Potential for Dietary ω3 Fatty Acids to Prevent Nonalcoholic Fatty Liver Disease and Reduce the Risk of Primary Liver Cancer

Donald B. Jump, Christopher M. Depner, Sasmita Tripathy and Kelli A. Lytle

Nutrition Program
School of Biological and Population Health Sciences
Linus Pauling Institute
Oregon State University
Corvallis Oregon, 97331

Corresponding Author:
Donald B. Jump, Ph.D.
107A Milam Hall
School of Biological and Population Health Sciences
Oregon State University
Corvallis, OR 97370
Phone: 541-737-4007
Email: Donald.Jump@oregonstate.edu

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ABSTRACT

Nonalcoholic fatty liver disease (**NAFLD**) has increased in parallel with central obesity and its prevalence is anticipated to increase as the obesity epidemic remains unabated. NAFLD is now the most common cause of chronic liver disease in developed countries and is defined as excessive lipid accumulation in the liver, i.e., hepatosteatosis. NAFLD ranges in severity from benign fatty liver to nonalcoholic steatohepatitis (**NASH**), where NASH is characterized by hepatic injury, inflammation, oxidative stress and fibrosis. NASH can progress to cirrhosis; and cirrhosis is a risk factor for primary hepatocellular carcinoma (**HCC**). The prevention of NASH will lower the risk of cirrhosis and NASH-associated HCC. Our studies have focused on NASH prevention. We developed a model of NASH using *Ldlr*$^{-/-}$ mice fed the western diet (**WD**). The WD induces a NASH phenotype in these mice that is similar to that seen in humans; and includes robust induction of hepatic steatosis, inflammation, oxidative stress and fibrosis. Using transcriptomic, lipidomic and metabolomic approaches, we examined the capacity of 2 dietary ω3 polyunsaturated fatty acids, eicosapentaenoic acid (20:5ω-3; **EPA**) and docosahexaenoic acid (22:6ω-3; **DHA**), to prevent WD-induced NASH. Dietary DHA was superior to EPA at attenuating WD-induced changes in plasma lipids and hepatic injury; and reversing WD effects on hepatic metabolism, oxidative stress, and fibrosis. The outcome of these studies suggests that DHA may be useful in the prevention of NASH and reducing the risk of HCC.

Key words:

Fatty liver disease, liver cancer, inflammation, oxidative stress, fibrosis, metabolomics, ω3 PUFAs

Introduction.

Primary hepatocellular carcinoma (HCC) is the 5th most common human cancer in men and the 7th most common cancer in women in the western societies; and HCC represents the 3rd most frequent cause of cancer deaths worldwide (1-3). High rates of HCC are seen in eastern and southeastern Africa and Asia and lower levels in western countries. Risk factors for HCC include age and gender (male), hepatitis virus infection (HBV, HCV), exposure to toxins (aflatoxin), chronic alcohol abuse, cirrhosis, tobacco, and genetic disorders (hereditary hemochromatosis, α1-antitrypsin deficiency and primary biliary cirrhosis) (1, 2).

The unabated increase in the incidence of obesity, type 2 diabetes and non-alcoholic fatty liver disease (NAFLD) (**Fig. 1**) is driving the concern for an increased HCC incidence in western societies (4). This is because NAFLD can progress to non-alcoholic steatohepatitis (NASH) and cirrhosis; cirrhosis is a risk factor for HCC. Chronic fatty liver disease sets the stage for poorly regulated regeneration of hepatic parenchymal cells resulting from hepatic inflammation, parenchymal cell death and fibrosis; thus increasing HCC risk. Current treatment options for HCC are limited to surgery and drugs like the multi-kinase inhibitor, sorafenib. Since diet is a major driver of NAFLD and NASH progression, our focus has been on developing nutritional strategies to prevent NASH. This report focuses on the use of dietary $C_{20\text{-}22}$ ω-3 polyunsaturated fatty acids (PUFAs) to prevent NASH.

NAFLD and NASH.

Current data from the CDC estimates that nearly 78.6 million obese adults and 12.7 million obese children (ages 2-19) are in the US (5, 6). Obesity is a risk factor for developing NAFLD and NASH. As such, the prevalence of NAFLD and NASH has increased in parallel with the incidence of central obesity in western societies (7, 8). NAFLD is the most common fatty liver disease in developed countries (9) and is defined as excessive lipid accumulation in the liver, i.e., hepatosteatosis (10, 11). NAFLD is the hepatic manifestation of metabolic syndrome (MetS) (12); and MetS risk factors include obesity, elevated plasma triacylglycerols (TAG) and LDL cholesterol, reduced HDL cholesterol, high blood pressure and fasting hyperglycemia (13). The prevalence of NAFLD in the general population is

estimated to range from 6% to 30% depending on the method of analysis and population studied (14) (**Fig. 1**).

