose	4.0	27.0	27.0	27.0	27.0
Saturated Fat	4.0	10.3	8.8	11.6	10.0
MUFA	3.0	17.8	15.5	14.9	15.3
(n-3) PUFA	0.2	0.2	2.5	2.5	2.5
(n-6) PUFA	6.3	2.9	3.3	2.1	2.7
Cholesterol, *weight%*	0.02	0.21	0.21	0.21	0.21
Fatty Acid, *Mole%*[2]					
14:0	1	9.3	5.0	12.2	7.7
16:0	16	30.2	32.3	30.1	31.6
18:0	4	12.0	12.7	12.2	12.8
18:1 (n-9)	20	33.5	26.2	26.3	26.8
18:2 (n-6)	50	6.1	7.3	4.7	6.1
18:3 (n-3)	0.4	0.4	0.5	0.3	0.4
20:5 (n-3)			5.2		2.6
22:6 (n-3)	1.1			5.3	2.7

[1]Values for energy composition and cholesterol content are from the supplier of the diet, NP, non-purified diet (Purina); WD western diet (Research Diets). Values for fat energy are corrected for the supplementation with olive oil (WD + OO), EPA (WD + EPA), DHA (WD + DHA) or EPA + DHA (WD + EPA + DHA).

[2]Fatty acid composition of the diet was determined by GC analysis and presented as Mol%.

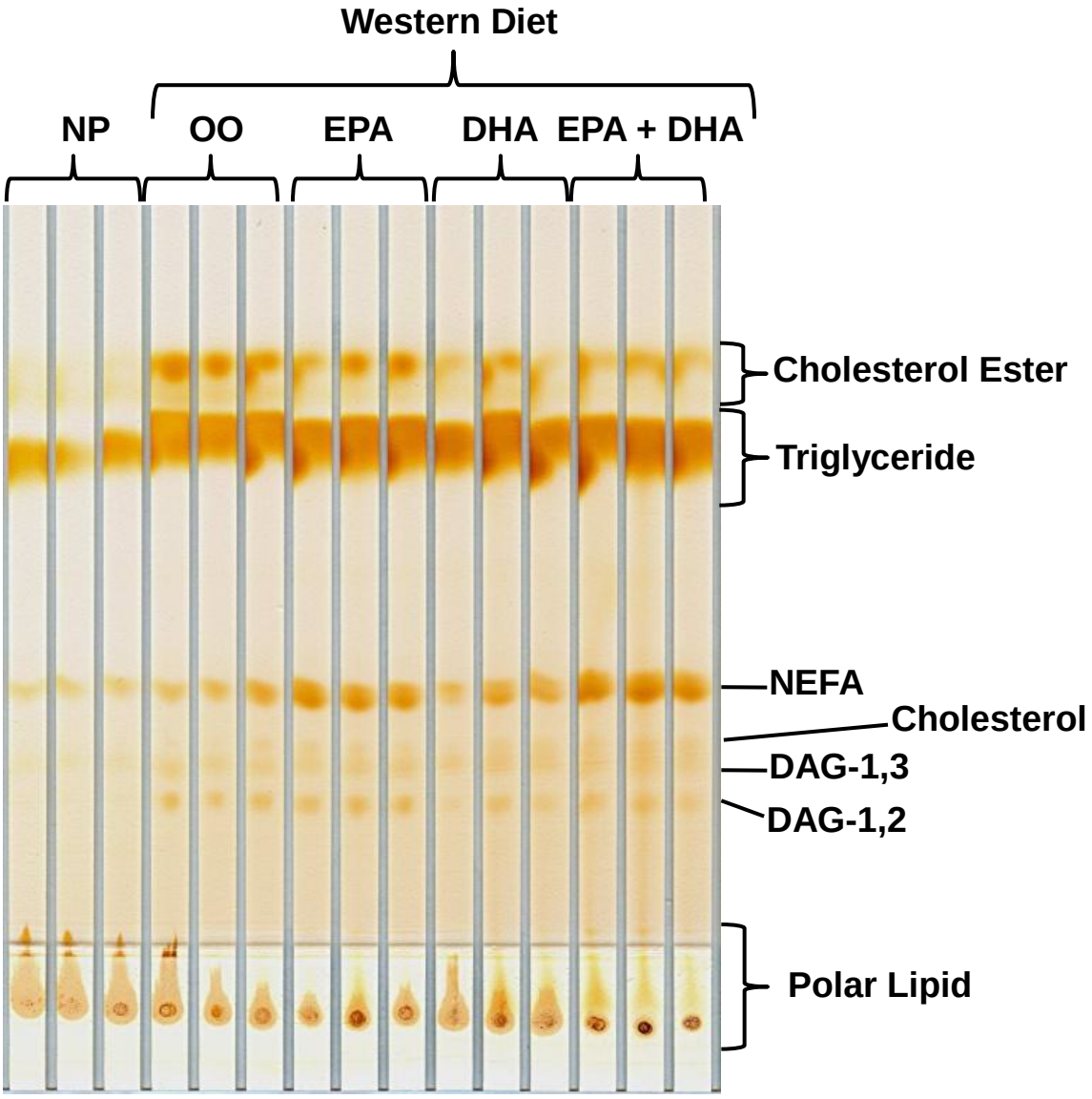

FIGURE 3.2. Analysis of hepatic lipids by thin-layer chromatography from *Ldlr*$^{-/-}$ mice fed the NP, WD + OO, WD + EPA, WD + DHA, and WD + EPA + DHA diets for 16 wk. Hepatic lipids were extracted and fractionated by thin-layer chromatography as described in Methods; n = 3 extracts per group. NP, non-purified diet; OO, western diet supplemented with olive oil; EPA, western diet supplemented with eicosapentaenoic acid; DHA, western diet supplemented with docosahexaenoic acid; EPA + DHA, western diet supplemented with eicosapentaenoic acid and docosahexaenoic acid; DAG, diacylglycerol; NEFA, non-esterified fatty acid.

TABLE 3.2. Mouse Primer Pairs for qRT-PCR

Target	Accession #	Forward	Reverse
Cyclophilin	NM_008907	CTTCTTGCTGGTCTTGCCATTCCT	GGATGGCAAGCATGTGGTCTTTG
Triglyceride Hydrolysis			
Adipose Triglyceride Lipase (*Atgl*)	NM_025802.3	TGTGGCCTCATTCCTCCTAC	AGAACTTCCTCTCGTCCAG
Triglyceride hydrolase (*Tgh*)	NM_053200.2	CTCAGAGACCCACAGAGCCCTTGTC	AAGCTGTGCACGCAGCAAGAGA
Arylacetamide deacetylase (*Aadac*)	NM_023383.1	TCCCACCCACGTCTGATGAGC	AAGCCCCGCCTGAGAGCCAT
Adipocyte differentiation related protein (*Adrp*)	NM_007408.3	TTGCCAGTGCCAGAGGTGCC	CCACGCTGCCAGTCACTGCT
Adiponutrin (*Adpn*)	NM_054088.3	ATTCCCCTCTTCTCTGGCCTA	ATGTCATGCTCACCGTAGAAAGG
Fatty Acid Synthesis			
Fatty acid synthase (*Fasn*)	NM_007988	CAAATACAATGGCACCCTGA	TGGCGAAGCCGTAGTTAGTT
Δ^5-desaturase (*Fads1*)	NM_146094	TGTGTGGGTGACACAGATGA	GTTGAAGGCTGATTGGTGAA
Δ^6-desaturase (*Fads2*)	NM_019699	CCACCGACATTTCCAACAC	GGGCAGGTATTTCAGCTTCTT
Fatty acid elongase-1 (*Elovl1*)	NM_001039175.1	GACTTCTCTCCGGCCCTGATCCCTT	GCCAACCAGACATCAGAAACCGC
Fatty acid elongase-2 (*Elovl2*)	NM_019423.2	ACGCTGGTCATCCTGTTCTT	GCCACAATTAAGTGGGCTTT
Fatty acid elongase-3 (*Elovl3*)	NM_007703.2	AGCCTGGACGCTCACTCGGTT	GCGGTTGAGCCCTCCTTCCCT
Fatty acid elongase-5 (*Elovl5*)	NM_134255.2	CGGGAGAATCCGATATGAAG	CATGGTAGCGTGGTGGTAGA
Fatty acid elongase-6 (Elovl6)	NM_130450.2	ACAATGGACCTGTCAGCAAA	GTACCAGTGCAGGAAGATCAGT
Fatty acid elongase-7 (*Elovl7*)	NM_029001.5	GGGCCGGTGAATCTTGCGGA	TGGTTTGTGGCAGAGGCGAGG
Stearoyl CoA desaturase-1 (*Scd1*)	NM_009127.4	TCAACTTCACCACGTTCTTCA	CTCCCGTCTCCAGTTCTCTT
Oxidant Stress			
Nuclear factor-E2-related factor-2 (*Nrf2*)	NM_010902.3	GCTGTTCCCAGCCCAGAAGCC	AGCGGAGGAAGGCATGCTGC
NADPH Oxidase-1, (*Nox1*)	NM_172203.1	ACCTGCTCATTTTGCAACCG	GCTGGAGAGAACAGAAGCGA
NADPH oxidase activator 1 (*Noxa1*)	NM_172204.4	AGACCGGAGGATTTGGACTT	AAGCATGGCTTCCACATAGG
NADPH oxidase organizer 1(*Noxo1*)	NM_027988.4	CATCAGGAAGCTTGGGAAGA	AGGAGCTGGGATGAGTTCAG
NADPH Oxidase-2, (*Nox2/Gp91phox*)	NM_007807.4	ACTGCGGAGAGTTTGGAAGA	ACTGTCCCACCTCCATCTTG
NADPH Oxidase-4, (N*ox4*)	NM_015760.4	TCTCAGGTGTGCATGTAGCC	CCGTCAGACCAGGAATGGTT
Cytochrome B-245 light chain (*p22phox*)	NM_007806.3	CGCTGGCGTCTGGCCTGATT	CCCCAGGATGGTGGCCAGGA
Neutrophil cytosolic factor 4 (*p40phox*)	NM_008677.2	TCAGAGAGCGACTTTGAGCA	GTTTTGCGCCCATGTAGACT
Neutrophil cytosolic factor 1 (*p47phox*)	NM_010876.3	TATCGCCGCAACAGCGTCCG	ACCGCGGGCTGTGGTTTGAC
Neutropil cytosolic factor 2 (*p67phox*)	NM_010877.4	GCAGTGGCCTACTTCCAGAG	CTTCATGTTGGTTGCCAATG
RAS-related C3 botulinum substrate 1 (*Rac1*)	NM_009007.2	CTGAAGTGCGACACCACTGT	CAGCAGGCATTTTCTCTTCC
Hemeoxygenase-1 (*Hmox1*)	NM_010442.1	GCCACCAAGGAGGTACACAT	GCTTGTTGCGCTCTATCTCC
Glutathione-S-transferase A1 (*Gsta1*)	NM_008181.3	CCTCTGGAGCTGGACTGTGAGCT	GCCAGGAGCCACCTGATGCA
Inflammation			
Cell Determination-68 (*Cd68*)	NM_009853.1	TCAAATCCGAATCCTATACCCA	ATGTAGGTCCTGTTTGAATCCA
C-type lectin domain family 4f (*Clec4f*)	NM_016751.3	CTGCGGCACGTCCAGGTCAT	CCAAGGCTGTGAAGCCACCACA
C-type lectin domain family 10a (*Clec10a*)	NM_001204252.1	GGATCCCAAAATTCCCAGTT	TGCTCCCTCTTCTCCACTGT
Monocyte chemoattractant protein-1 (*Mcp1*)	NM_011333.3	TCCCAATGAGTAGGCTGGAG	TCTGGACCCATTCCTTCTTG

Table 3.2. Continued

Interleukin 1-β□(*Il-1β*)	NM_008361.3	AGCCAAGCTTCCTTGTGCAAGTGT	GCTCTCATCAGGACAGCCCAGGT
Toll-like receptor-4 (*Tlr4*)	NM_021297.2	AGCAGCCGCTCTGGCATCAT	TGCCTCCCCAGAGGATTGTCCT
Toll-like receptor-2 (*Tlr2*)	NM_011905.3	CGGGACTTCGTTCCGGGCAA	TGGCCGCGTCGTTGTTCTCG
Toll-like receptor-9 (*Tlr9*)	NM_031178.2	ATAAGGCACAGAGCGCAGTT	ATCTCGGTCCTCCAGACACA
Monocyte differentiation antigen (*Cd14*)	NM_009841.3	TTGGTCGAACAAGCCCGTGGA	AAGCACACGCTCCATGGTCGGTA
Myeloid differentiation factor-2 (*Md-2*)	NM_001159711.1	TCCGATGGTCTTCCTGGCGAGT	TCCTTACGCTTCGGCAACTTTGGA
Tumor necrosis factor-α (*Tnfα*)	NM_013693.2	AGCCCCCAGTCTGTATCCTT	ACAGTCCAGGTCACTGTCCC
EGF-like module receptor 1 (*F4/80*)	NM_010130.4	GCTGTGAGATTGTGGAAGCA	CTGTACCCACATGGCTGATG
Myeloid differentiation primary response gene 88 (*Myd88*)	NM_010851.2	GCGAGCGTACTGGACGGCAC	TCCTCCGCCAGCAAGGTCCA
Fibrosis			
Procollagen 1α1 (*Procol1α1*)	NM_007742.2	GACATCCCTGAAGTCAGCTGC	TCCCTTGGGTCCCTCGAC
Transforming growth factor 1β (*Tgfβ1*)	NM_011577.2	TTGCCCTCTACAACCAACACAA	GCTTGCGACCCACGTAGTA
Tissue inhibitor of metalloproteinase (*Timp1*)	NM_011593.2	CCTTGCAAACTGGAGAGTGACA	AAGCAAAGTGACGGCTCTGGT
BMP and activin membrane-bound inhibitor homolog (*Bambi*)	NM_026505.2	CGCTGTTCGTGGCTTGTGCG	TCCATTGACGCCTCGCGCTC
Plasminogen activator inhibitor-1 (*Pai1*)	NM_008871.2	CCACCTCCAAGGGGCAACGG	AGCCAGGCACACACACGTCC

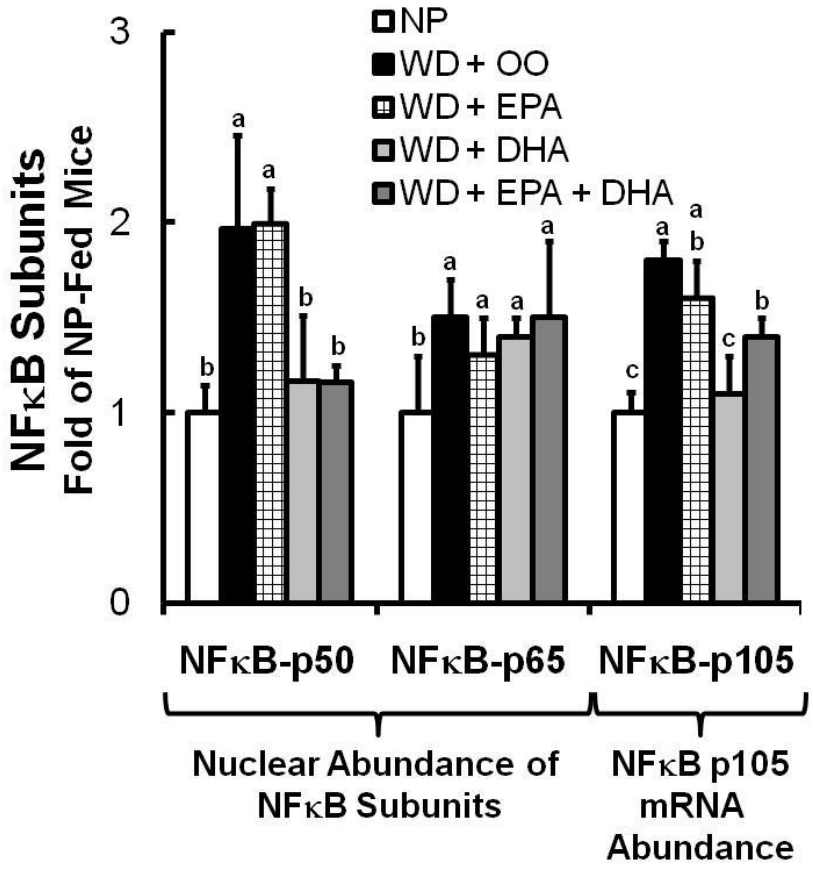

FIGURE 3.3. Effect of diet on hepatic NFκB-p50 and NFκB-p65 nuclear abundance and NFκB-p105 mRNA abundance in *Ldlr*$^{-/-}$ mice fed the NP, WD + OO, WD + EPA, WD + DHA, and WD + EPA + DHA diets for 16 wk. Protein results are expressed as nuclear protein abundance as fold of NP fed mice; mean ± SD, *n* = 8. TATA-binding protein (TBP) was the loading control. Hepatic RNA was extracted and transcript abundance was quantified by qRT-PCR (Methods). Data are mean ± SD, *n* = 8 per treatment group and are represented as fold of NP. Labeled means without a common letter differ, $P < 0.05$. NP, non-purified diet; WD + OO, western diet supplemented with olive oil; WD + EPA, western diet supplemented with eicosapentaenoic acid; WD + DHA, western diet supplemented with docosahexaenoic acid; WD + EPA + DHA, western diet supplemented with eicosapentaenoic acid and docosahexaenoic acid.

TABLE 3.3. Phenotypic comparison of *Ldlr*$^{-/-}$ mice fed the HFHC+ OO and WD + OO diets for 12 and 16 wk, respectively[1]

	HFHC + OO	WD + OO
Body Weight, *g*	40 ± 5	42 ± 2
Plasma Parameters		
Glucose, *mmol/L*	12 ± 5	11 ± 1
Triglycerides[2], *mg/dL*	352 ± 141	229 ± 51
Cholesterol[2], *mg/dL*	957 ± 252	1018 ± 54
ApoB[3], *mg/dL*	160 ± 70	101 ± 26
ApoC3[3], *mg/dL*	21 ± 6	31 ± 10
NEFA[4], *mEq/mL*	1.5 ± 0.6	1.1 ± 0.1
β-Hydroxybutyrate, *mmol/L*	2.9 ± 0.8	2.2 ± 0.4
ALT, *U/L*	38 ± 9	44 ± 9
AST[7], *U/L*	56 ± 10^{a}	36 ± 7^{b}
Liver Parameters		
Triglycerides[5,7], *ug/mg*	129 ± 13^{a}	328 ± 55^{b}
Cholesterol[6,7], *ug/mg*	17 ± 3^{a}	34 ± 5^{b}
Weight, *g*	2.0 ± 0.6	2.1 ± 0.3
% Body Weight	4.8 ± 0.9	5.2 ± 0.5

[1]Values are the mean ± SD, n = 8 per treatment group. Labeled means in a row with superscripts without a common letter differ, $P \leq 0.05$. ALT, alanine aminotransferase; AST, aspartate aminotransferase; NP, non-purified diet; HFHC + OO, high fat-high cholesterol diet supplemented with olive oil; WD + OO, western diet supplemented with olive oil. Data for the HFHC + OO fed group is taken from our previous study (15); Data for the WD + OO group is taken from Table 1 of this report.

[2]To convert from mg/dL to mmol/L, multiply by: 0.0113 for triglycerides; 0.02586 for cholesterol.

[3]To convert from mg/dL to g/L, multiply by: 0.01 for ApoB and ApoCIII.

[4]To convert from mEq/mL to mEq/L multiply by 1000 for NEFA.

[5]Data are expressed as μg triglyceride (TG) per mg protein (liver), to convert from μg/mg to mmol/g multiply by 0.00113.

[6]Data are expressed as μg cholesterol per mg protein (liver), to convert from μg/mg to mmol/g multiply by 0.002586

[7]t-test, $P < 0.05$.

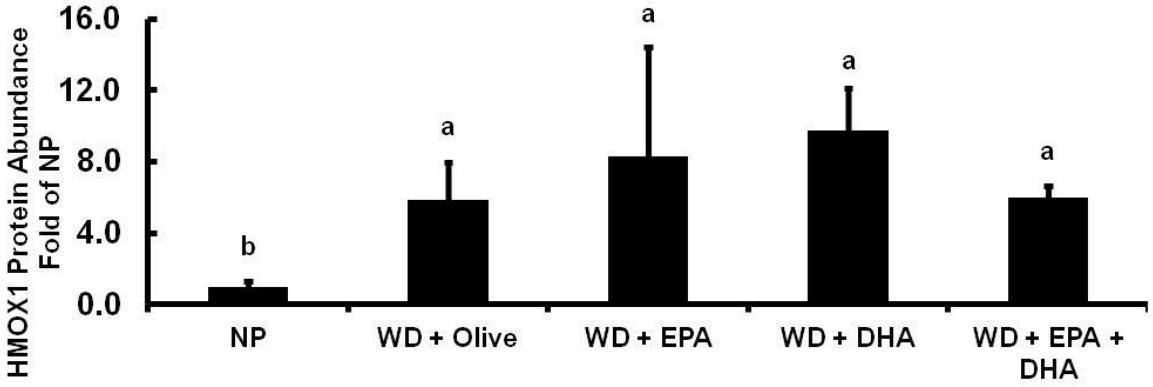

FIGURE 3.4. Effect of diet on hepatic HMOX1 protein abundance in *Ldlr*$^{-/-}$ mice fed the NP, WD + OO, WD + EPA, WD + DHA, and WD + EPA + DHA diets for 16 wk. Results are expressed as post-nuclear protein abundance as fold of NP fed mice; mean ± SD, n = 8; Na,K- ATPase served as a loading control. Labeled means without a common letter differ, $P < 0.05$. NP, non-purified diet; WD + OO, western diet supplemented with olive oil; WD + EPA, western diet supplemented with eicosapentaenoic acid; WD + DHA, western diet supplemented with docosahexaenoic acid; WD + EPA + DHA, western diet supplemented with eicosapentaenoic acid and docosahexaenoic acid.

TABLE 3.4. Hepatic gene expression in *Ldlr*$^{-/-}$ mice fed the HFHC + OO and WD + OO diet for 12 and 16 wk, respectively. Effects on mRNA abundance of proteins involved in inflammation, oxidative stress and fibrosis[1]

	HFHC + OO	WD + OO
	Fold of NP	
Inflammation		
Mcp1	28 ± 12	32 ± 11
Clec4f[2]	2.4 ± 0.5[a]	3.8 ± 0.9[b]
Oxidative Stress		
Hmox1	4.3 ± 1.1	5.0 ± 1.6
Fibrosis		
Procol1α1[2]	8.7 ± 5[a]	18 ± 6[b]
Tgfβ1[2]	1.6 ± 0.5[a]	2.5 ± 0.2[b]

[1]Values are the mean ± SD, n = 8 per treatment group. Labeled means in a row with superscripts without a common letter differ, $P \leq 0.05$. ALT, alanine aminotransferase; AST, aspartate aminotransferase; NP, non-purified diet; HFHC + OO, high fat-high cholesterol diet supplemented with olive oil; WD + OO, western diet supplemented with olive oil. Data for the HFHC + OO fed group is taken from our previous study (15); Data for the WD + OO group is taken from Table 4 of this report.
[2]t-test, $P < 0.05$.

TABLE 3.5. Phenotypic comparison of *Ldlr*$^{-/-}$ mice fed the NP, WD + OO, WD + EPA or WD + DHA diets for 16 wk[1]

	NP[2]	WD + OO	WD + EPA	WD + DHA	WD + EPA + DHA
Body Weight, *g*	31 ± 3^{b}	42 ± 2^{a}	48 ± 3^{a}	45 ± 3^{a}	47 ± 4^{a}
% Fat Mass	20 ± 4^{b}	42 ± 3^{a}	46 ± 3^{a}	45 ± 2^{a}	46 ± 3^{a}
Plasma variables					
Glucose, *mmol/L*	11 ± 3	11 ± 1	10 ± 1	9 ± 2	11 ± 1
TG[3], *mg/dL*	86 ± 19^{d}	229 ± 51^{a}	167 ± 59^{b,c}	128 ± 20^{c,d}	180 ± 44^{b}
Cholesterol[3], *mg/dL*	232 ± 30^{c}	1018 ± 54^{a}	880 ± 127^{a,b}	737 ± 139^{b}	809 ± 151^{b}
ApoB[4], *mg/dL*	20 ± 6^{c}	101 ± 26^{a}	52 ± 13^{b}	38 ± 8^{b}	41 ± 14^{b}
ApoC3[4], *mg/dL*	16 ± 6^{c}	31 ± 10^{a}	30 ± 8^{a}	19 ± 4^{b,c}	24 ± 6^{a,b}
NEFA[5], *mEq/mL*	0.7 ± 0.1^{c}	1.1 ± 0.1^{a}	0.8 ± 0.1^{b}	0.9 ± 0.1^{a,b}	0.8 ± 0.2^{b}
β-Hydroxybutyrate, *mmol/L*	0.9 ± 0.4^{b}	2.2 ± 0.4^{a}	2.0 ± 0.3^{a}	2.0 ± 0.3^{a}	1.7 ± 0.1^{a}
ALT, *U/L*	5 ± 1^{c}	44 ± 9^{a}	32 ± 8^{b}	29 ± 10^{b}	29 ± 10^{b}
AST, *U/L*	9 ± 3^{c}	36 ± 7^{a}	20 ± 4^{b}	20 ± 6^{b}	19 ± 5^{b}
Liver variables					
Weight, *g*	1.1 ± 0.1^{b}	2.1 ± 0.3^{a}	2.4 ± 0.4^{a}	1.7 ± 0.3^{a}	2.2 ± 0.4^{a}
% Body Weight	4.0 ± 0.5^{b}	5.2 ± 0.5^{a}	5.2 ± 0.7^{a}	4.2 ± 0.6^{b}	5.1 ± 0.8^{a}
TG[6], *μg/mg*	77 ± 23^{c}	328 ± 55^{b}	421 ± 103^{a,b}	387 ± 76^{b}	487 ± 39^{a}
Cholesterol[7], *μg/mg*	12 ± 4^{c}	34 ± 5^{a,b}	45 ± 14^{a}	31 ± 6^{b}	46 ± 9^{a}

[1]Values are the mean ± SD, n = 8 per treatment group. Labeled means in a row with superscripts without a common letter differ, $P \leq 0.05$. ALT, alanine aminotransferase; AST, aspartate aminotransferase; NP, non-purified diet; WD + OO, western diet supplemented with olive oil; WD + EPA, western diet supplemented with eicosapentaenoic acid; WD + DHA, western diet supplemented with docosahexaenoic acid; WD + EPA + DHA, western diet supplemented with eicosapentaenoic acid and docosahexaenoic acid.

[2]NP *Ldlr*$^{-/-}$ mice were used as the control group.

[3]To convert from mg/dL to mmol/L, multiply by: 0.0113 for triglycerides; 0.02586 for cholesterol.

[4]To convert from mg/dL to g/L, multiply by: 0.01 for ApoB and ApoCIII.

[5]To convert from mEq/mL to mEq/L multiply by 1000 for NEFA.

[6]Data are expressed as μg triglyceride (TG) per mg protein (liver), to convert from μg/mg to mmol/g multiply by 0.00113.

[7]Data are expressed as μg cholesterol per mg protein (liver), to convert from μg/mg to mmol/g multiply by 0.002586

TABLE 3.6. Hepatic fatty acid composition in *Ldlr*$^{-/-}$ mice fed the NP, WD + OO, WD + EPA, WD + DHA and WD + EPA + DHA diets for 16 wk[1]

	NP	WD + OO	WD + EPA	WD + DHA	WD + EPA + DHA
Fatty Acids			*μmol/g protein*		
14:0	5 ± 2^{c}	92 ± 11^{a}	57 ± 11^{b}	52 ± 12^{b}	56 ± 9^{b}
16:0	339 ± 77^{c}	1970 ± 284^{a}	1720 ± 320^{a,b}	1360 ± 327^{b}	1490 ± 206^{b}
16:1 (n-7)	30 ± 13^{c}	461 ± 57^{a}	445 ± 81^{a}	293 ± 82^{b}	363 ± 86^{a,b}
18:0	141 ± 11^{b}	178 ± 25^{a}	178 ± 18^{a}	153 ± 23^{a,b}	158 ± 14^{a,b}
18:1 (n-9)	196 ± 65^{d}	4140 ± 591^{a}	2930 ± 599^{b}	2030 ± 610^{c}	2460 ± 457^{b,c}
18:1 (n-7)	39 ± 9^{d}	847 ± 127^{a}	411 ± 104^{b}	204 ± 87^{c}	279 ± 103^{b,c}
18:2 (n-6)	320 ± 107^{a,b}	355 ± 46^{a,b}	413 ± 76^{a}	278 ± 44^{b}	333 ± 52^{a,b}
18:3 (n-3)	10 ± 5^{b,c}	9 ± 2^{c}	16 ± 4^{a}	11 ± 3^{a,b,c}	15 ± 4^{a,b}
20:0	4 ± 1^{c}	134 ± 20^{a}	74 ± 16^{b}	53 ± 22^{b}	62 ± 13^{b}
20:2 (n-6)	2.6 ± 0.3^{c}	8.1 ± 1.0^{a}	7.4 ± 1.7^{a}	2.3 ± 0.7^{c}	5.2 ± 0.9^{b}
20:3 (n-6)	8 ± 1^{c}	22 ± 3^{a}	24 ± 3^{a}	9 ± 1^{c}	16 ± 2^{b}
20:4 (n-6)	76 ± 8^{b}	142 ± 20^{a}	67 ± 8^{b}	31 ± 5^{c}	39 ± 5^{c}
20:5 (n-3)	8 ± 2^{c}	2 ± 1^{c}	115 ± 32^{a}	65 ± 8^{b}	92 ± 18^{a}
22:5 (n-6)	2 ± 1^{b}	26 ± 8^{a}	8 ± 1^{b}	6 ± 3^{b}	6 ± 3^{b}
22:5 (n-3)	7 ± 1^{d}	7 ± 2^{d}	126 ± 27^{a}	47 ± 13^{c}	77 ± 13^{b}
22:6 (n-3)	63 ± 11^{c}	55 ± 8^{c}	121 ± 21^{c}	424 ± 97^{a}	313 ± 84^{b}
Sum C20-22[2]					
C20-22 (n-3)	78 ± 14^{c}	64 ± 9^{c}	362 ± 78^{b}	536 ± 113^{a}	482 ± 109^{a}
C20-22 (n-6)	91 ± 8^{b,c}	211 ± 30^{a}	108 ± 13^{b}	51 ± 7^{d}	68 ± 7^{c,d}
Fatty Acid Ratios[3]					
20:4 (n-6)/18:2 (n-6)	0.26 ± 0.02^{b}	0.40 ± 0.04^{a}	0.16 ± 0.02^{c}	0.11 ± 0.02^{c}	0.12 ± 0.02^{c}
20:3 (n-6)/18:2 (n-6)	0.03 ± 0.003^{c}	0.06 ± 0.007^{a}	0.06 ± 0.007^{a,b}	0.03 ± 0.005^{c}	0.05 ± 0.004^{b}

[1] Values are the mean ± SD, n = 8 per treatment group. Labeled means in a row with superscripts without a common letter differ, $P \leq 0.05$. NP, non-purified diet; WD + OO, western diet supplemented with olive oil; WD + EPA, western diet supplemented with eicosapentaenoic acid; WD + DHA, western diet supplemented with docosahexaenoic acid; WD + EPA + DHA, western diet supplemented with eicosapentaenoic acid and docosahexaenoic acid.

[2]Sum C20-22 refers to the sum of all the 20 to 22 carbon fatty acids analyzed.

[3]Fatty acid ratios refers to the mole ratio of 20:4 (n-6) to 18:2 (n-6) or 20:3 (n-6) to 18:3 (n-6)

TABLE 3.7. Hepatic gene expression in *Ldlr*$^{-/-}$ mice fed the NP, WD + OO, WD + EPA, WD + DHA and WD + EPA + DHA diets for 16 wk; effects on mRNA abundance of enzymes involved in fatty acid synthesis and triglyceride hydrolysis[1]

	NP	WD + OO	WD + EPA	WD + DHA	WD + EPA + DHA
			Fold of NP		
Fatty Acid Synthesis					
Fasn	1.0 ± 0.4^{a}	0.6 ± 0.2^{a,b}	0.4 ± 0.1^{b,c}	0.2 ± 0.1^{c}	0.4 ± 0.1^{b,c}
Elovl1	1.0 ± 0.2^{c}	2.0 ± 0.4^{a}	1.8 ± 0.2^{a,b}	1.1 ± 0.2^{c}	1.5 ± 0.3^{b}
Elovl2	1.0 ± 0.4^{c,d}	1.5 ± 0.4^{a}	1.2 ± 0.3^{a,b}	0.6 ± 0.2^{d}	0.9 ± 0.2^{b,c}
Elovl3	1.0 ± 0.3^{a}	0.2 ± 0.1^{b}	0.3 ± 0.1^{b}	0.3 ± 0.1^{b}	0.3 ± 0.1^{b}
Elovl5	1.0 ± 0.3^{c}	2.1 ± 0.6^{a,b}	1.8 ± 0.4^{a,b}	1.0 ± 0.4^{c}	1.6 ± 0.5^{b,c}
Elovl6	1.0 ± 0.2^{b,c}	1.5 ± 0.4^{a}	1.4 ± 0.2^{a,b}	1.0 ± 0.4^{c}	1.3 ± 0.3^{a,b,c}
Elovl7	1.0 ± 0.2^{b}	5.5 ± 2.2^{a}	2.3 ± 1.6^{b}	2.1 ± 0.9^{b}	2.4 ± 0.9^{b}
Fads1	1.0 ± 0.3^{a,b}	1.1 ± 0.4^{a}	0.7 ± 0.2^{b,c}	0.2 ± 0.2^{d}	0.4 ± 0.2^{c,d}
Fads2	1.0 ± 0.2^{b,c}	2.0 ± 0.6^{a}	1.5 ± 0.2^{b}	0.8 ± 0.4^{c}	1.2 ± 0.2^{b,c}
Scd1	1.0 ± 0.7^{c}	6.0 ± 2.6^{a}	6.1 ± 2.6^{a}	2.4 ± 1.5^{b,c}	4.1 ± 1.5^{a,b}
Triglyceride Hydrolysis					
Atgl	1.0 ± 0.3	1.2 ± 0.3	1.2 ± 0.1	1.0 ± 0.3	1.3 ± 0.2
Tgh	1.0 ± 0.3^{b}	0.9 ± 0.4^{b}	1.2 ± 0.2^{a,b}	1.2 ± 0.5^{a,b}	1.5 ± 0.3^{a}
Aadac	1.0 ± 0.2	0.9 ± 0.3	0.9 ± 0.1	0.8 ± 0.3	0.9 ± 0.2
Adrp	1.0 ± 0.4	1.5 ± 0.3	1.4 ± 0.3	1.2 ± 0.4	1.3 ± 0.5
Adpn	1.0 ± 0.3^{b}	2.1 ± 0.9^{a}	1.3 ± 0.2^{b}	0.9 ± 0.2^{b}	1.4 ± 0.5^{b}

[1] Values are the mean ± SD, n = 8 per treatment group. Labeled means in a row with superscripts without a common letter differ, $P \leq 0.05$. NP, non-purified diet; WD + OO, western diet supplemented with olive oil; WD + EPA, western diet supplemented with eicosapentaenoic acid; WD + DHA, western diet supplemented with docosahexaenoic acid; WD + EPA + DHA, western diet supplemented with eicosapentaenoic acid and docosahexaenoic acid.

TABLE 3.8. Hepatic gene expression in *Ldlr*$^{-/-}$ mice fed the NP, WD + OO, WD + EPA, WD + DHA and WD + EPA + DHA diets for 16 wk; effects on mRNA abundance of proteins involved in inflammation, oxidative stress and fibrosis[1]