NAFLD ranges from benign hepatosteatosis to NASH (15), which is defined as hepatosteatosis with inflammation and hepatic injury (16). Approximately 30-40% of patients with steatosis develop NASH (17); representing ~3% to 5% in the general population (14). NAFLD and NASH have high prevalence ($\geq$60%) in the type 2 diabetic (T2D) population (18). The level of NAFLD and NASH in patients undergoing bariatric surgery is 93% and 26%, respectively (19). NASH patients have higher mortality rates than NAFLD patients; and both are higher than in the general population (20-22). Over a 10 year period, cirrhosis and liver related death occurs in 20% and 12% of NASH patients, respectively (23). Given the increasing prevalence of NASH and its adverse clinical outcome, NASH is rapidly becoming a significant public health burden. NASH can progress to cirrhosis and HCC (8, 17). By the year 2020, cirrhosis resulting from NASH is projected to be the leading cause of liver transplantation in the United States (24).

Multi-hit hypotheses for NASH development.

The development of NASH has been proposed to follow a multi-hit model (25-27). The "1st Hit" involves excessive neutral lipid accumulation in the liver which sensitizes the liver to the "2nd Hit" (26) (**Fig**. **2**). The "2nd Hit" is characterized by hepatic inflammation, oxidative stress and hepatic insulin resistance. These events promote hepatic damage which is associated with increased blood levels of hepatic enzymes/proteins (alanine aminotransferase [ALT], aspartate aminotransferase (AST), C-reactive protein, serum amyloid A1 and plasminogen activator inhibitor-1 (PIA1) (7, 8, 28). This pro-inflammatory state leads to hepatocellular death & necrosis (necroinflammation); and cell death promotes fibrosis, i.e., the "3rd Hit". Fibrosis is mediated by activation of hepatic stellate cells and myofibrillar cells; these cells produce extracellular matrix (ECM) proteins, such as collagen (*collagen 1A1, Col1A1*) and smooth muscle α2 actin (29). Dietary (excess fat, cholesterol, glucose and fructose), metabolic (plasma and hepatic fatty acid profiles, hepatic ceramide, oxidized LDL), endocrine/paracrine (insulin, leptin, adiponectin & TGFβ), gut (endotoxin, microbial metabolites) and genetic (e.g., patatin-

like phospholipase domain containing 3 [PNPLA3] polymorphisms) factors contribute to NASH progression (30-38).

Hepatosteatosis develops because of an imbalance of hepatic lipid metabolism leading to the accumulation of hepatic neutral lipids as TAG and diacylglycerols (DAG) and cholesterol esters (CE). Fatty acid sources of hepatic TAG and CE include non-esterified fatty acids (NEFA) mobilized from adipose tissue, *de novo* lipogenesis (DNL), and the diet via the portal circulation. Hepatic fatty acid oxidation (FAO) and very low density lipoprotein (VLDL) assembly and secretion represent two pathways for removal of fat from the liver. Hepatosteatosis develops when lipid storage exceeds lipid export and oxidation (39). In humans with NAFLD, ~60% of the fatty acids appearing in the liver are derived from circulating NEFA mobilized from adipose tissue; 26% are from DNL and 15% from diet (40). Both hepatic and peripheral insulin resistance also contribute to the disruption of these pathways and to the development of hepatosteatosis (39).

Patients with NASH consume a lower ratio of polyunsaturated fatty acid (PUFAs) to saturated fatty acid (SFA) when compared to the general population (41, 42). Consumption of a low ratio of ω3 PUFAs to ω6 PUFAs is also associated with NAFLD development, whereas increased dietary long-chain ω-3 PUFAs decreases hepatic steatosis (43-45). Mice fed a ω3 PUFA-deficient diet developed hepatosteatosis and insulin resistance (46). Livers of these mice exhibited a major decline in α-linolenic acid (ALA, 18:3ω-3), eicosapentaenoic acid (EPA, 20:5ω-3) and docosahexaenoic acid (DHA, 22:6ω-3), but no change in hepatic ω-6 PUFAs, such as linoleic acid (LA, 18:2ω-6) or arachidonic acid (ARA, 20:4ω-6). Depletion of hepatic ω-3 PUFAs lowered FAO, a peroxisome proliferator activated receptor α (PPARα)-regulated mechanism, and increased DNL and TAG accumulation; which are sterol regulatory element binding protein-1 (SREBP1), carbohydrate regulatory element binding protein (ChREBP), max-like factor X (MLX) regulated pathways. PPARα, SREBP1 and the ChREBP/MLX heterodimer are well established targets of $C_{20\text{-}22}$ ω-3 PUFAs control (47). While trans-fatty acid (TFA) consumption is associated with insulin resistance and cardiovascular disease, the impact of TFA consumption on NAFLD in humans is less clear (48). Studies utilizing mice suggest that TFA consumption is associated

with hepatic steatosis and injury (49, 50). Thus, reduced hepatic ω-3 PUFAs and increased levels of TFA may account for changes in hepatic lipid metabolism that promote NAFLD.

Excess dietary cholesterol contributes to NASH (51) by promoting hepatic inflammation (32, 52-54). In the *Ldlr*$^{-/-}$ mouse model, high fat-high cholesterol diets promote NASH (55). Kupffer cells, i.e., resident hepatic macrophage, become engorged with oxidized-LDL (ox-LDL) which induces inflammatory cytokine secretion. These locally secreted cytokines act on neighboring hepatic cells to promote a pro-inflammatory state leading to cell injury. Kupffer cells also secrete chemokines (monocyte chemoattractant protein-1, *MCP1*) that recruit monocytes to the liver further amplifying hepatic inflammation. Controlling hepatic inflammation is an attractive target for NASH management and therapy.

Excessive consumption of simple sugar has been implicated in hepatosteatosis and NASH progression. Over the last 30 years there has been a dramatic increase in obesity and NAFLD in the United States. While total fat consumption has remained steady, carbohydrate and total caloric intake have increased (56-60). As such, elevated carbohydrate, and specifically fructose consumption, has been linked to NAFLD and NASH progression (61-63). The liver expresses the fructose-specific transporter (*Glut5*). Moreover, the liver metabolizes up to 70% of dietary fructose (62, 63); and fructose metabolism is independent of insulin regulation. When compared to glucose, fructose more readily enters the pathways for DNL and TAG synthesis. Fructose promotes all aspects of MetS including hepatosteatosis, insulin resistance, dyslipidemia, hyperglycemia, obesity and hypertension. In contrast to fructose, hepatic glucose metabolism is well-regulated by insulin in healthy individuals; and glucose is converted to glycogen for storage. Excess glucose consumption does not promote hepatosteatosis as aggressively as excess fructose consumption. Fructose also affects several biochemical events that exacerbate NASH development, including formation of advanced glycation end-products (AGEP) and reactive oxygen species (ROS), (64-67).

Development of mouse models of NASH.

Several mouse models of NAFLD and NASH have been developed. Four such models include the genetic models (*ob/ob* and *db/db* mice), a dietary model (methionine-choline deficient diets) and chemically-induced model (intraperitoneal carbon tetrachloride) (68, 69). These models recapitulate some aspects of human NAFLD/NASH, but not other aspects of the disease. Mice with global ablation of the low density lipoprotein receptor (*Ldlr*$^{-/-}$) develop hypercholesteremia due to elevated plasma VLDL and LDL when fed a high cholesterol diet (70). While *Ldlr*$^{-/-}$ mice have been used to study atherosclerosis, we and others observed that when *Ldlr*$^{-/-}$ mice are fed high fat-high cholesterol diet, like the western diet, mice develop a NASH phenotype similar to that seen in humans (32, 36, 54, 71-74). Since humans and *Ldlr*$^{-/-}$ mice develop NAFLD and NASH in a context of obesity and insulin resistance, these mice appear to be a useful preclinical model to investigate the development, progression and remission of NASH.

The western diet (WD; Research Diets, D12079B) used in our studies is moderately high in saturated and trans-fat (41% total calories), sucrose (30% total calories) and cholesterol (0.15 g%, w/w); and is similar to the "fast-food" diet (75) and human diets linked to obesity in the US (76, 77). Both the WD and "fast food" mouse models induced a NASH phenotype that recapitulates many of the clinical features of human NASH with MetS, including dyslipidemia, hyperglycemia, hepatosteatosis, hepatic damage (plasma ALT & AST), hepatocyte ballooning, induction of hepatic markers of inflammation (*MCP1*), oxidative stress (*NOX2* and other *NOX* components) and fibrosis (*TGFβ1, proCol1A1, TIMP1*) (54, 73, 75, 78-80) (**Fig. 3**). Moreover, NASH is associated with a major enrichment of both plasma and liver with saturated (SFAs) and monounsaturated fatty acids (MUFAs) and depletion of hepatic ω3 PUFAs (54, 73, 78). The development of this phenotype has been attributed to a diet high in saturated and trans-fat, sucrose and cholesterol (62, 67, 81-83).

Potential for dietary C_{20-22} ω3 PUFAs to prevent NASH.

C_{20-22} ω3 PUFAs are pleiotropic regulators of cell function; they have well established effects on membrane structure, cell signaling, gene expression, lipid and carbohydrate metabolism and inflammation (84). As such, these fatty acids appear to be an ideal bioactive nutrient to combat NASH. A meta-analysis of 9 clinical studies indicated that dietary supplementation with C_{20-22} ω-3 PUFAs decreased liver fat (85) and clinical trials suggest C_{20-22} ω-3 PUFAs may lower liver fat in children and adults with NAFLD (86-91). Of 235 clinical trials (119) assessing NASH and NASH therapies, 23 trials used C_{20-22} ω3 PUFAs as a treatment strategy. In most trials, diets were supplemented with fish oil or a combination of EPA + DHA; few studies used EPA or DHA alone.