	NP	WD + OO	WD + EPA	WD + DHA	WD + EPA + DHA
			Fold of NP		
Inflammation					
Mcp1	1.0 ± 0.4^{c}	32 ± 11^{a}	13 ± 4^{b}	9.0 ± 3^{b,c}	10 ± 4^{b}
Cd68	1.0 ± 0.2^{d}	7.6 ± 1.3^{a}	5.0 ± 1.4^{b,c}	3.5 ± 1.0^{c}	5.5 ± 1.5^{b}
Clec4f	1.0 ± 0.3^{c}	3.8 ± 0.9^{a}	2.6 ± 0.8^{b}	1.5 ± 0.4^{c}	2.4 ± 0.5^{b}
Clec10a	1.0 ± 0.4^{b}	2.7 ± 1.6^{a}	1.9 ± 0.6^{a,b}	1.5 ± 1.0^{a,b}	2.7 ± 1.0^{a}
F4/80	1.0 ± 0.4^{c}	4.1 ± 0.7^{a}	3.6 ± 0.9^{a}	2.2 ± 0.9^{b}	3.4 ± 1.0^{a}
Il-1β	1.0 ± 0.6^{c}	3.9 ± 0.7^{a}	2.5 ± 0.6^{b}	1.7 ± 0.2^{b,c}	2.0 ± 0.7^{b}
Tnf-α	1.0 ± 0.4^{c}	8.6 ± 2.0^{a}	4.2 ± 1.3^{b}	3.8 ± 1.2^{b}	4.6 ± 1.7^{b}
Tlr2	1.0 ± 0.3^{c}	5.6 ± 1.3^{a}	3.5 ± 1.1^{b}	2.6 ± 0.5^{b}	3.3 ± 0.9^{b}
Tlr4	1.0 ± 0.3^{c}	4.0 ± 1.6^{a}	2.9 ± 0.9^{a,b}	1.7 ± 0.4^{b,c}	2.7 ± 0.7^{b}
Tlr9	1.0 ± 0.3^{c}	7.7 ± 1.8^{a}	6.2 ± 2.3^{a,b}	4.2 ± 1.1^{b}	5.9 ± 1.7^{a,b}
Cd14	1.0 ± 0.3^{c}	6.1 ± 1.5^{a}	3.4 ± 1.5^{b}	2.4 ± 0.5^{b,c}	3.7 ± 1.4^{b}
Md-2	1.0 ± 0.3	1.4 ± 0.4	1.4 ± 0.5	1.6 ± 0.9	1.0 ± 0.3
Myd88	1.0 ± 0.2^{b}	1.6 ± 0.4^{a}	1.4 ± 0.3^{a,b}	1.0 ± 0.3^{b}	1.3 ± 0.2^{a,b}
Oxidative Stress					
Nrf2	1.0 + 0.2^{c}	1.9 + 0.5^{a}	1.9 + 0.4^{a,b}	1.4 + 0.4^{b,c}	1.7 + 0.3^{a,b}
Gsta1	1.0 ± 0.4^{b}	1.9 ± 0.6^{b}	3.6 ± 0.9^{a}	1.9 ± 0.8^{b}	3.6 ± 1.9^{a}
Hmox1	1.0 ± 0.3^{c}	5.0 ± 1.6^{a}	3.5 ± 1.9^{b}	2.7 ± 0.5^{b}	3.7 ± 1.1^{a,b}
Nox1	1.0 ± 1.5	1.8 ± 0.6	1.8 ± 1.2	0.8 ± 1.2	0.8 ± 0.3
Noxa1	1.0 ± 0.5^{b}	6.2 ± 2.5^{a}	3.9 ± 1.7^{a}	3.9 ± 1.2^{a}	3.5 ± 1.7^{a}
Noxo1	1.0 ± 0.3^{b}	1.6 ± 0.4^{a}	2.0 ± 0.3^{a}	1.7 ± 0.5^{a}	1.9 ± 0.3^{a}
Nox2 (Gp91phox)	1.0 ± 0.3^{b}	6.7 ± 0.3^{a}	5.9 ± 2.2^{a}	2.5 + 0.9^{b}	5.1 ± 1.9^{a}
Nox4	1.0 ± 0.2	0.9 ± 0.3	1.1 ± 0.2	0.7 ± 0.3	0.8 ± 0.2
P22phox	1.0 ± 0.3^{c}	5.7 ± 1.2^{a}	3.7 ± 1.1^{b}	2.9 ± 0.8^{b}	3.4 ± 1.1^{b}
P40phox	1.0 ± 0.3^{d}	5.6 ± 1.3^{a}	4.5 ± 1.6^{a,b}	2.8 ± 0.6^{c}	3.9 ± 1.1^{b,c}
P47phox	1.0 ± 0.3^{c}	3.9 ± 1.2^{a}	2.9 ± 0.8^{a,b}	2.0 ± 0.3^{b,c}	3.0 ± 0.9^{a,b}
P67phox	1.0 ± 0.6^{b}	7.3 ± 4.4^{a}	2.3 ± 1.2^{b}	1.0 ± 0.7^{b}	3.1 ± 2.5^{b}
Rac1	1.0 ± 0.2^{b}	1.0 ± 0.2^{b}	1.0 ± 0.2^{b}	0.6 ± 0.1^{a}	0.9 ± 0.2^{b}
Fibrosis					
Procol1α1	1.0 ± 0.4^{b}	18 ± 6^{a}	16 ± 11^{a}	6.1 ± 3^{b}	10 ± 5^{a,b}
Tgfβ1	1.0 ± 0.1^{c}	2.5 ± 0.2^{a}	2.0 ± 0.5^{a,b}	1.8 ± 0.4^{b}	1.8 ± 0.5^{b}
Bambi	1.0 ± 0.2	1.2 ± 0.3	1.1 ± 0.2	0.9 ± 0.3	1.0 ± 0.2
Timp1	1.0 ± 0.5^{c}	39 ± 11^{a}	20 ± 10^{b}	16 ± 10^{b}	19 ± 10^{b}

Table 3.8. Continued

Pai1	1.0 ± 0.5[c]	7.4 ± 1.6[a]	4.6 ± 1.4[b]	3.9 ± 1.0[b]	4.3 ± 1.6[b]

[1] Values are the mean ± SD, n = 8 per treatment group. Labeled means in a row with superscripts without a common letter differ, $P \leq 0.05$. NP, non-purified diet; WD + OO, western diet supplemented with olive oil; WD + EPA, western diet supplemented with eicosapentaenoic acid; WD + DHA, western diet supplemented with docosahexaenoic acid; WD + EPA + DHA, western diet supplemented with eicosapentaenoic acid and docosahexaenoic acid.

Chapter 4

Metabolomic analysis of the development and progression of NASH in western diet fed *Ldlr*$^{-/-}$ mice and the impact from eicosapentaenoic acid and docosahexaenoic acid supplementation.

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4.1. Abstract

Non-alcoholic steatohepatitis (NASH) is the progressive form of non-alcoholic fatty liver disease and can advance to liver failure. Mechanisms contributing to NASH progression are incompletely understood. We previously reported that EPA (20:5 (n-3)) and DHA (22:6 (n-3)) supplementation in *Ldlr*$^{-/-}$ mice attenuates western diet (WD)-induced NASH without reducing hepatosteatosis. To expand our understanding of NASH progression, and the effect of EPA and DHA supplementation, we conducted a metabolomics analysis on livers from *Ldlr*$^{-/-}$ mice fed the WD for 16 weeks without/with EPA and DHA supplementation. WD feeding induced a severe NASH phenotype with hepatosteatosis, inflammation, oxidative stress, and fibrosis. Development of NASH had wide-reaching effects perturbing all major metabolic pathways analyzed including lipid, amino acid, and carbohydrate metabolism. WD feeding elevated ARA (20:4 (n-6)) and depleted EPA in the hepatic polar lipid fraction, altered hepatic lysophospholipids in parallel with the polar lipid fraction, elevated expression of enzymes regulating the Land's cycle (PLA2g6 & LPCAT1-4), and elevated palmitoyl-sphingomyelin (16:0-SML). As such, there was strong evidence of membrane phospholipid remodeling in WD fed mice with NASH. Changes in the hepatic polar lipid fatty acid profile correlated with increased (n-6) and depressed (n-3) derived oxidized PUFA (oxPUFA). We hypothesize these WD-induced changes in phospholipids and oxPUFA are major drivers of NASH progression. WD supplementation with DHA more effectively than EPA resulted in favorable changes in these observed metabolic derangements. Since DHA supplementation most potently attenuated NASH progression, we speculate that membrane phospholipid remodeling and the corresponding changes in oxPUFA associated with DHA consumption plays a

major role in attenuating inflammation, fibrosis, and oxidative stress associated with WD-induced NASH in $LDLR^{-/-}$ mice.

4.2. Introduction

By the year 2020, it is estimated that non-alcoholic steatohepatitis (NASH) will be the leading cause of liver transplantation in the United States [12]. NASH is the progressive form of non-alcoholic fatty liver disease (NAFLD) and is defined as hepatic steatosis with inflammation and hepatic injury [2]. Once developed, NASH can progress to hepatic fibrosis, cirrhosis, hepatocellular carcinoma, and end stage liver disease [3]. Approximately 30% to 40% of individuals with hepatic steatosis progress to NASH [3]. In the general population the prevalence of NASH ranges from 3% to 5% [5]. Given the clinical severity and increasing prevalence of NASH, it is quickly becoming a significant public health burden.

The current model for the development of NASH is a two-hit hypothesis proposed by Day and James [21]. The “first hit” comes from excess lipid accumulation in the liver. This is hypothesized to sensitize the liver to the “second hit” consisting of oxidative stress, inflammation, and insulin resistance. While the two-hit hypothesis is helpful in understanding processes that contribute to development and progression of NASH, our overall understanding of NASH is incomplete. This has limited the development of therapies specifically targeted to NAFLD/NASH. The standard of care for patients diagnosed with NAFLD or NASH is to treat for liver disease and the associated co-morbidities including obesity, T2D, hyperlipidemia, and the metabolic syndrome [2,13,14]. As such, we need a more complete understanding of NASH to address its imminent public health burden.

We recently reported that the capacity of dietary DHA to suppress markers of hepatic inflammation (Clec4f, F4/80, Tlr4), oxidative stress (NADPH oxidase subunits Nox2, p22phox, p40phox, p47phox, and p67phox), and fibrosis (Procol1α1, Tgfβ1) is greater than dietary EPA using an *Ldlr*$^{-/-}$ mouse model of WD-induced NASH [197]. While dietary DHA provided significant benefit in preventing progression of NASH, neither EPA nor DHA had the capacity to prevent hepatosteatosis. Using a less severe model, we previously demonstrated that menhaden oil rich in C20-22 (n-3) PUFA has the capacity to prevent high-fat diet (60% calories as fat) induced hepatosteatosis [168]. The WD used here was 39% calories as carbohydrate, with 27% calories as sucrose. The high level of simple carbohydrate in this diet contributes to a more aggressive NASH phenotype than feeding a typical high-fat diet with lower carbohydrate content. Thus we attribute the inability of EPA and DHA to prevent hepatosteatosis in WD fed *Ldlr*$^{-/-}$ mice to the severity of the model. Accordingly, WD feeding to *Ldlr*$^{-/-}$ mice is a strong model for the study of diet-induced NASH and the therapeutic potential of EPA and DHA supplementation.

To advance our understanding of both the progression of NASH and the impact from EPA and DHA supplementation, we conducted an untargeted global metabolomics analysis on livers from *Ldlr*$^{-/-}$ mice fed the WD for 16 weeks with and without EPA and or DHA supplementation. A major advantage of this study is that it provided analysis of perturbed hepatic metabolism associated with advanced diet-induced NASH, the most clinically detrimental stage of NAFLD. Multiple studies have previously employed omics strategies in effort to identify new biomarkers of NAFLD/NASH [198,199,200,201]. A few studies have used metabolomics specifically in the liver to assess changes associated with NAFLD or NASH [202,203,204]. However, this is the first study we are aware of that

uses metabolomics to identify pathways involved in diet-induced NASH and the impact from EPA and DHA supplementation on those pathways. Our overall goal was to identify potential pathways implicated in the attenuation of NASH progression by C20-22 (n-3) PUFA supplementation, ultimately establishing targets for future therapies designed to combat NAFLD/NASH. In all, we identified several metabolic targets of C20-22 (n-3) PUFA supplementation potentially contributing to attenuated progression of NASH including methylglyoxal (MG) detoxification, one-carbon and phospholipid metabolism, membrane remodeling, and an altered oxidized PUFA (oxPUFA) profile.

4.3. Materials and methods

4.3.1) Animals and diets

All procedures for the use and care of animals for laboratory research were approved by the Institutional Animal Care and Use Committee at Oregon State University. Male *Ldlr*$^{-/-}$ mice [on the C57BL/6J background] (Jackson Laboratories) at 2 months of age were fed one of the following five diets *ad libitum* for 16 weeks; each group consisted of 8 male mice. The control diet was Purina chow 5001 consisting of 13.5% energy as fat and 58.0% energy as carbohydrates. The western diet (WD) (D12709B, Research Diets] was used to induce NAFLD/NASH; it consists of 17% energy as protein, 43% energy as carbohydrate and 41% energy as fat; cholesterol was at 0.2% w/w. The WD was supplemented with olive oil (WD + OO), EPA (WD + EPA), DHA (WD + DHA) or EPA plus DHA (WD + EPA + DHA). Supplementation of the WD with OO, EPA, DHA or EPA + DHA increased total fat energy to 44.7% and reduced protein and carbohydrate energy to 15.8 and 39.5%, respectively. Olive oil was added to the WD to have a uniform level of fat energy in all WD groups. Preliminary studies established that the addition of olive oil had no effect on diet-induced fatty liver disease

in *Ldlr*$^{-/-}$ mice. For the WD + EPA, WD + DHA and WD + EPA + DHA diets, the C20-22 (n-3) PUFA consisted of 2% total calories. A detailed analysis and description of the diets was reported previously [197]. At the end of the 16 wk feeding period, all mice were fasted overnight (18:00 to 08:00 the next day) then euthanized (isoflurane anesthesia and exsanguination) at 08:00 for blood and liver harvesting as described [168].

4.3.2) RNA extraction and qRT-PCR

Total RNA was extracted from liver and target mRNA were quantified by qRT-PCR as previously described [205]. Primers for each target mRNA are listed in **(Table 4.1)**. Cyclophilin was used as the internal control for all target genes.

4.3.3) Urinary F2- and F3-isoprostanes

After 15 weeks of feeding, mice were placed in metabolic cages for 24 hour urine collections. Urine was stored at -80ºC until analyzed for F2- and F3-isoprostanes. Results were normalized to urinary creatinine as previously described [128,130,168].

4.3.4) Lipid extraction and analysis

Hepatic total lipid extracts were prepared as previously described [205]. Hepatic polar and neutral lipids were then separated from the total lipid extract by solid phase extraction using established methods [206]. Briefly, an Alltech aminopropyl disposable cartridge column was equilibrated with 2 column volumes of hexane. Total lipid extract in chloroform was loaded onto the column. Neutral lipids were washed off with chloroform-2-propanol (2:1). Non-esterified fatty acids were washed off with diethyl ether-2%acetic acid. Lastly, polar lipids were washed off with methanol. Thin-layer chromatography was used to confirm separation of lipid classes as previously described [197]. From the polar and neutral lipid fractions fatty acid methyl esters were prepared and quantified by gas chromatography [205].

4.3.5) Metabolomic analysis

The non-targeted global metabolomic analysis was carried out by Metabolon, Inc (Durham, NC) on ~100mg of liver tissue. The sample preparation process was carried out using the automated MicroLab STAR® system from Hamilton Company. Recovery standards were added prior to the first step in the extraction process for QC purposes. Sample preparation was conducted using a proprietary series of organic and aqueous extractions to remove the protein fraction while allowing maximum recovery of small molecules. The resulting extract was divided into two fractions; one for analysis by LC and one for analysis by GC. Samples were placed briefly on a TurboVap® (Zymark) to remove the organic solvent. Each sample was then frozen and dried under vacuum. Samples were then prepared for the appropriate instrument, either LC/MS or GC/MS.

The LC/MS portion of the platform was based on a Waters ACQUITY UPLC and a Thermo-Finnigan LTQ mass spectrometer, which consisted of an electrospray ionization (ESI) source and linear ion-trap (LIT) mass analyzer. The GC column was 5% phenyl and the temperature ramp is from 40° to 300° C in a 16 minute period. Samples were analyzed on a Thermo-Finnigan Trace DSQ fast-scanning single-quadrupole mass spectrometer using electron impact ionization. Identification of known chemical entities was based on comparison to metabolomic library entries of purified standards. A detailed description of this platform has been published previously [202].

Detailed bioinformatic analysis was carried out using MetaboAnalyst (http://www.metaboanalyst.ca). The data set for all metabolomic analysis used the original data normalized to protein abundance/sample. Principle component analysis was conducted to confirm samples in the individual dietary treatments clustered together

and there was separation between treatment groups **(Fig. 4.2)**. Volcano plots **(Table 4.2 & Fig. 4.3)** were used to identify the most significantly different metabolites between groups. BioVenn (venn diagrams) (http://www.cmbi.ru.nl/cdd/biovenn/) was used to identify and sort metabolites significantly impacted by specific treatment groups. MultiExperiment Viewer (heat map) (http://www.tm4.org) was used to represent the metabolomic data. Using these approaches, metabolites and pathways of interest were identified and analyzed statistically as described below.

4.3.6) Statistical analysis

For all analysis including metabolomic data, one-way ANOVA was used to detect significant differences between groups. Data were analyzed for homogenous variances by the Levine test. If unequal variances were detected, data were log-transformed. ANOVA analysis was performed on both transformed and untransformed data. A *P*-value < 0.05 was considered significantly different. Values are reported as mean ± SD.

4.4. Results

4.4.1) Development and progression of diet-induced NAFLD/NASH

Refer to our previous report for a comprehensive analysis of NAFLD/NASH in *Ldlr*$^{-/-}$ mice fed the WD for 16 wk without and with added C20-22 (n-3) PUFA [197]. Briefly, WD + OO feeding induced a severe NASH phenotype characterized by hepatosteatosis, hepatic damage (ALT/AST), inflammation (MCP1), oxidative stress (HO-1 & NOX2), and fibrosis (COL1A1). WD + C20-22 (n-3) PUFA feeding did not prevent hepatosteatosis but attenuated markers of hepatic damage, inflammation, oxidative stress, and fibrosis, establishing that a reduction of hepatosteatosis is not required for attenuation of markers of NASH. The attenuation of these markers of

disease progression was more robust with DHA versus EPA supplementation making DHA more attractive for combating NASH.

4.4.2) Overall hepatic metabolomic analysis

All 320 known metabolites sorted into 8 metabolic categories consisting of amino acid, carbohydrate, peptide, energy, lipid, nucleotide, vitamins and cofactors, and xenobiotics as identified by Metabolon were analyzed. Importantly, the lipid and amino acid pathways were most extensively analyzed consisting of 136 (43%) and 78 (24%) of the 320 metabolites, respectively. A representation of all metabolites expressed as fold-change relative to control (chow) mice is displayed as a heat map sorted by pathways in **Fig. 4.1A**. Over 50% of the metabolites in every pathway analyzed were affected by the treatments **(Fig. 4.1B)**. In all, the various treatment effects were wide-reaching impacting 238 (74%) of the 320 metabolites analyzed relative to control mice. Specifically, WD + OO feeding significantly altered 167 metabolites relative to control mice, 32 of which were unique to only WD + OO fed mice and not altered in WD + C20-22 (n-3) PUFA fed mice. Alternatively, WD + C20-22 (n-3) PUFA feeding impacted 206 metabolites relative to control mice with 71 metabolites exclusively impacted by WD + C20-22 (n-3) PUFA feeding. 135 of the metabolites affected were common to all treatments **(Fig. 4.1C)**. Principal component analysis illustrating segregation of the treatments is presented in **(Fig. 4.2)**.

4.4.3) Breadth of NAFLD/NASH development

The lipid and amino acid categories were the first and second most abundantly perturbed by WD + OO feeding, respectively. The top 3 sub-pathways impacted within lipid metabolism were long-chain fatty acids, lysophospholipids, and oxPUFA **(Fig. 4.3)**. A volcano plot was used to identify and rank the metabolites significantly altered in WD +

OO fed mice relative to controls **(Fig. 4.4A & Table 4.2)**. Ranked by *P*-value, palmitoyl-sphingomyelin (16:0-SML), of lipid metabolism was the metabolite most perturbed by WD + OO feeding. Other metabolites outside lipid metabolism that ranked in the top-ten perturbed by WD + OO feeding consisted of α-tocopherol (vitamin E), ascorbate (vitamin C), and 5-methyltetrahydro-folate (5-MeTHF) **(Table 4.2)**. 5-MeTHF is a component of one-carbon metabolism, other metabolites involved in one-carbon metabolism perturbed by WD + OO feeding consisted of dimethylglycine (DMG), homocysteine (HCY), and S-adenosyl homocysteine (SAH) **(Table 4.3)**. S-lactoylglutathione, an intermediate in the detoxification of MG was the amino acid metabolite most perturbed by WD + OO feeding relative to controls and was the 11th overall perturbed metabolite **(Table 4.2)**. All together, the metabolomic analysis revealed the wide-reaching impacts of WD + OO induced NASH on all major pathways of hepatic metabolism analyzed. The most dramatically impacted pathways and metabolites consisted of long-chain fatty acids, lysophospholipids, oxPUFA, 16:0-SML, anti-oxidants (vitamins E and C), one-carbon metabolism, and MG detoxification.

4.4.4) Scope of C20-22 (n-3) PUFA supplementation on NAFLD/NASH development

Out of 320 metabolites analyzed, 155 (48%) were significantly altered in WD + (n-3) PUFA fed mice relative to WD + OO fed mice, including all pathways except peptides. In general, the various (n-3) PUFA diets had similar impacts on hepatic metabolism with some changes unique to each diet. Of these 155 metabolites, 74 were common to all 3 (n-3) PUFA diets with 16, 18, and 25 exclusive to WD + EPA, WD + DHA, and WD + EPA + DHA fed mice, respectively **(Fig. 4.5)**.