Preclinical assessment of the efficacy of ω3 PUFA supplementation to prevent NASH in Ldlr$^{-/-}$ mice.

Diets supplemented with fish oil, EPA or DHA prevent high fat diet-induced NASH to varying degrees (54, 73, 78, 84). The level of EPA and DHA in these high fat diets was at ~2% of total calories. This dose of C_{20-22} ω-3 PUFAs is comparable to the dose consumed by patients taking Lovaza™ (GlaxoSmithKline) for the treatment of dyslipidemia (92). Humans consuming EPA + DHA ethyl esters (4 g/d for 12 wks) exhibited increased plasma EPA + DHA from 5.5 mol% before treatment to 16.2 mol% after treatment (93). Supplementing human diets with a DHA-enriched fish oil (6 g/day for 8 wks) increased plasma DHA from 4 mol% before treatment to 8 mol% after treatment (94, 95). Plasma levels of DHA and total C_{20-22} ω-3 PUFA [EPA, docosapentaenoic acid (DPA, 22:5ω-3) and DHA] in *Ldlr*$^{-/-}$ mice fed a western diet for 16 wks was 4.3 and 6.7 mol%, respectively. Feeding *Ldlr*$^{-/-}$ mice a western diet containing DHA (at 2% total calories) for 16 wks increased plasma DHA and total C_{20-22} ω-3 PUFA to 9 and 15.2 mol%, respectively. Our protocol for C_{20-22} ω-3 PUFA supplementation of diets yields a change in blood C_{20-22} ω3 PUFAs that is comparable to that seen in humans consuming 4-6 g/d of C_{20-22} ω-3 PUFA.

Dietary ω3 PUFAs do not prevent WD-induced systemic inflammation.

Systemic inflammation is a major driver of NASH. Inflammatory signals affecting NASH progression include: gut-derived microbial products, e.g., endotoxin/LPS, oxidized LDL (ox-LDL) (34, 55, 80, 96); adipokines (leptin & adiponectin) & cytokines (TNFα) (97) and products from hepatocellular death (27, 98) (**Fig. 2**). Supplementation of the WD with either EPA or DHA fails to attenuate WD-induced endotoxinemia (78). The appearance of endotoxin in the plasma of WD-fed *Ldlr*$^{-/-}$ mice (99) may represent a problem with gut physiology such as microbial overgrowth, increased gut permeability (leaky gut), or co-transport of microbial lipids with chylomicron (34, 100, 101). A link between the gut microbiome and NAFLD has been established (34, 102, 103).

ω3 PUFAs attenuate hepatic inflammation.

Despite the absence of an effect of $C_{20\text{-}22}$ ω-3 PUFAs on systemic inflammation markers, like endotoxin, gene expression analyses showed that DHA was more effective than EPA at attenuating WD-induced expression of hepatic toll-like receptor (*TLR*) subtypes (*TLR2, TLR4, TLR9*), *CD14* (binds endotoxin), downstream targets of TLRs; like NFκB (p50 subunit) nuclear abundance and downstream targets of NFκB like chemokines (*MCP1*), cytokines (*IL1β),* inflammasome components (*NLRP3*) and oxidative stress (*NOX2*, and its subunits) markers (73, 78). These studies suggest that EPA and DHA attenuate the hepatic (cellular) response to plasma inflammatory factors by down-regulating key cellular mediators of inflammation, like *TLRs, CD14* (binds LPS, effect on *CD14* mRNA and protein), NFκB-p50 nuclear abundance.

ω3 PUFAs have selective effects on hepatic oxidative stress.

Hepatic oxidative stress increases with NASH and is reflected by a significant increase in gene expression and metabolite markers of oxidative stress that appear in liver and urine (54, 73). A response to increased oxidative stress is the induction of nuclear factor (erythroid-derived 2)-like 2 (*Nrf2*), a key transcription factor involved in the antioxidant response (78). Nrf2 regulates the expression of multiple transcripts linked to the anti-oxidant stress response, such as *Hmox1, Gst1α* and

several *NOX* subunits. Adding EPA or DHA to the WD did not prevent the WD-mediated increase in hepatic nuclear content of *Nrf2* or expression of *Hmox1 or Gst1*α. The EPA- and DHA-containing diets, however, significantly lowered WD-mediated induction of multiple NOX subunits [*Nox2*, *P22phox*, *P40phox* and *P67phox]* (73). *NOX* subtypes are a major source of superoxide and hydrogen peroxide. As such, the NOX pathway is a major target of WD and $C_{20\text{-}22}$ ω3 PUFAs.

ω3 PUFAs attenuate hepatic fibrosis.

Hepatic fibrosis (scarring) develops as a result of cell death and activation of hepatic stellate cells and myofibrillar cells to produce extracellular matrix (ECM) proteins. Key regulators of fibrosis include transforming growth factor (TGFβ), connective tissue growth factor (CTGF), platelet-derived growth factor (PDGF), NOX, inflammatory mediators (endotoxin, TLR agonist), and leptin (38, 80, 104). A fibrotic liver can progress to a cirrhotic liver (**Fig. 1**); and 90% of HCCs arise from cirrhotic livers (105).

Addition of DHA to the WD attenuated the WD-mediated fibrosis as quantified by suppression of expression of *Col1A1*, tissue inhibitor of metalloprotease-1 (*TIMP1*), *TGFβ1*, plasminogen activated inhibitor-1 (*PIA1*) and staining of liver for fibrosis using trichrome, a collagen stain (54, 73). Interestingly, EPA did not prevent WD-induced fibrosis. Based on these studies, DHA is the preferred ω-3 PUFA to prevent NASH-associated fibrosis.

The WD and $C_{20\text{-}22}$ ω3 PUFAs affect all major hepatic metabolic pathways.

Additional insight into the impact of the WD and $C_{20\text{-}22}$ ω-3 PUFAs on liver metabolism was gained by using a global non-targeted metabolomic approach. The analysis identified 320 known biochemicals (78). When compared to chow-fed mice, both the WD + olive oil- and WD + DHA-containing diets significantly affected the abundance of metabolites in all major hepatic metabolic pathways including amino acids & peptides, carbohydrate and energy, lipid, nucleotide and vitamins & cofactors. Our studies have identified gene expression and metabolite signatures for NASH (73, 78).

The gene expression signature for NASH includes increased expression of chemokines (*MCP1)*, Kupffer cell surface marker (*CD68*), TLRs and their components (*TLR4, CD14),* enzymes involved in oxidative stress *(NOX2),* stearoyl CoA desaturase (*SCD1)* and collagen (*Col1A1).* The metabolomic signature for NASH includes increased hepatic content of palmitoyl-sphingomyelin, MUFA (16:1ω-7; 18:1ω-7 and 18:1ω-9), α-tocopherol (vitamin E), 5-methyl tetrahydrofolate (5MeTHF); and decreased hepatic content of EPA, DHA and oxidized lipids derived from EPA, specifically 18-hydroxyeicosapentaenoic acid [18-HEPE] and 17,18-dihydroxyeicosatetraenoic acid [17,18-DiHETE]). A volcano plot of the metabolomic and gene expression data illustrates the impact of diet on the hepatic level of these molecules (**Fig. 4**). The metabolites and mRNAs that comprise the metabolomic and gene expression signature were changed dramatically by the WD + olive oil diet, when compared to mice fed the chow diet. These changes were reversed in mice fed the WD + DHA diet.

The oxidized lipids identified in these studies are generated by enzymatic and non-enzymatic processes. 18-HEPE is a resolvin (RvE1) precursor; and resolvins are anti-inflammatory oxidation products of EPA (106). 17,18-DiHETE is an oxidized lipid generated first by CYP2C-catalyzed formation of 17,18-epoxy-eicosatetraenoic acid from EPA; this epoxy fatty acid is converted to the di-hydroxy fatty acid by a epoxide hydrolase to form 17,18-DiHETE. The metabolomic analysis did not detect the 17,18-epoxyETA suggesting that this lipid does not accumulate as a non-esterified lipid. When compared to chow-fed mice, WD + olive oil-fed mice have >60% reduction in hepatic content of 18-HEPE and 17,18-DiHETE. When compared to WD + Olive oil-fed mice hepatic, levels of 18-HEPE and 17,18-DiHETE increased ≥40-fold in mice fed the WD containing EPA or DHA. These dramatic changes in oxidized derivatives of EPA are inversely associated with the severity of NASH. A recent report suggest the Cyp450 epoxygenase pathway may play a key role in regulating hepatic inflammation in fatty liver disease (107). As such, the generation of these oxidized ω3 PUFAs may be hepatoprotective.

Can ω3 PUFA be used to treat human NASH?

Therapeutic strategies for human NASH start with life style management (diet and exercise) and treating the co-morbidities associated with NASH, i.e., obesity, T2D, dyslipidemia. The best strategy for managing NASH, however, has not been established (108). Some clinical approaches to manage NASH included: 1) reduce overall body weight through diet management, exercise or bariatric surgery; 2) pharmaceutical & dietary supplements, i.e., metformin, fibrates, thiazolididiones, statins, ω3 PUFAs; 3) suppress inflammation using TLR modifiers or ω-3 PUFAs); and 4) suppress oxidative stress using vitamin E, silybin and other antioxidants (86, 109-114). Therapeutic regulators of fibrosis, however, are less well-defined (80, 115).

Several clinical trials have reported that ω3 PUFAs lower hepatic fat in obese children and adults with NAFLD (86-91, 116, 117), while others report that fish oil (116) and EPA-ethyl esters (117) do not attenuate the histological features of the disease, like fibrosis. As such, human studies using ω3 PUFAs to treat NAFLD/NASH have yielded mixed results.