To identify potential effects of (n-3) PUFA supplementation contributing to attenuated NASH, we identified metabolites that were significantly different in WD + OO

fed mice versus chow, but not different in WD + C20-22 (n-3) PUFA fed mice versus chow. 32 metabolites met these criteria **(Fig. 4.6)**. Within these 32 metabolites, lipid metabolism was most represented with glycerolipid metabolism being the most represented sub-pathway. These metabolites are intermediates in phospholipid synthesis, breakdown, or both, suggesting membrane turnover was attenuated by C20-22 (n-3) PUFA supplementation. Additionally, we probed volcano plots comparing the various C20-22 (n-3) PUFA containing diets versus WD + OO **(Fig. 4.4B - D)**. For all 3 comparisons, the top-ten significantly different metabolites included (n-3) and (n-6) long-chain PUFA, EPA derived oxidized fatty acids (18-hydroxyeicosapentaenoic acid (18-HEPE), 17,18-dihydroxyeicosatetraenoic acid (17,18-DiHETE)), vitamin E, and S-lactoylglutathione. Since these major targets of C20-22 (n-3) PUFA supplementation fell within the same major pathways perturbed by WD + OO induced NASH, we focused the metabolomic analysis on the following pathways and metabolites: phospholipid fatty acid remodeling and 16:0-SML, oxPUFA and anti-oxidants, one-carbon metabolism linked with glycerolipid metabolism, and MG detoxification.

4.4.5) Phospholipid fatty acid remodeling

Lysophospholipids analyzed consisted of lysophosphocholines (LPC), lysophosphoethanolamines (LPE), and lysophosphoinositols (LPI) **(Table 4.3)**. WD feeding, regardless of (n-3) PUFA supplementation, significantly ($P < 0.05$) elevated most 18:1(n-9) containing lysophospholipids. A few 18:1(n-9)-LPCs were only trending ($0.05 < P < 0.10$) upward in WD fed mice. 1-20:4(n-6)-LPC was less abundant than 2-20:4(n-6)-LPC across all groups. There was a trend ($0.05 < P < 0.10$) for elevated 1-20:4(n-6)-LPC in WD + OO fed mice relative to controls. Only DHA containing diets (WD + DHA and WD + EPA + DHA) reduced 1- and 2-20:4(n-6)-LPC accumulation (~84% (*P*

< 0.05)) relative to WD + OO fed mice. 1-20:4(n-6)-LPE changed similarly but was attenuated by all C20-22 (n-3) PUFA diets. 1-20:4(n-6)-LPI was more abundant than 2-20:4(n-6)-LPI across all groups. WD + OO feeding elevated 1-20:4(n-6)-LPI by 140% (P < 0.05) compared to control mice. WD + C20-22 (n-3) PUFA feeding attenuated 1-20:4(n-6)-LPI by at least 46% (P < 0.05) relative to WD + OO fed mice. There were no changes in 2-20:4(n-6)-LPI. Accumulation of 2-22:6(n-3)-LPC was greater than 1-22:6(n-3)-LPC in all groups. 1- and 2-22:6(n-3)-LPC was elevated 140% and 80% (P < 0.05), respectively, only by WD + DHA feeding relative to control mice. 2-22:6(n-3)-LPE was elevated 100% (P < 0.05) only by WD + DHA feeding. In all, only WD + DHA feeding had the capacity to elevate DHA containing lysophospholipids. Combined, these results are indicative of significant phospholipid fatty acid remodeling in WD fed mice.

To further investigate changes in lysophospholipids we analyzed expression of genes involved in remodeling phospholipid fatty acids (Land's Cycle) **(Table 4.4)** [207,208]. Phospholipases (PLA) hydrolyze phospholipids releasing fatty acids and the associated lysophospholipid. PLA2g6 was elevated (P < 0.05) in all WD fed mice relative to control mice. iPLA2g expression did not change. Lysophosphotidylcholine acyltransferases (LPCAT) reacylate lysophospholipids, re-forming phospholipids and thus contributing to phospholipid fatty acid remodeling. WD + OO feeding elevated LPCAT1-4 relative to control mice, LPCAT2 was most responsive with a 300% (P < 0.05) increase. WD + DHA feeding attenuated the LPCAT response most robustly with a 38% (P < 0.05) decrease in LPCAT2 expression relative to WD + OO fed mice **(Table 4.4)**. Elevated expression of genes regulating the Land's cycle in WD fed mice are likely contributing to phospholipid fatty acid remodeling.

To confirm the evidence of phospholipid fatty acid remodeling, we analyzed the fatty acid profile in hepatic polar and neutral lipid fractions **(Table 4.5)**. Separation of lipid fractions was confirmed by thin-layer chromatography **(Fig. 4.7)**. On a mole percent basis, long-chain PUFA accumulation was greater in the polar versus neutral lipid fraction. Specifically, WD + OO feeding elevated ARA by 44% ($P < 0.05$) relative to control mice in the polar fraction. DHA containing diets most effectively reduced ARA in the polar fraction by up to 85% ($P < 0.05$) compared to WD + OO fed mice. ARA in the neutral lipid fraction was depressed ~76% ($P < 0.05$) in all WD fed mice regardless of (n-3) PUFA content. EPA was not detected in the polar or neutral lipid fractions in WD + OO fed mice. The (n-3) PUFA diets equally elevated ($P < 0.05$) EPA in the polar lipid fraction whereas WD + EPA feeding most effectively elevated EPA in the neutral lipid fraction by 100% ($P < 0.05$) compared to control mice. DHA accumulation in the polar fraction was not impacted in WD + OO fed mice versus controls. However, WD + DHA feeding most robustly elevated DHA in the polar fraction by 90% ($P < 0.05$) compared to WD + OO fed mice. In the neutral lipid fraction, DHA was suppressed 95% ($P < 0.05$) by WD + OO feeding. WD + DHA feeding most effectively reversed this suppression by elevating DHA 5550% ($P < 0.05$) in the neutral lipid fraction compared to WD + OO fed mice. These dramatic changes in PUFA accumulation in the polar lipid fraction parallel changes observed in lysophospholipids and confirm the evidence of phospholipid fatty acid remodeling in (n-3) PUFA supplemented mice.

Sphingomyelin (SML) is an abundant sphingolipid in mammalian cells and an important component of plasma membranes and lipid raft formation [209]. As such, SML abundance can dramatically impact membrane dynamics. Since 16:0-SML was the metabolite most significantly altered by WD + OO feeding **(Table 4.2)**, it was further

investigated. 16:0-SML was elevated 216% ($P < 0.05$) in WD + OO fed mice relative to controls **(Table 4.3)**. All (n-3) PUFA supplemented diets attenuated WD-induced 16:0-SML accumulation by ~38% ($P < 0.05$). The key enzymes regulating sphingomyelin synthesis are serine palmitoyltransferase (SPTLC) and sphingomyelin synthase (SGMS) [210,211]. SPTLC1 was elevated 49% ($P < 0.05$) in WD + OO fed mice relative to controls **(Table 4.4)**. Only addition of DHA to the WD completely prevented this induction. SPTLC2 was elevated at least 69% ($P < 0.05$) in all WD fed mice relative to controls with no significant impact from C20-22 (n-3) PUFA supplementation. SGMS1 was elevated 56% ($P < 0.05$) in WD + OO fed mice compared to controls. Again, only addition of DHA to the WD completely prevented this induction **(Table 4.4)**. SGMS2 was not impacted by any of the treatments. Overall, while WD + DHA feeding most robustly attenuated the expression of enzymes regulating 16:0-SML synthesis, all (n-3) PUFA containing diets effectively attenuated WD-induced accumulation of 16:0-SML.

4.4.6) Oxidized PUFA, anti-oxidants, and lipid peroxidation

Phospholipid fatty acid remodeling can potentially alter fatty acid availability for production of eicosanoids and other oxPUFA. Supporting this, multiple oxPUFA were among the top metabolites altered in C20-22 (n-3) PUFA fed mice compared to WD + OO fed mice **(Fig. 4.4B – D)**. As such, we investigated the array of oxPUFA from the metabolomics analysis in detail **(Fig. 4.8)**. WD + OO feeding elevated 6-keto-prostaglandin F1α (6-keto-PGF1α) by 243% ($P < 0.05$) relative to all other groups. Thus, inclusion of (n-3) PUFA in the WD completely prevented induction of 6-keto-PGF1α. 12-Hydroxyeicosatetraenoic acid (12-HETE) was elevated about 2-fold in WD + OO fed mice compared to controls, but this was not a statistically significant change ($P = 0.11$). Conversely, WD + C20-22 (n-3) PUFA depleted ($P < 0.05$) 12-HETE accumulation. WD

+ DHA feeding was most effective with an 86% reduction ($P < 0.05$) in 12-HETE relative to WD + OO fed mice. Both 17,18-DiHETE and 18-HEPE were depressed by 74% and 93% ($P < 0.05$) respectively, in WD + OO fed mice compared to control mice. All (n-3) PUFA supplemented diets elevated 17,18-DiHETE and 18-HEPE, but WD + EPA was most effective with 1219% and 5514% inductions ($P < 0.05$), respectively, relative to WD + OO feeding.

We also assessed expression of genes involved in the synthesis of several of these oxPUFA **(Table 4.4)**. COX-1 and COX-2 were elevated 150% and 1370% respectively, by WD + OO feeding relative to control mice. WD + DHA most effectively attenuated COX-1 and COX-2 by 52% and 70% respectively, compared to WD + OO fed mice. These changes in COX expression likely contribute to changes in 6-keto-PGF1α accumulation. The expression of other genes analyzed were either not impacted or did not change in parallel with the oxPUFA and thus do not appear to be driving the altered oxPUFA profile **(Table 4.4)**. We conducted a correlation analysis of oxPUFA with their corresponding precursor fatty acid abundance **(Table 4.6)**. For all oxPUFA analyzed except 9,10-DHOME, precursor fatty acid abundance in the polar lipid fraction most significantly correlated with oxPUFA abundance. In all, these results suggest the oxPUFA profile is being driven more by mass action of precursor fatty acid abundance versus altered gene expression.

To complement the oxPUFA analysis we investigated hepatic vitamins E and C. When normalized to hepatic TAG content, WD + OO feeding did not impact hepatic vitamin E accumulation relative to control mice **(Fig. 4.9A)**. WD + (n-3) PUFA feeding reduced hepatic vitamin E content by ~68% ($P < 0.05$) relative to WD + OO fed mice.

Hepatic vitamin C content correlated (R^2 = 0.84; P < 0.05) with hepatic vitamin E content. The F2- and F3-isoprostanes are non-enzymatic oxidation products of ARA and EPA, respectively. We analyzed urinary F2- and F3-isoprostanes to assess whole body lipid peroxidation **(Fig. 4.9B)**. WD + OO feeding had no impact on F2- or F3-isoprostanes relative to control mice. WD + (n-3) PUFA elevated urinary F2- and F3-isoprostanes by ~275% and ~488% (P < 0.05) relative to control mice, respectively. This suggests (n-3) PUFA supplementation elevated whole body lipid peroxidation, likely accounting for reduced hepatic vitamin E and C in (n-3) PUFA fed mice.

4.4.7) One-carbon and glycerolipid metabolism

WD + OO feeding suppressed HCY and SAH by 64% and 47% (P < 0.05) relative to controls, respectively. Alternatively, the [DMG] / [betaine] ratio and 5-MeTHF were induced in WD + OO fed mice by 101% and 183% (P < 0.05), respectively, relative to controls. All these WD + OO induced changes were prevented or attenuated with C20-22 (n-3) PUFA supplementation **(Table 4.3)**. The glycerolipid metabolites choline phosphate, glycerol, phosphoethanolamine, and glycerophospholrylcholine were elevated 32%, 57%, 143%, and 76% (P < 0.05), respectively, in WD + OO fed mice relative to controls. C20-22 (n-3) PUFA supplementation completely prevented these WD + OO induced perturbations **(Fig. 4.6B – D)**. One-carbon metabolism and phosphatidylcholine (PC) metabolism are linked (see discussion). Accordingly, these results suggest C20-22 (n-3) PUFA prevented the WD-induced perturbation of these pathways.

4.4.8) MG detoxification

S-lactoylglutathione (MG detoxification) was depressed 83% (P < 0.05) in WD + OO fed mice relative to controls. C20-22 (n-3) PUFA supplementation elevated S-

lactoylglutathione by at least 441% ($P < 0.05$) compared to WD + OO fed mice **(Table 4.3)**. This outcome suggests the addition of C20-22 (n-3) PUFA to the WD helped maintain proper MG detoxification.

4.5. Discussion

The overall goal of this study was to identify potential mechanisms of C20-22 (n-3) PUFA supplementation contributing to attenuation of NASH progression in WD fed *Ldlr*$^{-/-}$ mice. We previously established that supplementing the WD with EPA and or DHA for 16 weeks at a physiological dose (2% calories) attenuates progression of NASH without preventing hepatosteatosis [197]. In comparing EPA versus DHA supplementation, we concluded that the capacity of DHA to suppress WD-induced hepatic markers of inflammation (Clec4f, F4/80, Tlr4), fibrosis (Procol1α1, Tgfβ1), and oxidative stress (NADPH oxidase subunits Nox2, p22phox, p40phox, p47phox, and p67phox) was significantly greater than EPA [197]. To advance our understanding of both the progression of WD-induced NASH and the impact from C20-22 (n-3) PUFA supplementation, we conducted a global untargeted metabolomics analysis on livers from *Ldlr*$^{-/-}$ mice fed the WD for 16 weeks with and without EPA and DHA supplementation.

Overall, the metabolomic analysis revealed wide-reaching impacts on hepatic metabolism resulting from the development of WD-induced NASH. Every major group of metabolite analyzed consisting of lipid, amino acid, carbohydrate, vitamins and cofactors, nucleotide, peptide, energy, and xenobiotics was perturbed in WD + OO fed mice **(Fig. 4.1)**. This analysis represents the vast complexity and array of metabolic derangements associated with development of advanced NAFLD/NASH. This

complexity is one of the major contributing factors to our incomplete understanding of NASH progression and limited success in developing treatments specific to NAFLD. Surprisingly, supplementation with C20-22 (n-3) PUFA prevented or reversed many of these WD-induced perturbations in every category of metabolite analyzed except xenobiotics **(Fig. 4.1A)**. The impacts of C20-22 (n-3) PUFA on hepatic fatty acid metabolism are well established [91,92]. As such, the large impact on hepatic lipid metabolites from EPA and DHA supplementation was anticipated, however the range of impacts on metabolites in the other categories analyzed were unanticipated. Demonstration of these wide-reaching effects, combined with our previous work [168,197], further establish the C20-22 (n-3) PUFA, and especially DHA as potential therapeutic agents to combat NAFLD/NASH in obese humans.

S-lactoylglutathione, an intermediate produced by detoxification of MG, was dramatically reduced (~7-fold) in WD + OO fed mice relative to chow, implicating defective MG detoxification in NASH [212]. WD + EPA feeding normalized S-lactoylglutathione while both DHA containing diets (WD + DHA and WD + EPA + DHA) elevated S-lactoylglutathione above levels in control mice suggesting restored MG detoxification with C20-22 (n-3) PUFA supplementation **(Table 4.3)**. MG is a highly reactive precursor to advanced glycation end products implicated in the pathophysiology of diabetes and known to induce cell growth arrest, mitochondrial dysfunction, and apoptosis [213,214]. Hyperglycemia and increased glycolytic flux, such as from a fructose bolus, are known to elevate MG formation [214]. Thus, accumulation of MG has been proposed to mediate fructose induced hepatic stress signaling, providing another potential link between fructose consumption and progression of NASH [49,215]. With 26.6% calories as sucrose, the WD has relatively high fructose content. We speculate

that impaired MG detoxification induced by WD feeding is contributing to progression of NASH. Preserved MG detoxification by C20-22 (n-3) PUFA supplementation is a potential mechanism of NASH attenuation.

Several metabolites involved in one-carbon metabolism were perturbed by WD + OO feeding **(Table 4.3)**. One-carbon metabolism and specifically the methionine cycle is the primary pathway for generation of S-adenosylmethionine (SAM), a critical methyl donor for transmethylation reactions. Disrupted one-carbon metabolism is associated with fatty liver, liver injury, and hepatocellular carcinoma [216]. SAM was not included in the metabolomic analysis, but its precursor methionine remained constant across all groups. In line with previous reports, depressed HCY and elevated [DMG] / [betaine], as in WD + OO fed mice, is indicative of enhanced HCY remethylation by betaine-homocysteine methyltransferase to maintain sufficient methionine levels [217,218]. The methyl group in this pathway is derived from choline, likely through breakdown of PC, as previously identified in high-fat diet fed mice [217,218]. Supporting this concept, glycerophosphorylcholine and glycerol, intermediates of PC breakdown were elevated in WD + OO fed mice compared to controls. Phosphoethanolamine, also an intermediate of phospholipid metabolism, was elevated in WD + OO fed mice. Phosphoethanolamine is a precursor to phosphatidylethanolamine (PE), which can be converted to PC via methylation by phosphatidylethanolamine *N*-methyltransferase using SAM as the methyl donor, linking PC synthesis and one-carbon metabolism [216,219,220]. Given the elevated phosphoethanolamine in WD + OO fed mice, it is possible PE synthesis was elevated to replenish and maintain sufficient PC levels. Since choline is required for sphingomyelin synthesis, it is possible that elevated sphingomyelin synthesis in WD + OO fed mice is depleting the choline pool used for methyl donation in one-carbon

metabolism cycles. Thus, we speculate elevated sphingomyelin synthesis in WD + OO fed mice is linked to disrupted homeostasis of one-carbon metabolism and is likely contributing to development of NASH. This outcome is similar to others and in line with the known hepatic toxicity of methionine-choline deficient diets [73,217,218]. WD supplementation with C20-22 (n-3) PUFA prevented or attenuated sphingomyelin synthesis and all the WD-induced abnormalities associated with one-carbon and phospholipid metabolism. The capacity of C20-22 (n-3) PUFA to maintain proper one-carbon and phospholipid metabolism is one of many potential mechanisms contributing to their protective effects against NASH. Future studies specifically focusing on the impacts of WD feeding on hepatic one-carbon metabolism are required to better define cause and affect mechanisms and the implications of sphingomyelin synthesis.