The *Ldlr*$^{-/-}$ mouse studies described above suggest that ω3 PUFAs may be an attractive dietary supplement to combat NAFLD and NASH, with the added benefit of preventing NASH-associated HCC. These fatty acids have well-defined effects on hepatic lipid metabolism and inflammation (84, 118); and more recently hepatic fibrosis (54, 73, 119). While several human studies have provided evidence in support of using supplemental ω-3 PUFAs to treat NAFLD (86-91, 116, 117), some studies suggest there may be limitations to the use of ω-3 PUFAs to treat NASH (116, 117). For example, in a recent double-blind, placebo-controlled trial, NAFLD patients received placebo or Lovaza™ at 4 g/d (~50:50 mix of EPA- and DHA-ethyl esters) for 15-18 months. When compared to the placebo-treated group, the Lovaza™ -treated group showed a significant reduction in liver fat without a significant reduction in fibrosis scores.

Since DHA attenuates fibrosis in two separate rodent models of liver injury, i.e., WD-induced fibrosis in mice and BDL-induced fibrosis in rats (54, 73, 119), we speculate that failure of $C_{20\text{-}22}$ ω-3 PUFAs to decrease hepatic fibrosis in humans may be explained by study design. Likely explanations

include the type and amount of ω-3 PUFAs used in the trial. Our studies established that DHA is more effective than EPA at attenuating the onset and progression of NASH (73). Human studies, however, have examined the impact of ω-3 PUFAs on patients with pre-existing disease (86-91, 116, 117). We are unaware of preclinical rodent studies that have assessed the impact of ω3 PUFAs to promote remission or regression of NASH or hepatic fibrosis. As such, more preclinical studies are required to establish the capacity of ω-3 PUFAs to attenuate NASH at various stages in the disease process.

Conclusions and key unanswered questions.

To date, several human studies have indicated that ω-3 PUFAs may be useful in reducing liver fat in obese patients with NAFLD. Moreover, preclinical studies in mice have established that DHA can prevent NASH and NASH-associated fibrosis. It remains unclear whether dietary ω3 PUFAs have the capacity to reverse the NASH, cirrhosis or HCC phenotypes once these diseases are established. Equally important is defining the molecular mechanisms for DHA control of hepatic fibrosis. Finally, changes in hepatic EPA and DHA content significantly impact oxidized lipids derived from ω-3 and ω-6 PUFAs. These oxidized lipids likely play a role in inflammation and will affect the onset and progression of NASH. Whether these oxidized lipids impact the development of NASH, cirrhosis or HCC remains to be determined.

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Figure Legends:

Figure 1: **Transition from normal liver to primary hepatocellular carcinoma (HCC).**

Figure 2: **Factors contributing to the onset and progression of NASH.**

Figure 3: **Effects of the western diet and C_{20-22} ω-3 PUFAs on the prevention of NASH *Ldlr*$^{-/-}$ mice.** The size of the arrow indicated effect size. "No effect" indicates no changes from western diet + olive oil-fed mice. Olive oil was added to the WD to keep all diets isocaloric.

Figure 4: Volcano plots of western diet effects on hepatic metabolites. A metabolomic and transcriptomic analysis was carried out as described (78). Over 300 hepatic metabolites and 6 mRNAs markers of NASH were examined using MetaboAnalyst 3.0 [http://www.metaboanalyst.ca/MetaboAnalyst/] (120). The outcome of this analysis provided a volcano plot. Results are plotted as log2 Fold Change versus –log10 p-value. Several metabolites and RNA transcripts are labeled to illustrate the impact of diet on hepatic abundance of these molecules. Panel A is the comparison of hepatic molecules from Chow-fed versus WD + olive oil-fed *Ldlr*$^{-/-}$ mice. Panel B is the comparison of hepatic molecules from WD + Olive oil-fed mice versus WD + DHA-fed *Ldlr*$^{-/-}$.

Abbreviations:

AGEP, advanced glycation end products; ALA, α-linolenic acid; ALT, alanine aminotransferase; ARA, arachidonic acid; AST, aspartate aminotransferase; CE, cholesterol ester; ChREBP, carbohydrate regulatory element binding protein; Col1A1, collagen 1A1; CTGF, connective tissue growth factor; DAG, diacylglycerol; 17,18-DiHETE, 17,18-dihydroxy-eicosatetraenoic acid; DHA; docosahexaenoic acid; DNL, *de novo* lipogenesis; ECM, extracellular matrix; EPA, eicosapentaenoic acid; FAO, fatty acid oxidation; GLUT, glucose transporter; HMOX1, hemeoxygenase 1; 18-HEPE, 18-hydroxy-eicosapentaenoic acid; IL1β, interleukin-1β; LA, linoleic acid; LDLR, low density lipoprotein receptor; MCP1, monocyte chemoattractant protein-1; 5MeTHF, 5-methyl tetrahydrofolate; MetS, metabolic syndrome; MLX, max-like factor X; MUFA, monounsaturated fatty acids; NAFLD, non-alcoholic fatty liver disease; NASH, non-alcoholic steatohepatitis; NEFA, non-esterified fatty acid; NFκB, nuclear factor κB; NLRP3, NACHT, LRR and PYD domains-containing protein 3; NOX, NADPH oxidase; Nrf2, nuclear factor (erythroid-derived 2)-like 2; p-βOx, peroxisomal β-oxidation; PIA1, plasminogen activator inhibitor-1; PPAR, peroxisome proliferator activated receptor; PDGF, platelet-derived growth factor; PUFAS, polyunsaturated fatty acids; ROS, reactive oxygen species; SCD1, stearoyl CoA desaturase-1; SFA, saturated fatty acids; SREBP, sterol regulatory element binding protein; TAG, triacylglycerol; T2D, type 2 diabetes; TGFβ, transforming growth factor-β; TLR, toll-like receptor; TNFα, tumor necrosis factor-α; VLDL, very low density lipoprotein; WD, western diet.

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Figure 1

Parallels the incidence of obesity & T2D in the US; 6-30% of the general population