Cell membranes mainly consist of glycerophospholipids, sphingolipids, and cholesterol. Glycerophospholipids are formed *de novo* by the Kennedy pathway and then undergo acyl chain remodeling via the Land's cycle [221,222]. The dramatic changes in lysophospholipids in all WD fed mice are indicative of membrane remodeling via the Land's cycle **(Table 4.3)**. Elevated 18:1(n-9) containing lysophospholipids are likely the result of elevated total hepatic 18:1(n-9) associated with hepatosteatosis as previously reported [168,197]. Overall, inclusion of DHA in the WD most effectively reduced ARA and elevated DHA containing lysophospholipids. Interestingly, 1-22:6(n-3)-LPC has been reported to have anti-inflammatory properties in RAW 264.7 cells and mice [223]. 1-22:6(n-3)-LPC was only elevated in the WD + DHA fed mice, this could be one contributing factor to the superior hepatic anti-inflammatory properties of DHA versus EPA supplementation in WD-induced NASH previously reported [197]. To confirm the changes in lysophospholipids paralleled changes in membrane lipids, we

analyzed the fatty acid profile in the hepatic polar lipid fraction **(Table 4.5)**. In the polar lipid fraction, DHA containing diets most robustly suppressed ARA accumulation while also elevating both EPA and DHA accumulation. LPCAT2 has a high affinity for ARA and its expression is up-regulated in inflammatory cells [224]. DHA supplementation most effectively attenuated WD-induction of LPCAT2 expression, likely contributing to reduced membrane ARA content in WD + DHA fed mice. Importantly, WD + EPA feeding did not elevate DHA in the polar lipid fraction. Membrane fatty acid composition can critically impact membrane dynamics and function [101]. The double bonds of PUFA are highly flexible allowing for altered conformations and interactions with other membrane components, including sphingomyelin and cholesterol, and can ultimately alter membrane domains [103]. The lipid raft is one such domain, highly enriched in cholesterol and sphingolipids, and considered critical for the optimal function of many cell signaling proteins [225]. With 6 double bonds, DHA is the most unsaturated PUFA incorporated into membranes and thus can highly alter the composition and function of lipid rafts. Recently, it was proposed that DHA is more than twice as likely as EPA to be incorporated into and disrupt membrane raft organization [226]. This aspect of DHA is hypothesized to be one of the driving factors of the wide-reaching health benefits associated with dietary C20-22 (n-3) PUFA [103,226]. Toll-like receptor 4 (TLR4) signaling is dependent on receptor dimerization in lipid rafts and is implicated in development of hepatic inflammation and fibrosis associated with NASH [227,228]. We previously reported that WD + DHA feeding has more robust anti-inflammatory and anti-fibrotic effects than WD + EPA feeding [197]. We propose the superior ability of dietary DHA to attenuate NASH progression is, in part due to the enrichment of hepatic

membrane DHA content derived from DHA consumption. This concept is supported by recent findings suggesting DHA specifically inhibits TLR4 signaling [106].

Another important component of membranes, 16:0-SML was elevated in WD + OO fed mice with NASH and attenuated with dietary C20-22 (n-3) PUFA **(Table 4.3)**. WD feeding elevated expression of the enzymes regulating SML synthesis **(Table 4.4)**. However, C20-22(n-3) PUFA regulation of 16:0-SML abundance appears to involve other mechanisms yet to be investigated. SML and its precursor ceramide (CER) have been implicated in development of atherosclerosis, insulin resistance, diabetes, and obesity and are known to be elevated in the liver upon high-fat diet feeding [229,230]. Disrupted SML synthesis inhibits excess hepatic triglyceride accumulation in mice [231]. In line with this, all WD fed mice in our study had hepatosteatosis and elevated 16:0-SML accumulation compared to controls. CER are known second messengers associated with insulin resistance, oxidative stress, inflammation, and apoptosis, and therefore have been implicated in development of NASH [28]. While we did not analyze CER, CER is the direct precursor to SML and thus CER levels likely parallel the attenuated SML with C20-22 (n-3) PUFA supplementation. While more work is needed in this area, depressed SML is a potential mechanism of action of the C20-22 (n-3) PUFA.

Non-esterified fatty acids released from the sn-2 position of phospholipids are substrates for cyclooxygenases (COX), lipooxygenases, and cytochrome P450 monooxygenases [101]. Since our treatments induced large alterations in the polar fatty acid profile (phospholipids), we further investigated the oxPUFA from the metabolomic data **(Fig. 4.8)**. In general, dietary C20-22 (n-3) PUFA depressed accumulation of (n-6)

derived oxPUFA and dramatically elevated accumulation of (n-3) derived oxPUFA. These changes are in part explained by depressed COX2 expression but are mainly driven by mass action of the precursor fatty acid abundance in the polar lipid fraction **(Table 4.6)**. Most often, (n-3) derived oxPUFA promote resolution of inflammation [232]. 18-HEPE is a precursor, and pathway marker for the formation of the resolvin-E1 series with well established anti-inflammatory properties [232,233]. The physiological function of 17,18-DiHETE in the liver has not been assessed but its precursor 17,18-epoxyeicosatetraenoic acid (17,18-EpETE), and the DHA derived 19,20-epoxydocosapentaenoic acid (19,20-EpDPE) have known benefits in cardiovascular studies [234]. Preliminary data from our lab indicate 17,18-EpETE and 19,20-EpDPE were elevated only in C20-22 (n-3) PUFA supplemented mice (not shown). These epoxy fatty acids may provide physiological benefit against NASH progression. Overall, the ability of C20-22 (n-3) PUFA supplementation to suppress (n-6) derived oxPUFA and dramatically elevate anti-inflammatory (n-3) derived oxPUFA is a key mechanism in the attenuation of inflammation and likely fibrosis associated with NASH.

Lastly, we assessed the anti-oxidants vitamin E and C **(Fig. 4.9A)**. Vitamin E has been suggested to have benefit against NAFLD in humans [154]. Addition of C20-22 (n-3) PUFA to the WD caused a significant decline in hepatic vitamin E content normalized to hepatic triglycerides. Dietary vitamin E was assessed to confirm changes in hepatic vitamin E were not due to altered vitamin E consumption (not shown). Hepatic vitamin C was directly correlated to hepatic vitamin E content. This was accompanied by elevated urinary F2- and F3-isoprostanes **(Fig. 4.9B)**. As such, we hypothesize that supplementation with C20-22(n-3) PUFA elevated lipid peroxidation and therefore caused the decline in hepatic vitamin E and C. This phenomenon was not associated

with progression of NASH in this model. However, since the physiological function of the F3-isoprostanes is largely unknown, more research is required to determine if they have hepato-protective effects. Additionally, more work is needed to assess the relationship between hepatic vitamin E status and NASH, and if there is potential benefit in restoring hepatic vitamin E to control levels in C20-22 (n-3) PUFA supplemented mice.

In conclusion, we previously established that DHA supplementation is more effective than EPA supplementation for attenuating WD-induced hepatic inflammation, oxidative stress, and fibrosis associated with NASH [197]. Here we used a metabolomics approach to expand those findings demonstrating that WD-induced NASH impacted all major pathways of hepatic metabolism analyzed consisting of lipid, amino acid, carbohydrate, vitamins and cofactors, nucleotide, peptide, energy, and xenobiotics. Furthermore, supplementation with C20-22 (n-3) PUFA prevented or attenuated many of the WD-induced perturbations in all categories of metabolites analyzed except xenobiotics. Thus, the impacts on hepatic metabolism from C20-22 (n-3) PUFA supplementation are very wide-reaching. We identified several potential mechanisms of protection from C20-22 (n-3) PUFA including MG detoxification, one-carbon and phospholipid metabolism, membrane fatty acid remodeling, SML metabolism, and an altered oxPUFA profile. A key difference between EPA and DHA supplementation is the ability of dietary DHA to suppress ARA and elevate DHA accumulation in polar lipids. This is likely a critical difference contributing to the superior ability of DHA to attenuate NASH progression. Ultimately, this report further confirms and defines the potential benefit of EPA and DHA supplementation to combat diet-induced NASH. Even though C20-22 (n-3) did not prevent WD-induced hepatosteatosis, their benefit on overall hepatic metabolism was wide-reaching. As such, C20-22 (n-3) PUFA supplementation

alone or in combination with other therapy is likely to provide benefit to obese humans with NAFLD/NASH.

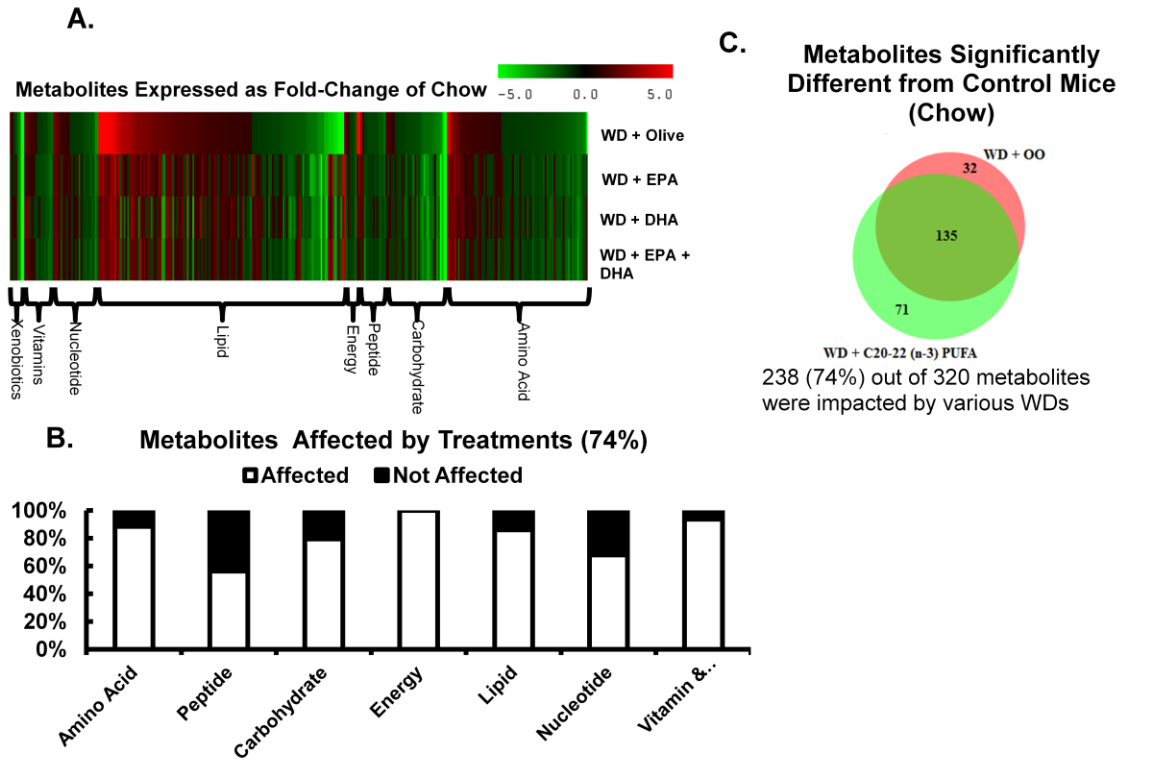

FIGURE 4.1. Impact from all dietary treatments on the 320 metabolites assessed in the metabolomics analysis. A) Heat map representing fold-change for each metabolite relative to control mice (chow), sorted by category and fold-change in the WD + OO group. **B)** Total percent of metabolites significantly impacted in each category by all treatments combined. **C)** Venn-diagram representing all metabolites significantly different (increased and decreased) in the WD + OO and WD + C20-22 (n-3) PUFA diets relative to control mice (chow).

Table 4.1. Mouse Primer Pairs for qRT-PCR

Target	Accession #	Forward	Reverse
Cyclophilin	NM_008907	CTTCTTGCTGGTCTTGCCATTCCT	GGATGGCAAGCATGTGGTCTTTG
Lands Cycle			
PLA2g6	NM_001199025.1	AAACCCAAGGTGATGCTGAC	CGGTGGCTTCAGGTTAATGT
iPLA2	NM_026164.2	CAGCTGCTCCAGGCTACTTT	CTCTCATACCGTCCTGTGCC
LPCAT1	NM_145376.5	CACGAGCTGCGACTGAGC	ATGAAAGCAGCGAACAGGAG
LPCAT2	NM_173014.1	ACCTGTTTCCGATGTCCTGA	CCAGGCCGATCACATACTCT
LPCAT3	NM_145130.2	AGCCTTAACAAGTTGGCGAC	ATGCCGGTAAAACAGAGCC
LPCAT4	NM_207206.2	GAGTTACACCTCTCCGGCCT	GGCCAGAGGAGAAAGAGGAC
Oxidation of FAs			
COX1	NM_008969.3	GCTTGAAGCCTTACACCTCT	TTCTTCAGTGAGGCTGTGTT
COX2	NM_011198.3	CATATTTGATTGACAGTCCACC	GCTCCTTATTTCCCTTCACAC
5-LOX	NM_009662.2	GCGGGCTGTAGCGAGAAGCA	CTCGCCCTCGCCTGTGATCC
12-LOX	NM_007440.4	CGCCCACCAGCAAGGACGAC	GCGACCCAGGTGCCATGTGA
15-LOX	NM_009660.3	CCCCGGAGACCAGGGATCGG	TCGCCGCCACGTTCAGGATT
CYP2C29	NM_007815.3	TCAGGTCTTTATCCACATCCCTCCC	TGCTGGGTCTTGAGAGAAGAGGGT
CYP2C37	NM_010001.2	ACACGAGGCGTTTCTCACTC	AGGGCTGCTCAGAATCTTTGT
CYP2C38	NM_010002.3	CACTATGGAGACAGAGGTCTA	CCAAATACAGAGTGAAAACG
CYP2C40	NM_010004.2	CAAAGATGCCCAAACGCAA	CAGAGTGAACACAGGGCCATA
CYP2C44	NM_001001446.3	TACGCTTGCTCTCCTGGTTT	GGTCCTCTCCCAAGGAACTC
CYP2J5	NM_010007.4	GAAATGCAAAAGGACCCTGA	GGACTCTCGGTCAGACAAGC
EPHX1	NM_010145.2	CCCCAAGACCCACGGCCTGA	CCTCGCAGTGGCCACCGAAT
EPHX2	NM_007940.4	GCATTGTCACCAACAACTGG	CTGTGTTGTGGACCAGGATG
SML Synthesis			
SPTLC1	NM_009269.2	CCTCCAACCCACAACATCGT	TGCCAGGCGCTCTTCTAAAT
SPTLC2	NM_011479.3	ATTGGCGCCTTTGGAAGAGA	GAGCACCAGAAAGGCTCAGT
SGMS1	NM_001168525.1	GAAATGAGGCGAACGAATGT	CACCTTCTTGGGTGACCAGT
SGMS2	NM_028943.5	ACACGGCTGTTTTGGTGGTA	CACCAAGCCCGAGACAAGAA

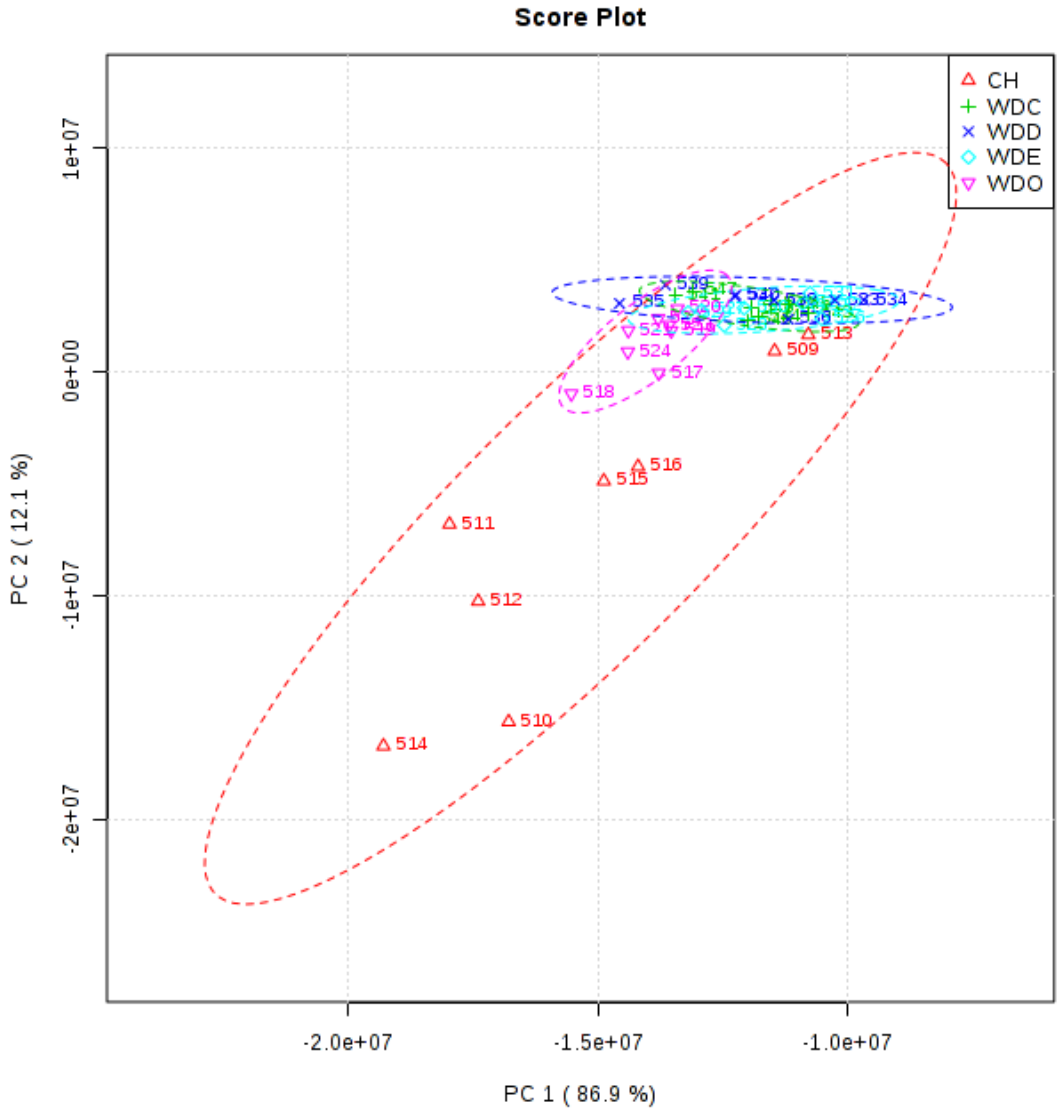

FIGURE 4.2. Principle component analysis. Principle component analysis was conducted using MetaboAnalyst (http://www.metaboanalyst.ca), data for this analysis included all 320 metabolites and used the original metabolomic data normalized to protein abundance as described in the methods section. Principle component 1 on the "x" axis explains 86.9% of the variability in the samples and principle component 2 on the "y" axis explains 12.1% of the variability in the samples. All WD fed mice are clustered similarly but there is clear visual separation of WD + OO and WD + C20-22 (n-3) PUFA fed mice, confirming broad impacts from dietary C20-22 (n-3) PUFA supplementation.

Table 4.2. Metabolites significantly different in WD + OO fed mice versus controls, ranked by *P*-value, as represented in Fig. 4.4A.

Metabolite	Fold-Change (FC)	log2(FC)	p.value	Rank
palmitoyl sphingomyelin	4.2829	2.0986	1.77E-09	1
alpha-tocopherol	7.2246	2.8529	7.75E-08	2
arachidonate (20:4n6)	3.3754	1.755	1.42E-06	3
methyl palmitate (15 or 2)	6.254	2.6448	1.42E-06	4
18-HEPE	0.092544	-3.4337	1.44E-06	5
squalene	2.7676	1.4687	1.74E-06	6
ascorbate (Vitamin C)	6.8833	2.7831	1.78E-06	7
palmitoleate (16:1n7)	3.7563	1.9093	3.31E-06	8
5-methyltetrahydrofolate (5MeTHF)	4.2131	2.0749	5.83E-06	9
succinylcarnitine	3.393	1.7626	7.98E-06	10
S-lactoylglutathione	0.17153	-2.5434	1.09E-05	11
docosapentaenoate (n3 DPA; 22:5n3)	0.29355	-1.7683	1.17E-05	12
N1-methyladenosine	2.2145	1.147	1.18E-05	13
myristate (14:0)	7.169	2.8418	1.45E-05	14
9,10-hydroxyoctadec-12(Z)-enoic acid	0.20828	-2.2634	1.92E-05	15
adenine	3.0883	1.6268	2.06E-05	16
adrenate (22:4n6)	2.2006	1.1379	2.40E-05	17
xanthosine	0.48692	-1.0383	3.00E-05	18
docosatrienoate (22:3n3)	703.71	9.4588	3.67E-05	19
13-HODE + 9-HODE	0.38815	-1.3653	3.70E-05	20
10-nonadecenoate (19:1n9)	10.53	3.3964	4.92E-05	21
5-methylthioadenosine (MTA)	2.8317	1.5017	5.49E-05	22
17-methylstearate	9.326	3.2213	6.16E-05	23
dihydrocholesterol	2.1636	1.1135	6.74E-05	24
equol sulfate	0.057539	-4.1193	6.80E-05	25
dimethylglycine	3.6371	1.8628	7.47E-05	26
gamma-glutamylleucine	2.7776	1.4739	8.02E-05	27
p-cresol sulfate	6.3195	2.6598	8.83E-05	28
oleoyltaurine	6.4774	2.6954	0.00012051	29
eicosenoate (20:1n9 or 11)	8.1603	3.0286	0.0001424	30
cysteine-glutathione disulfide	3.8586	1.9481	0.00015413	31
17,18-dihydroxy-5Z,8Z,11Z,14Z-eicosatetraenoate	0.22938	-2.1242	0.00015852	32
7-alpha-hydroxycholesterol	0.28161	-1.8282	0.00018182	33
dihomo-linoleate (20:2n6)	4.8317	2.2725	0.0001888	34
chenodeoxycholate	3.3469	1.7428	0.00019882	35
13-octadecenoate (18:1n5)	10.426	3.3821	0.00023709	36
phosphoethanolamine	3.3338	1.7372	0.00027943	37
cis-vaccenate (18:1n7)	10.445	3.3848	0.00028743	38
eicosapentaenoate (EPA; 20:5n3)	0.33508	-1.5774	0.0002927	39
2-oleoylglycerophosphoethanolamine*	3.0625	1.6147	0.00035139	40
laurate (12:0)	2.1046	1.0736	0.00040346	41
gamma-glutamylisoleucine*	2.5685	1.3609	0.00043259	42
S-methylglutathione	2.0734	1.052	0.00057558	43
1-oleoylglycerophosphoethanolamine	2.8432	1.5075	0.00071175	44
inosine	0.45322	-1.1417	0.00081215	45
docosadienoate (22:2n6)	5.1494	2.3644	0.00099236	46
stachydrine	0.0156	-6.0023	0.0011255	47
myristoleate (14:1n5)	3.8517	1.9455	0.0011778	48
pentadecanoate (15:0)	3.5463	1.8263	0.0012624	49
dihomo-linolenate (20:3n3 or n6)	3.0585	1.6128	0.0013938	50
2-linoleoylglycerophosphoethanolamine*	0.32311	-1.6299	0.0017849	51
2'-deoxyguanosine	2.0802	1.0567	0.0018641	52
sphinganine	5.865	2.5521	0.0021593	53
6-keto prostaglandin F1alpha	32.07	5.0031	0.0024431	54
margarate (17:0)	2.0661	1.0469	0.0025807	55
oleate (18:1n9)	6.5884	2.7199	0.0028047	56
12-dehydrocholate	0.088323	-3.5011	0.0029954	57
guanosine	3.2981	1.7216	0.0033264	58

Table 4.2 Continued

1-palmitoylplasmenylethanolamine*	7.5202	2.9108	0.0033998	59
maltose	0.48453	-1.0453	0.0034186	60
pyruvate	0.4469	-1.162	0.0050461	61
gamma-glutamylvaline	2.0554	1.0394	0.005303	62
1-arachidonoylglycerophosphoethanolamine*	2.1413	1.0985	0.005932	63
1-oleoylglycerophosphoinositol*	3.4701	1.795	0.0063064	64
glucosamine	0.48555	-1.0423	0.0077682	65
stearidonate (18:4n3)	0.23135	-2.1118	0.0078105	66
linolenate [alpha or gamma; (18:3n3 or 6)]	0.49805	-1.0057	0.0081469	67
maltotriose	0.18987	-2.3969	0.0085355	68
propionylcarnitine	0.46787	-1.0958	0.009335	69
sphingosine	7.5769	2.9216	0.010146	70
6-beta-hydroxylithocholate	2.3609	1.2393	0.010745	71
beta-muricholate	2.2944	1.1981	0.011431	72
glycerol	2.1852	1.1278	0.01155	73
1-arachidonoylglycerophosphoinositol*	3.3301	1.7356	0.011681	74
taurochenodeoxycholate	2.5985	1.3777	0.012078	75
maltotetraose	0.13083	-2.9342	0.01495	76
maltopentaose	0.054211	-4.2053	0.016119	77
glycerophosphorylcholine (GPC)	2.4769	1.3085	0.017763	78
campesterol	0.00099251	-9.9766	0.018322	79
2-oleoylglycerophosphocholine*	4.1772	2.0625	0.020474	80
aspartylleucine	2.4208	1.2755	0.02211	81
1-myristoylglycerophosphocholine	2.6535	1.4079	0.023167	82
1-arachidonoylglycerophosphocholine*	4.3743	2.1291	0.02524	83
1-linoleoylglycerophosphoinositol*	0.49936	-1.0018	0.027116	84
1-oleoylglycerophosphocholine	3.1232	1.643	0.030184	85
1-eicosatrienoylglycerophosphocholine*	3.223	1.6884	0.038191	86
maltohexaose	0.11134	-3.167	0.038929	87
homocysteine	0.41611	-1.265	0.03904	88
tagatose	2.0565	1.0402	0.039954	89
12-HETE	2.7137	1.4402	0.040529	90
tauroursodeoxycholate	2.3678	1.2435	0.043967	91
1-pentadecanoylglycerophosphocholine*	2.8612	1.5166	0.047812	92
2-palmitoleoylglycerophosphocholine*	2.6769	1.4206	0.048112	93

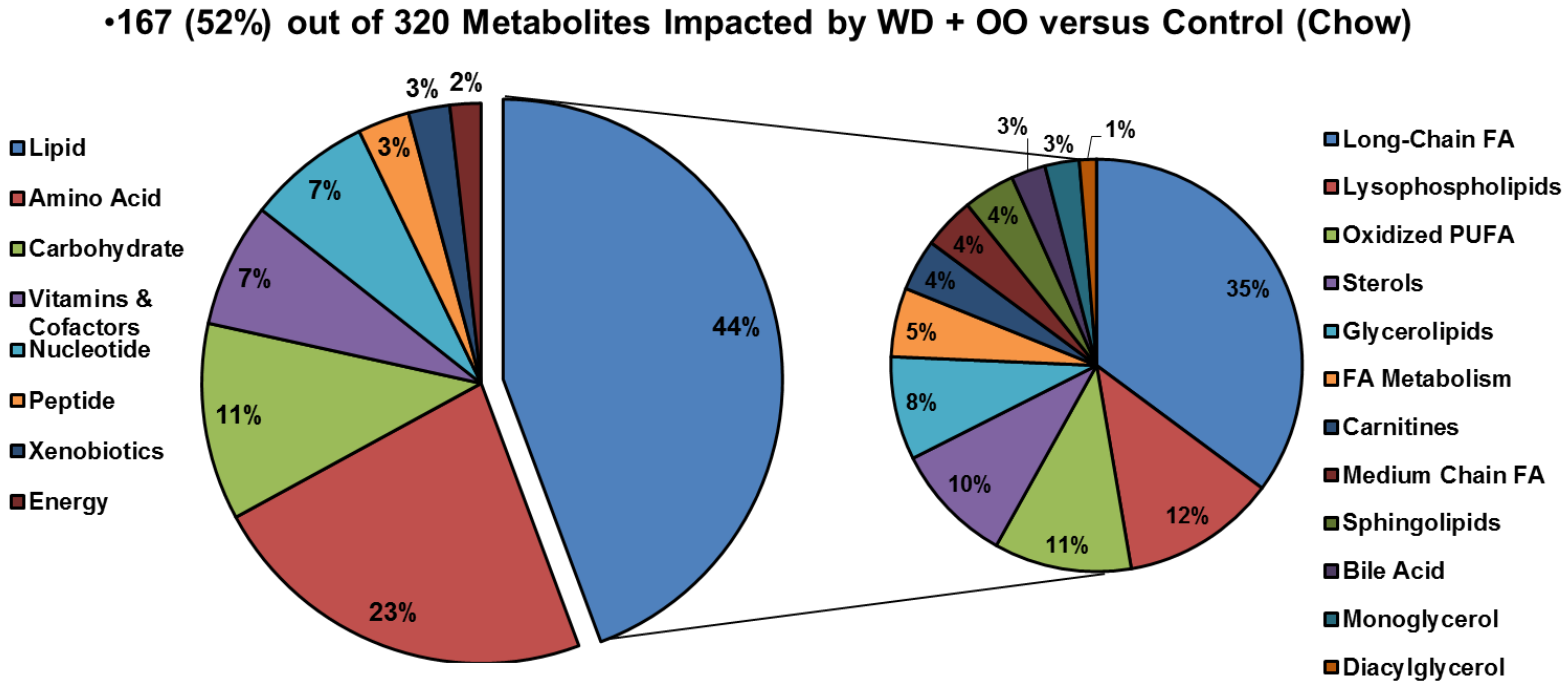

FIGURE 4.3. Metabolites impacted by WD + OO versus control mice, distributed by category. The sub-categories impacted within the lipid metabolites are represented since this was the largest category impacted.

Table 4.3. Hepatic lysophospholipids, sphingomyelin, one-carbon metabolites, and S-lactoylglutathione as analyzed by metabolomics.

	CH	WD + OO	WD + EPA	WD + DHA	WD + EPA + DHA
			Relative Units		
LPCs					
1-18:1 (n-9)-LPC	1.0 ± 1.2	2.4 ± 1.6	2.5 ± 1.9 *	2.1 ± 1.0 *	1.9 ± 1.5
2-18:1 (n-9)-LPC	1.0 ± 1.2	3.1 ± 2.3	2.4 ± 1.6 *	3.1 ± 1.5 *	2.1 ± 1.5
1-20:4 (n-6)-LPC	1.0 ± 1.0	3.3 ± 2.9	0.9 ± 0.8	0.5 ± 0.2 #	0.4 ± 0.3 #
2-20:4 (n-6)-LPC	1.0 ± 1.1	2.0 ± 1.6	0.6 ± 0.4	0.3 ± 0.1 #	0.3 ± 0.2 #
1-22:6 (n-3)-LPC	1.0 ± 1.0	1.5 ± 1.2	1.2 ± 0.8	2.8 ± 1.0 *	1.4 ± 1.1
2-22:6 (n-3)-LPC	1.0 ± 1.1	1.0 ± 0.8	0.7 ± 0.6	1.8 ± 0.8 *	1.2 ± 1.0
LPEs					
1-18:1 (n-9)-LPE	1.0 ± 0.6	2.0 ± 0.7 *	2.3 ± 0.8 *	2.1 ± 0.6 *	2.4 ± 0.7 *
2-18:1 (n-9)-LPE	1.0 ± 0.5	2.2 ± 0.7 *	1.9 ± 0.6 *	2.0 ± 0.6 *	2.0 ± 0.6 *
1-20:4 (n-6)-LPE	1.0 ± 0.5	1.6 ± 0.7	0.5 ± 0.3 *#	0.3 ± 0.1 *#	0.3 ± 0.1 *#
2-22:6 (n-3)-LPE	1.0 ± 0.9	1.1 ± 0.8	1.0 ± 0.6	2.0 ± 0.6 *	1.4 ± 1.0
LPIs					
1-18:1 (n-9)-LPI	1.0 ± 1.1	2.4 ± 1.5 *	2.7 ± 2.5 *	2.2 ± 1.0 *	3.6 ± 1.8 *
1-20:4 (n-6)-LPI	1.0 ± 0.5	2.4 ± 1.7 *	1.3 ± 1.21 #	0.8 ± 0.4 #	1.0 ± 0.6 #
2-20:4 (n-6)-LPI	1.0 ± 0.7	1.5 ± 0.7	1.9 ± 2.5	1.1 ± 0.9	1.5 ± 1.3
Sphingolipids					
16:0-SML	1.0 ± 0.2	3.2 ± 0.4 *	2.2 ± 0.3 *#	1.8 ± 0.3 *#	2.0 ± 0.8 *#
One-carbon metabolism					
Homocysteine	1.0 ± 0.5	0.4 ± 0.3 *	0.6 ± 0.3	0.9 ± 0.5	0.9 ± 0.3 #
S-adenosylhomocysteine	1.0 ± 0.1	0.5 ± 0.2 *	0.8 ± 0.2 *#	0.8 ± 0.1 *#	0.8 ± 0.1 *#
Dimethylglycine (DMG)	1.0 ± 0.3	2.5 ± 0.7 *	2.1 ± 0.5 *	2.0 ± 0.8 *	2.0 ± 0.7 *
[DMG] / [Betaine] Ratio	1.0 ± 0.3	2.0 ± 0.5 *	1.6 ± 0.3	1.6 ± 0.5	1.6 ± 0.6
5-methyltetrahydrofolate	1.0 ± 0.8	2.8 ± 0.7 *	1.6 ± 0.8 #	1.9 ± 1.3	1.0 ± 0.4 #
Methionine	1.0 ± 0.1	1.0 ± 0.2	0.9 ± 0.1	0.9 ± 0.2	0.8 ± 0.1
S-Lactoylglutathione	1.0 ± 0.3	0.2 ± 0.1 *	0.9 ± 0.2 #	1.4 ± 0.3 *#	1.3 ± 0.4 #

*, $P < 0.05$ versus control mice (chow). $^{\#}$, $P < 0.05$ versus WD + OO fed mice.

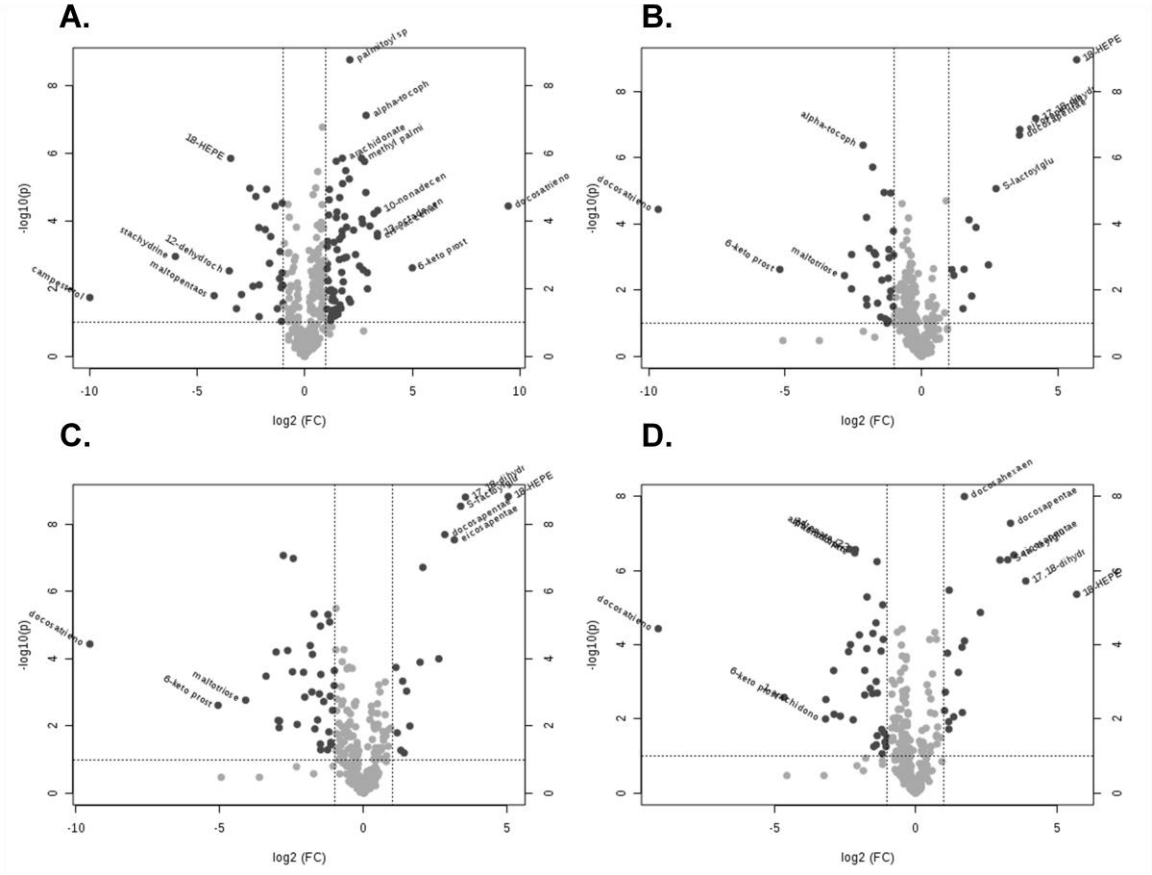

FIGURE 4.4. Volcano plots. Data used to construct volcano plots included all 320 metabolites analyzed using the original metabolomic data normalized to protein abundance for each sample as described in the methods section. The "x" axis represents log2 fold-change and the "y" axis represents log10 *P*-value. As such, all metabolites of interest with significant *P*-values ($P \geq 0.1$) and fold change ≥ 2.0 (increase or decrease) fall outside the dashed lines and are colored black. Metabolites of most interest to this report were further analyzed by one-way ANOVA as described in the methods section **A)** WD + OO versus controls (Chow). **B)** WD + EPA versus WD + OO. **C)** WD + DHA versus WD + OO. **D)** WD + EPA + DHA versus WD + OO.
Fold-change (FC); *P*-value (p).

Table 4.4. Hepatic mRNA abundance of proteins involved in the Land's Cycle, oxidation of fatty acids, and sphingomyelin synthesis.

	CH	WD + OO	WD + EPA	WD + DHA	WD + EPA + DHA
			Relative to CH		
Lands Cycle					
PLA2g6	1.0 ± 0.1^{a}	2.3 ± 0.4^{b}	3.4 ± 0.3^{c}	2.7 ± 0.7^{b,d}	3.3 ± 0.3^{c,d}
iPLA2	1.0 ± 0.1	1.0 ± 0.1	1.1 ± 0.1	1.1 ± 0.2	1.1 ± 0.1
LPCAT1	1.0 ± 0.1^{a}	2.9 ± 0.3^{b}	2.3 ± 0.4^{c}	1.4 ± 0.3^{a}	1.9 ± 0.5^{c}
LPCAT2	1.0 ± 0.1^{a}	4.0 ± 0.3^{b}	3.5 ± 0.8^{b}	2.5 ± 0.4^{c}	3.3 ± 0.7^{b}
LPCAT3	1.0 ± 0.1^{a}	1.4 ± 0.1^{b}	1.8 ± 0.1^{c}	1.3 ± 0.3^{a,b}	1.6 ± 0.2^{b}
LPCAT4	1.0 ± 0.1^{a}	1.9 ± 0.1^{b}	1.9 ± 0.2^{b}	1.3 ± 0.1^{c}	1.6 ± 0.2^{c}
Oxidation of FAs					
COX1	1.0 ± 0.2^{a}	2.5 ± 0.3^{b}	1.9 ± 0.4^{c}	1.2 ± 0.3^{a}	1.9 ± 0.4^{c}
COX2	1.0 ± 0.9^{a}	14.7 ± 6.0^{b}	5.4 ± 2.3^{a,c}	4.4 ± 1.3^{a,c}	7.1 ± 4.6^{c}
5-LOX	1.0 ± 0.2^{a}	3.0 ± 0.4^{b}	3.1 ± 0.8^{b}	2.0 ± 0.4^{c}	2.8 ± 0.8^{b,c}
12-LOX	1.0 ± 0.1^{a}	1.4 ± 0.2^{b}	1.4 ± 0.2^{b}	1.3 ± 0.3^{a,b}	1.4 ± 0.2^{b}
15-LOX	1.0 ± 0.4^{a}	6.6 ± 3.6^{b}	3.8 ± 2.0^{a,b}	2.7 ± 1.1^{a}	4.0 ± 1.4^{a,b}
CYP2C29	1.0 ± 0.2^{a}	0.5 ± 0.2^{b}	1.2 ± 0.4^{a}	0.7 ± 0.2^{a,b}	1.1 ± 0.2^{a}
CYP2C37	1.0 ± 0.2^{a}	0.4 ± 0.1^{b}	0.7 ± 0.2^{c}	0.4 ± 0.1^{b}	0.7 ± 0.2^{c}
CYP2C38	1.0 ± 0.6	0.7 ± 0.3	0.7 ± 0.5	0.3 ± 0.2	1.0 ± 0.5
CYP2C40	1.0 ± 0.4^{a}	0.6 ± 0.3^{b}	0.4 ± 0.2^{b}	0.3 ± 0.2^{b}	0.4 ± 0.2^{b}
CYP2C44	1.0 ± 0.2^{a}	0.6 ± 0.2^{b}	0.6 ± 0.2^{b}	0.3 ± 0.1^{b}	0.5 ± 0.2^{b}
CYP2J5	1.0 ± 0.2^{a}	1.2 ± 0.2^{a,b}	1.4 ± 0.1^{b}	1.3 ± 0.3^{a,b}	1.4 ± 0.2^{b}
EPHX1	1.0 ± 0.1^{a}	1.1 ± 0.1^{a}	1.5 ± 0.1^{b}	1.4 ± 0.2^{b}	1.5 ± 0.1^{b}
EPHX2	1.0 ± 0.3^{a}	1.3 ± 0.2^{a,c}	1.7 ± 0.2^{b}	1.6 ± 0.4^{b,c}	1.8 ± 0.4^{b}
SML Synthesis					
SPTLC1	1.0 ± 0.2^{a}	1.5 ± 0.1^{b}	1.5 ± 0.1^{b}	1.0 ± 0.3^{a}	1.4 ± 0.2^{b}
SPTLC2	1.0 ± 0.1^{a}	1.9 ± 0.2^{b}	2.0 ± 0.2^{b}	1.7 ± 0.3^{b}	2.0 ± 0.2^{b}
SGMS1	1.0 ± 0.1^{a}	1.6 ± 0.2^{b}	1.6 ± 0.2^{b}	1.1 ± 0.4^{a,c}	1.5 ± 0.2^{b,c}
SGMS2	1.0 ± 0.2	1.0 ± 0.2	1.0 ± 0.1	0.8 ± 0.2	0.9 ± 0.1

Labeled means without a common letter differ, $P < 0.05$.

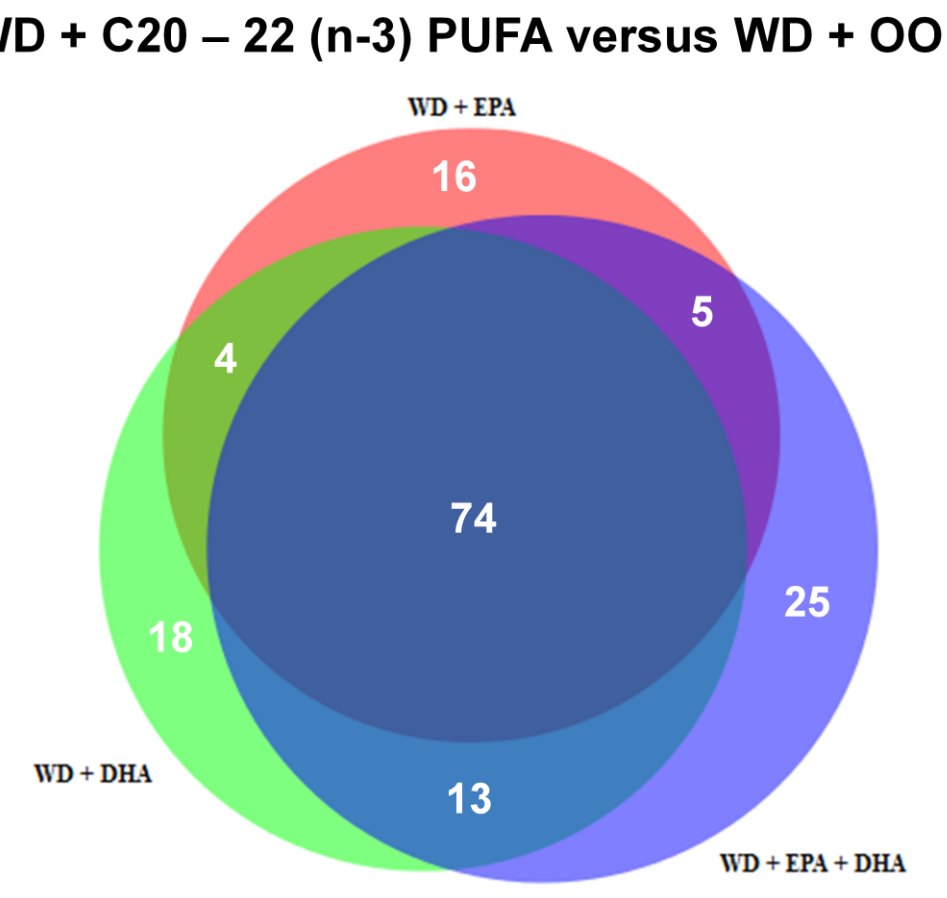

FIGURE 4.5. Distribution of metabolites significantly different in each of the WD + C20 – 22 (n-3) PUFA fed mice versus WD + OO fed mice.

Table 4.5. Fatty acid profile in hepatic neutral and polar lipid fractions.

	CH	WD + OO	WD + EPA	WD + DHA	WD + EPA + DHA
			Mol%		
Neutral Fraction					
16:0	24.5 ± 0.6^{a}	24.8 ± 0.8^{a,b}	25.7 ± 1.1^{a,b}	26.3 ± 0.8^{a,b}	26.7 ± 1.1^{b}
16:1 (n-7)	2.8 ± 0.6^{a}	5.7 ± 0.7^{b}	7.4 ± 0.4^{c}	6.1 ± 0.4^{b}	6.3 ± 0.5^{b,c}
18:0	3.4 ± 1.1^{a}	1.4 ± 0.1^{b}	1.4 ± 0.1^{b}	1.8 ± 0.3^{b}	2.1 ± 1.1^{a,b}
18:1 (n-9)	22.3 ±3.1^{a}	52.3 ± 0.9^{b}	45.2 ± 0.8^{c}	40.7 ± 2.5^{c}	44.3 ± 3.8^{c}
18:1 (n-7)	1.9 ± 0.4^{a}	7.1 ± 0.6^{b}	3.6 ± 0.4^{c}	1.8 ± 0.7^{a,c}	3.1 ± 1.0^{a,c}
18:2 (n-6)	31.4 ± 3.1^{a}	4.1 ± 0.9^{b}	6.3 ± 0.7^{b}	5.8 ± 0.9^{b}	5.5 ± 1.4^{b}
20:3 (n-6)	0.5 ± 0.2^{a}	0.2 ± 0.1^{b}	0.2 ± 0.1^{b}	0.1 ± 0.1^{b}	0.1 ± 0.1^{b}
20:4 (n-6)	2.1 ± 0.4^{a}	0.5 ± 0.1^{b}	0.3 ± 0.1^{b}	0.3 ± 0.1^{b}	0.4 ± 0.4^{b}
20:5 (n-3)	1.4 ± 0.1^{a}	0.0 ± 0.0^{b}	2.8 ± 0.5^{c}	1.8 ± 0.2^{a}	1.8 ± 0.5^{a}
22:5 (n-3)	1.0 0.1^{a}	0.1 0.1^{b}	2.6 ± 0.3^{c}	1.1 ± 0.1^{a,d}	1.5 ± 0.3^{d}
22:6 (n-3)	4.2 ± 0.2^{a}	0.2 ± 0.1^{b}	1.4 ± 0.2^{a,b}	11.3 ± 2.4^{c}	4.8 ± 1.9^{a}
Polar Fraction					
16:0	28.1 ± 0.9^{a}	30.1 ± 2.6^{a,b}	29.5 ± 0.7^{a,b}	32.8 ± 1.6^{b}	29.2 ± 2.5^{a,b}
16:1 (n-7)	0.8 ± 0.1^{a}	1.3 ± 0.2^{b,c}	1.5 ± 0.1^{b}	1.4 ± 0.1^{b}	1.1 ± 0.3^{a,c}
18:0	15.5 ± 0.3^{a}	12.3 ± 1.6^{b}	13.3 ± 0.4^{a}	13.1 ± 1.6^{a}	12.5 ± 1.2^{b}
18:1 (n-9)	7.4 ± 0.4^{a}	16.8 ± 1.6^{b}	13.6 ± 0.2^{c}	14.0 ± 0.3^{c}	12.2 ± 1.8^{c}
18:1 (n-7)	1.7 ± 0.2^{a}	4.4 ± 0.9^{b}	2.4 ± 0.4^{a}	1.2 ± 0.4^{a}	1.9 ± 0.8^{a}
18:2 (n-6)	17.4 ± 0.9	6.6 ± 1.2	10.3 ± 0.8	10.3 ± 2.6	10.9 ± 3.5
20:3 (n-6)	1.7 ± 0.7^{a}	1.7 ± 0.2^{a}	2.0 ± 0.1^{a}	0.9 ± 0.1^{b}	1.3 ± 0.2^{a,b}
20:4 (n-6)	11.7 ± 0.4^{a}	16.8 ± 2.5^{b}	6.5 ± 0.3^{c}	2.6 ± 0.8^{d}	3.6 ± 0.5^{d}
20:5 (n-3)	1.2 ±0.1^{a}	0.0 ± 0.0^{a}	7.3 ± 0.4^{b}	5.6 ± 0.8^{b}	6.1 ± 1.6^{b}
22:5 (n-3)	0.6 ± 0.1^{a}	0.5 ± 0.1^{a}	2.3 ± 0.1^{b}	0.7 ± 0.1^{a}	0.8 ± 0.1^{a}
22:6 (n-3)	13.2 ± 1.2^{a,b}	9.1 ± 2.0^{b}	11.0 ± 0.7^{b,c}	17.3 ± 3.4^{a}	14.8 ± 3.2^{a,c}

Labeled means without a common letter differ, $P < 0.05$.

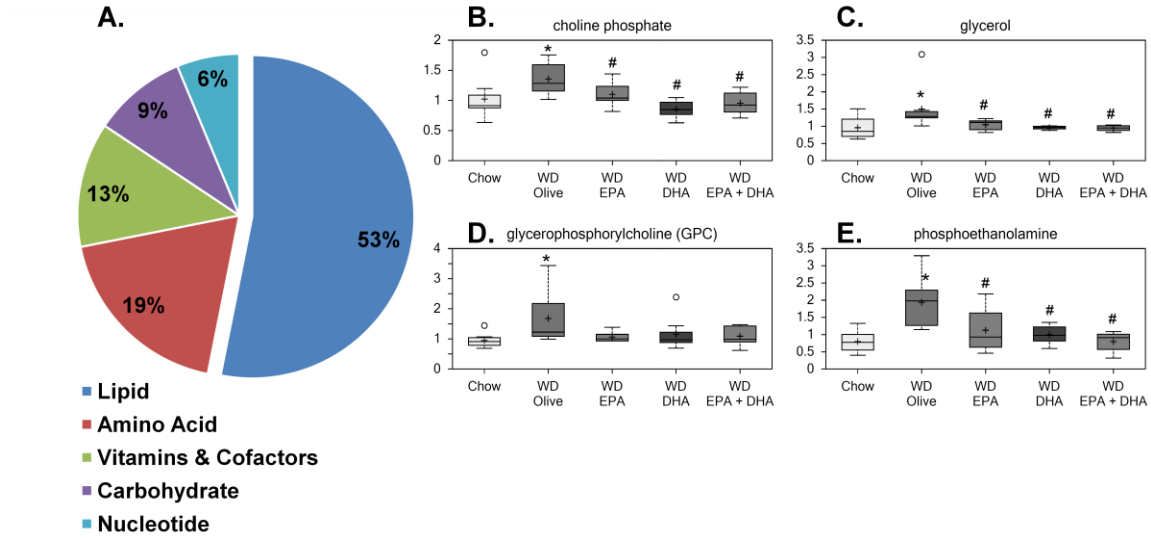

FIGURE 4.6. Metabolites perturbed by WD + OO feeding relative to controls (chow) that were prevented with C20 – 22 (n-3) PUFA supplementation. A) Categorical distribution of metabolites, lipid metabolites was the largest category with glycerolipid metabolites being the most represented sub-category. The glycerolipid metabolites altered by WD + OO that were prevented with C20 – 22 (n-3) PUFA supplementation were: **B)** choline phosphate, **C)** glycerol, **D)** glycerophosphorylcholine, and **E)** phosphoethanolamine. *, $P < 0.05$ versus control mice (chow). $^{\#}$, $P < 0.05$ versus WD + OO fed mice.

Table 4.6. Correlation analysis of oxidized fatty acids and their respective precursor fatty acids in the hepatic neutral and polar lipid fractions.

Oxidized FA	Precursor FA	Polar Fraction Correlation Coefficient	Neutral Fraction Correlation Coefficient
6-keto-PGF1α	20:4 (n-6)	0.63*	0.03
5-HETE	20:4 (n-6)	0.75*	0.53*
12-HETE	20:4 (n-6)	0.63*	0.11
15-HETE	20:4 (n-6)	0.74*	0.25
13 + 9-HODE	18:2 (n-6)	0.55*	0.81*
9,10-DHOME	18:2 (n-6)	0.45	0.27
17,18-DiHETE	20:5 (n-3)	0.92*	0.80*
18-HEPE	20:5 (n-3)	0.92*	0.82*

*, $P < 0.05$

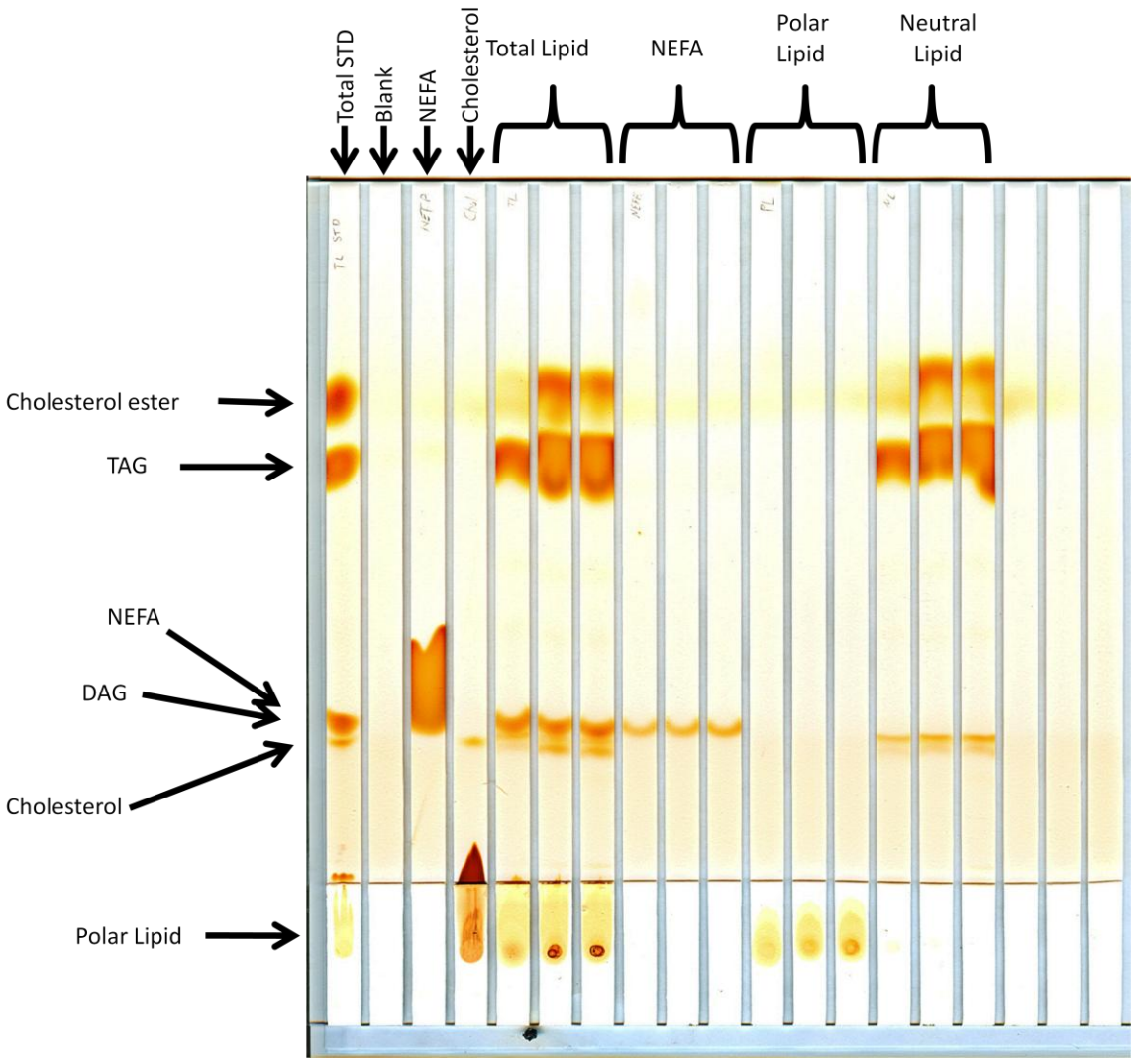

FIGURE 4.7. Confirmation of separated hepatic neutral and polar lipid fractions by thin-layer chromatography.

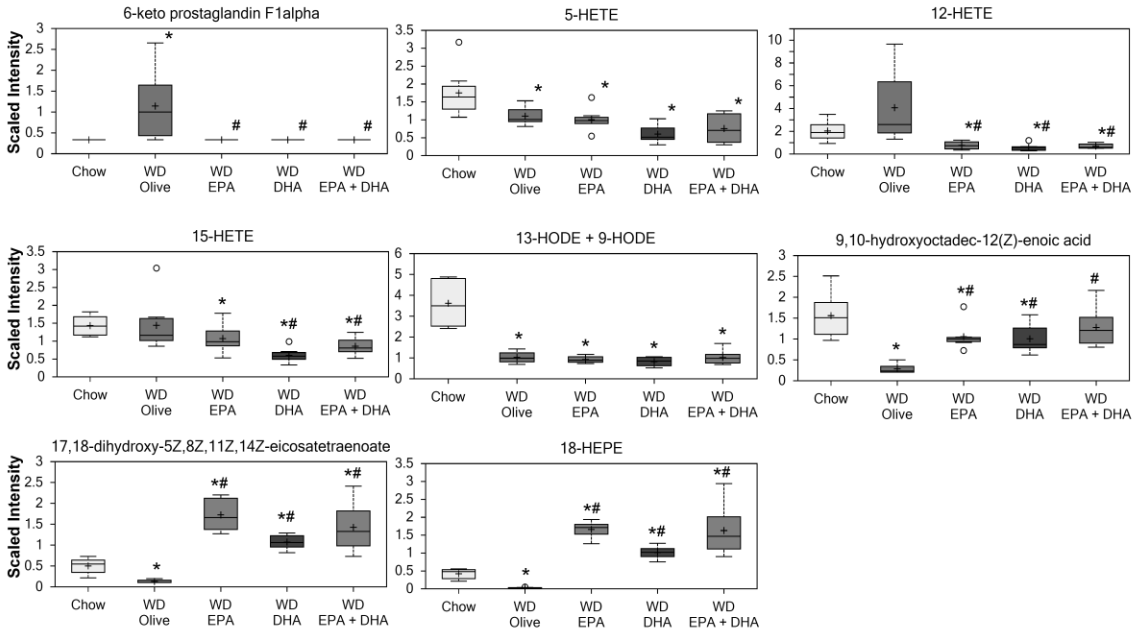

FIGURE 4.8. Oxidized PUFA analyzed by metabolomics.

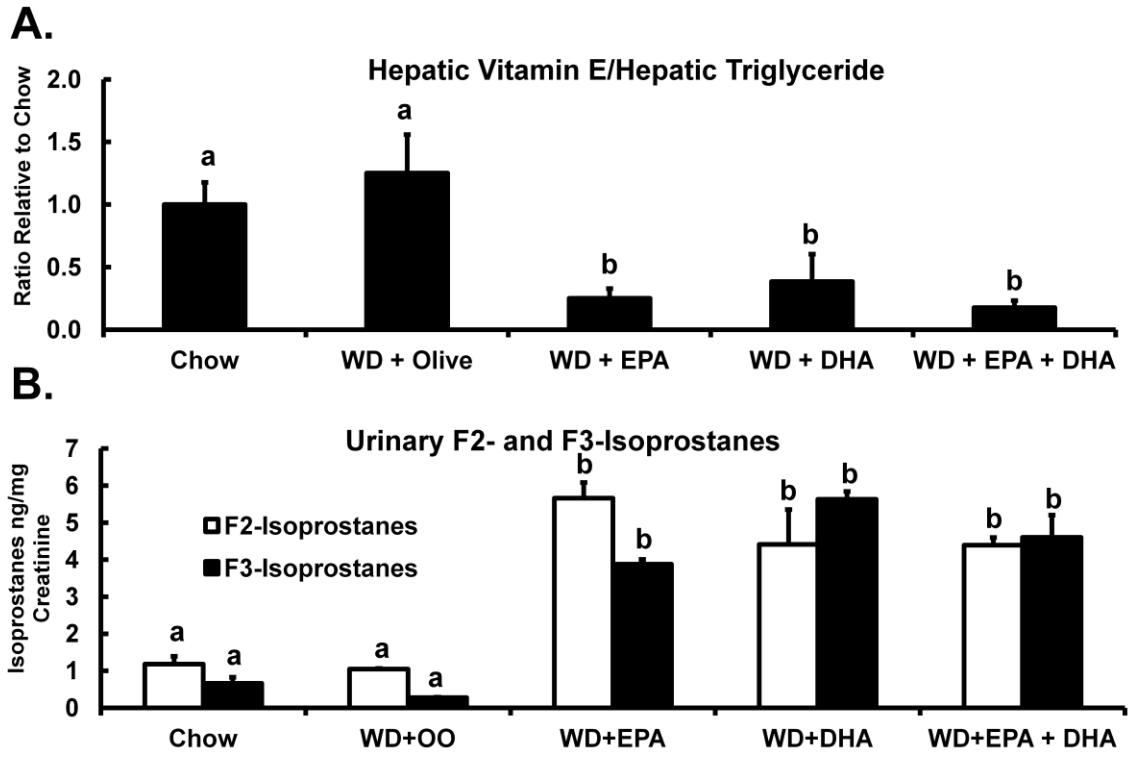

FIGURE 4.9. Analysis of the hepatic anti-oxidant vitamin E and urinary markers of lipid peroxidation F2- and F3-isoprostanes. A) Hepatic vitamin E from the metabolomic analysis normalized to hepatic triglyceride content (all WD fed mice had elevated hepatic triglyceride accumulation regardless of C20 – 22 (n-3) PUFA). **B)** F2- and F3-isoprostanes analyzed from 24 hour urine collections. Labeled means without a common letter differ, $P < 0.05$.

Chapter 5

Conclusion

NAFLD is a highly complex disease involving excess hepatic lipid accumulation mainly in the form of neutral lipids (triglyceride, diacylglycerol, cholesterol ester). In general, simple steatosis is considered a benign condition. However, the progressive form of the disease, NASH, is characterized by hepatic steatosis with inflammation and liver damage and can develop to fibrosis, cirrhosis, advanced liver disease, and hepatocellular carcinoma. It is estimated that NASH will be the leading cause of liver transplants in the United States by the year 2020. As such, NAFLD/NASH is rapidly becoming a major public health burden.

Currently there are no treatments specifically directed against NAFLD/NASH. Recommendations are to treat for liver disease and the associated co-morbidities including obesity, T2D, hyperlipidemia, and the MetS. One of the major limitations to developing effective therapies targeted at NAFLD/NASH is our incomplete understanding of the development and progression of the disease. While we have identified several lifestyle factors that contribute to development of NAFLD such as diet and physical activity, the progression of NASH is very poorly understood. A more complete understanding of the progression of NASH and specific factors that drive its progression will hopefully lead to new treatment approaches.

One potential therapeutic agent warranting further investigation is the long-chain C20-22 (n-3) PUFA. Dietary C20-22 (n-3) PUFA have well established anti-inflammatory properties and benefits on hepatic lipid metabolism. Furthermore, C20-22 (n-3) PUFA are currently used clinically for the treatment of hypertriglyceridemia and thus are well tolerated with minimal side-effects. Preliminary studies from our lab suggested that C20-22 (n-3) PUFA supplementation has the capacity to prevent development of NAFLD in HFD fed mice. Accordingly, my overall goal was to establish the potential therapeutic

effectiveness of C20-22 (n-3) PUFA to combat NAFLD/NASH. The following aims were completed to accomplish this goal.

Aim 1: Develop a mouse model of diet induced NASH closely resembling the human pathophysiology. The hypothesis was that *Ldlr*$^{-/-}$ mice fed a WD for 16 weeks would develop a phenotype resembling human NASH and provide a model for future studies investigating NASH.

Aim 2: Determine if EPA and DHA are equally effective at preventing diet-induced NASH. My preliminary data indicated there may be differences in the effectiveness of EPA versus DHA. Accordingly, I hypothesized that supplementation with DHA would prevent the progression of NASH in mice fed the WD for 16 weeks more robustly than supplementation with EPA.

Aim 3: Identify metabolic pathways perturbed by diet-induced NASH and the impact from EPA and DHA supplementation on those pathways. This was a hypothesis generating aim to identify mechanisms of NASH progression and targets of dietary EPA and DHA.

To accomplish aim 1, I used several diet and genotype combinations to induce NAFLD/NASH with the goal of establishing a model closely resembling the human pathophysiology. The models I investigated consisted of feeding HFD, HFHC, and the WD to both WT and *Ldlr*$^{-/-}$ mice. Ultimately, feeding the WD to *Ldlr*$^{-/-}$ mice resulted in the most severe phenotype with fibrosis reminiscent of that in the human condition. As hepatic cholesterol content correlated with NASH severity across all models, this established hepatic cholesterol as a driver of NASH progression. Additionally, since the WD had the highest simple carbohydrate content, these results further confirm the role of simple carbohydrate consumption, and notably fructose in the progression of NASH. Given these outcomes, it is clear that multiple dietary factors are involved in NASH

progression. In all, my studies were integral in establishing the utility of feeding the WD to *Ldlr*$^{-/-}$ mice for the study of NAFLD/NASH. As such, my major C20-22 (n-3) PUFA feeding studies utilized this model.

To determine if EPA and DHA are equally effective at preventing diet-induced NASH, *Ldlr*$^{-/-}$ mice were fed the WD for 16 weeks supplemented with either olive oil, EPA, DHA, or EPA + DHA as described in chapter 3. Even though the C20-22 (n-3) PUFA diets did not prevent hepatosteatosis, they all attenuated WD-induced hepatic inflammation, damage, and fibrosis. However, the capacity of dietary DHA was much greater than dietary EPA to suppress several markers of inflammation (*Clec4F, F4/80, Tlr4, Tlr9, CD14, Myd88*), fibrosis (*Col1a1*), and oxidative stress (NADPH oxidase subunits *Nox2, p22phox, p40phox, p47phox, p67phox*). These changes paralleled significantly reduced hepatic ARA content in WD + DHA fed mice. Thus, regulation of hepatic ARA content by dietary DHA is likely a key contributing factor to the superior ability of DHA compared to EPA for the attenuation of NASH in WD fed *Ldlr*$^{-/-}$ mice. The other key outcome of this study was that NASH can be dramatically attenuated without any impact on hepatosteatosis. Since fibrosis associated with NASH is the most detrimental clinical aspect of the disease, new therapeutic approaches targeted specifically to NASH progression will potentially have a large clinical and public health benefit.

Lastly, to further explore pathways and mechanisms contributing to NASH and impacts from EPA and DHA supplementation, I conducted a metabolomics analysis on livers from *Ldlr*$^{-/-}$ mice fed the WD for 16 weeks with/without EPA and DHA supplementation as described in chapter 4. The metabolomics analysis revealed the development of NASH has wide-reaching impacts on all major pathways of hepatic

metabolism. The most dramatic changes associated with NASH were elevated ARA and depressed EPA in the hepatic polar lipid (phospholipid) fatty acid profile, elevated 16:0-SML, and increased (n-6) and depressed (n-3) derived oxPUFA. The changes in phospholipid fatty acids and SML are likely altering membrane dynamics and raft domains in such a way that allows for progression of NASH. Furthermore, the oxPUFA profile was highly correlated with the polar lipid fatty acid profile and thus mass action of the polar lipid fatty acids appear to be driving the accumulation of oxPUFA. Since (n-3) derived oxPUFA promote resolution of inflammation, the imbalance of (n-6) and (n-3) derived oxPUFA in mice with NASH is likely further promoting progression of NASH. These changes derived from altered membrane organization are likely a major driving factor in NASH progression. WD + DHA feeding most effectively decreased ARA and elevated both EPA and DHA in the hepatic polar lipid fraction, depressed 16:0-SML, and decreased (n-6) and elevated (n-3) derived oxPUFA. These changes in membrane lipids induced by DHA supplementation most likely re-organize membrane structure and function in a manner that interrupts progression of NASH. Furthermore, the elevated (n-3) derived oxPUFA are known to attenuate inflammation, likely dampening all aspects of NASH progression. Thus, the dramatic impacts of dietary DHA on membrane dynamics and oxPUFA is likely a major contributing factor to the attenuated NASH progression observed with DHA supplementation. Another major impact of the C20-22 (n-3) PUFA treatments was depressed hepatic vitamin E. While I hypothesize this is due to elevated lipid peroxidation, the physiological and disease outcome impacts of reduced hepatic vitamin E remain to be established.

Figure 5.1 illustrates the major proposed pathways of WD-induced NASH progression and the impacts from C20-22 (n-3) PUFA supplementation identified

throughout all my studies. The evidence is clear that there is not one single factor driving NASH progression but there are multiple insults to liver metabolism occurring in parallel. It is the combined effect of all these insults that is ultimately driving progression of NASH. The C20-22 (n-3) PUFA are attractive therapeutic agents because of their pleiotropic actions. My data supports this concept illustrating several potential mechanisms through which the C20-22 (n-3) PUFA attenuate progression of NASH.

While this series of studies was very enlightening and helped make significant progress in the area of C20-22 (n-3) PUFA and NAFLD, many questions still exist. My studies and others have repeatedly identified hepatic cholesterol as a major driver of NASH progression. However, the mechanism behind this is poorly understood. One hypothesis is that cholesterol accumulation is disrupting mitochondrial membrane integrity and the electron transport chain, ultimately elevating reactive oxygen species and promoting NASH. Future studies are needed to further investigate this potential mechanism of hepatic cholesterol accumulation. Another major area yet to be addressed is the mechanisms at play in specific cell types within the liver. Several potential mechanisms and targets of C20-22 (n-3) were identified using whole liver extracts. Future studies are needed to identify what specific cell types such as Kupffer Cells (resident macrophages) and hepatic stellate cells (responsible for fibrosis) these mechanisms are impacting. Concerning C20-22 (n-3) PUFA and NAFLD, all my studies have been prevention trials where the mice were supplemented with C20-22 (n-3) PUFA throughout the entire feeding period. Future studies where C20-22 (n-3) PUFA supplementation is not initiated until after NASH is established are needed to assess the efficacy of C20-22 (n-3) PUFA to resolve inflammation, oxidative stress, and fibrosis associated with NASH.

While this series of studies was very enlightening, they are not without limitations. Even though WD feeding to *Ldlr*$^{-/-}$ mice induces a phenotype similar to human NASH, there are still inherent differences between humans and mice that cannot be overcome. Very simply, the inbred nature of mouse models combined with highly controlled laboratory conditions and 100% adherence to treatments calls to question the relevancy of using mice to study human disease. Furthermore, while mice are similar to humans, differences in cellular composition of various organs can contribute to differing molecular responses between mice and humans [235]. Additionally, all my studies were based on a single endpoint analysis. As such, I cannot determine if the order of events promoting disease progression paralleled what is observed in humans. Time course studies are needed to address this weakness. Overall, while there are weaknesses, all my C20-22 (n-3) PUFA feeding trials had significant benefit in attenuating progression of NASH. Given the minimal side-effects of C20-22 (n-3) PUFA consumption, I believe my data warrants investigations into the potential benefit of dietary EPA and DHA to combat NASH in humans.

In conclusion, this work has provided 3 major outcomes. In the first aim, the utility of feeding the WD to *Ldlr*$^{-/-}$ mice for the study of NAFLD and NASH was established, while identifying hepatic cholesterol as a driver of NASH. In the second aim, the superior ability of dietary DHA over EPA to attenuate progression of hepatic inflammation, oxidative stress, and fibrosis associated with NASH was established. In the third aim, several mechanisms of NASH progression and targets of C20-22 (n-3) PUFA were identified with the most dramatic effects on membrane lipid re-organization and oxPUFA. In all, these studies have established dietary DHA as a potential therapeutic agent to

combat NASH in humans, and identified several potential mechanisms of both NASH progression and dietary C20-22 (n-3) PUFA in the prevention of NAFLD.

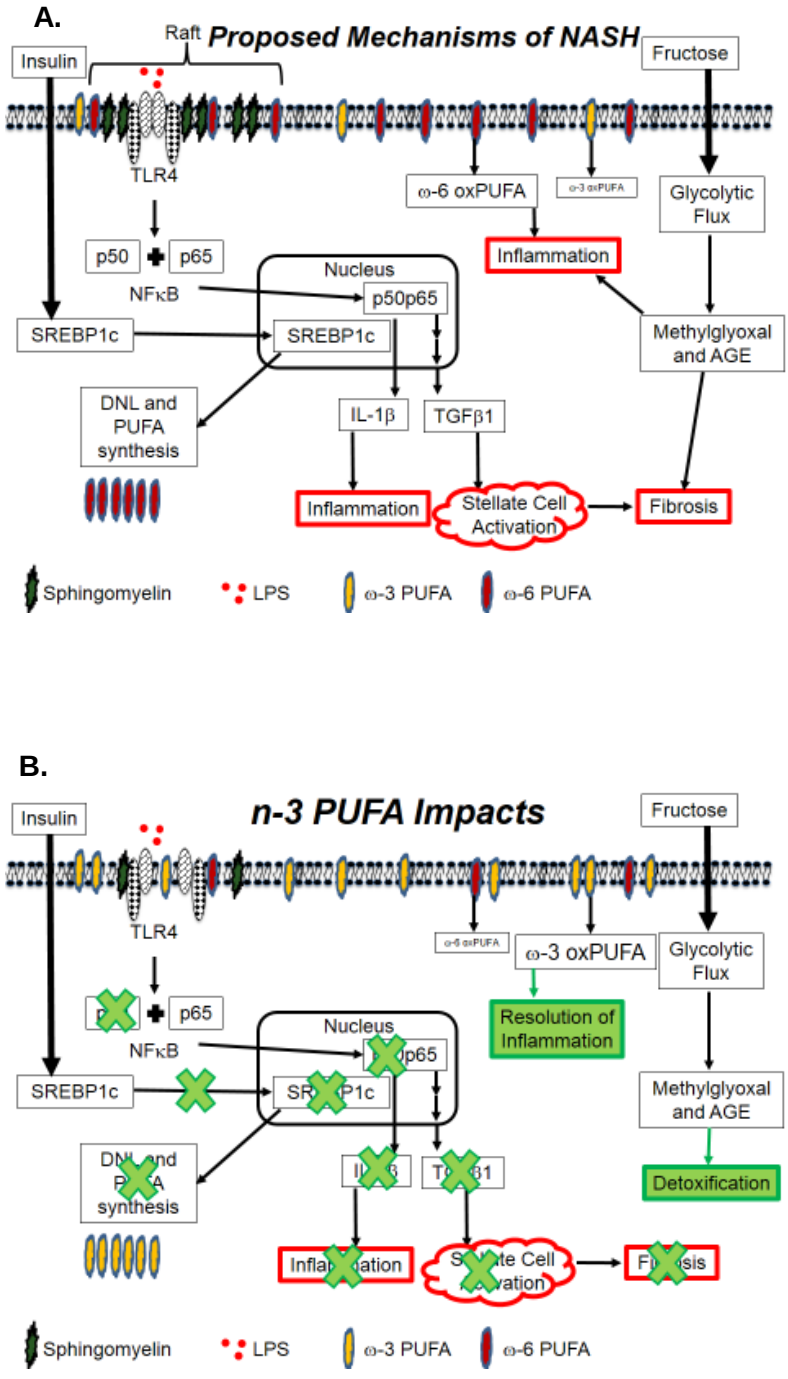

FIGURE 5.1. Proposed mechanisms of WD-induced NASH progression and impacts from C20-22 (n-3) PUFA supplementation. A) WD feeding elevated DNL and PUFA synthesis through SREBP-1c nuclear translocation leading to accumulation of (n-6) PUFA that are incorporated into membrane phospholipids. Altered lipid metabolism promotes SML accumulation and lipid raft organization allowing optimal functioning of raft proteins such as TLR4. TLR4 activation leads to an inflammatory response and

promotes stellate cell activation and fibrosis. Elevated (n-6) membrane PUFA result in elevated (n-6) derived oxPUFA and thus further enhance the inflammatory response. The high fructose component of the WD promotes glycolytic flux and thus MG formation that further promotes inflammation and fibrosis. **B)** Several potential targets of C20-22 (n-3) PUFA supplementation were identified. C20-22 (n-3) PUFA decreased nuclear SREBP-1c thus depressing DNL and PUFA synthesis, allowing for accumulation and incorporation of C20-22 (n-3) into membranes. Altered lipid metabolism resulted in depressed SML accumulation. Combined, these impacts likely disrupted lipid raft formation and thus impeded TLR4 function. This was evidenced by attenuated NFkB-p50 expression and nuclear translocation, ultimately dampening the inflammatory response and activation of stellate cells and fibrosis. Accumulation of membrane (n-3) PUFA allowed for elevated production of (n-3) PUFA derived oxPUFA that promote resolution of inflammation. Lastly, preserved MG detoxification with C20-22 (n-3) PUFA supplementation further attenuated the inflammatory and fibrotic responses to WD feeding.

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