3-5% of the general population develop NASH with hepatic inflammation & fibrosis

10-30% of NASH patients develop cirrhosis

2-4% of NASH patients develop HCC

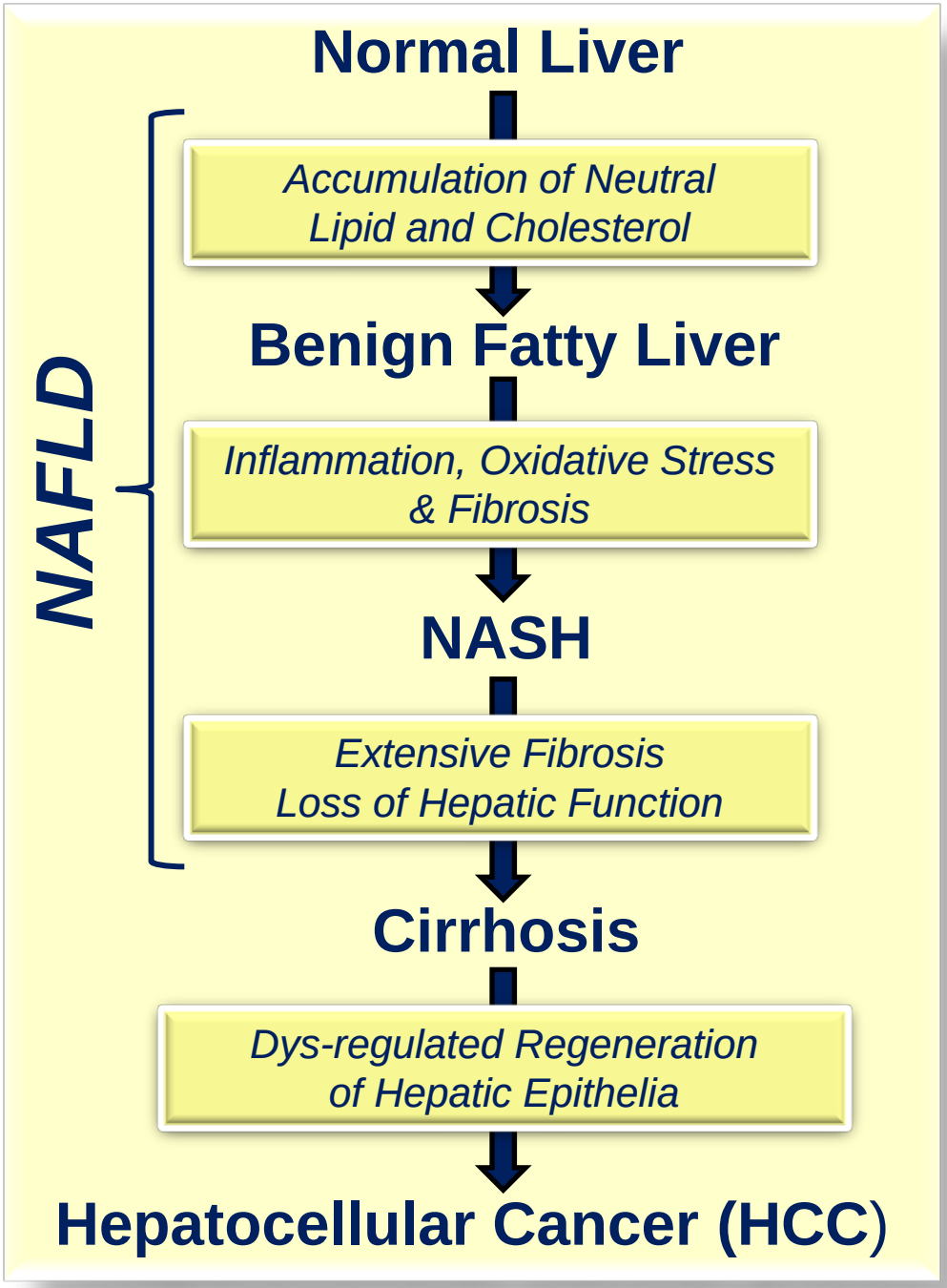


Figure 2

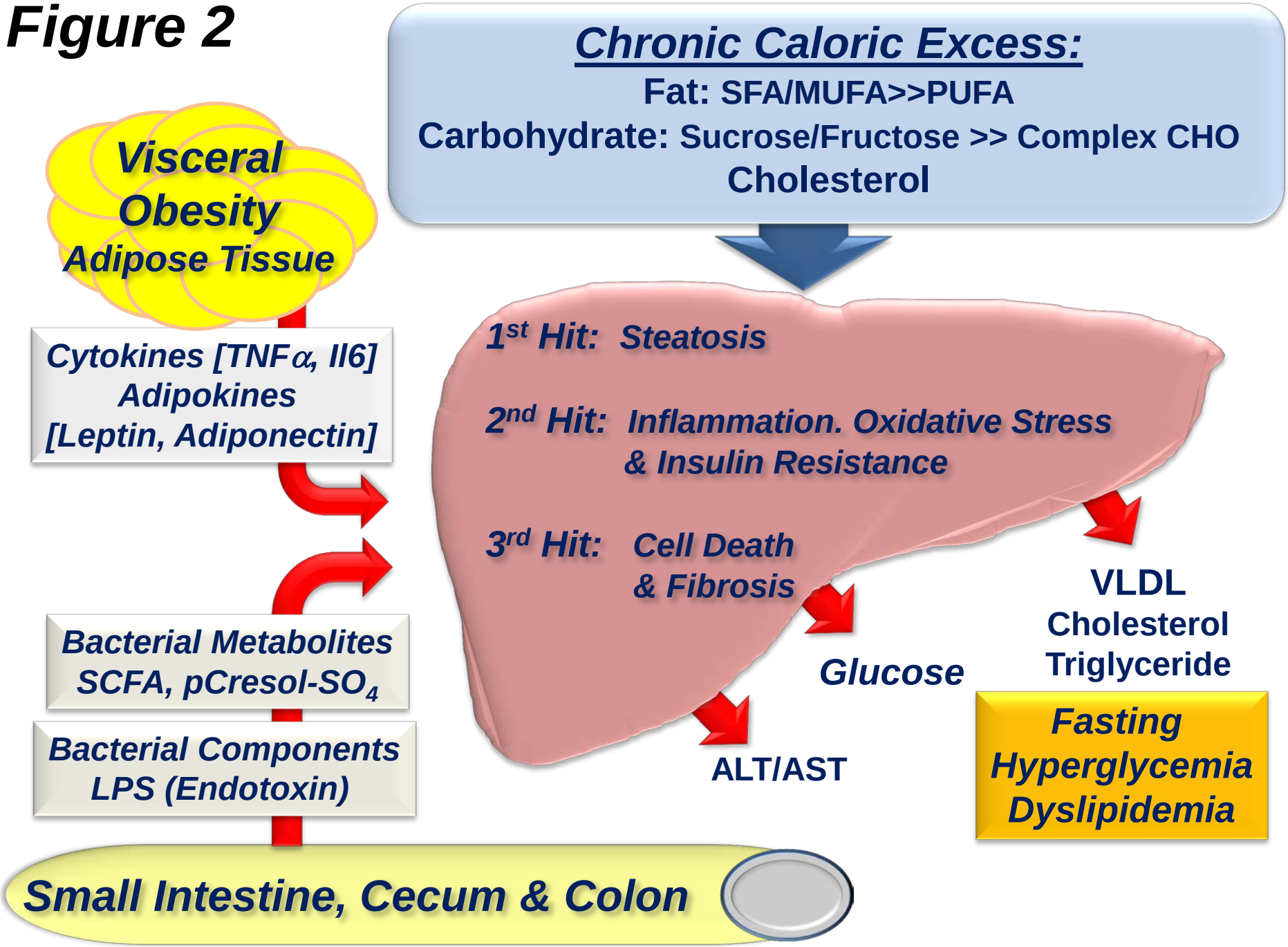

Chronic Caloric Excess:
Fat: SFA/MUFA>>PUFA
Carbohydrate: Sucrose/Fructose >> Complex CHO
Cholesterol
Visceral Obesity
Adipose Tissue
Cytokines [TNFα, Il6]
Adipokines
[Leptin, Adiponectin]
Bacterial Metabolites
SCFA, pCresol-SO4
Bacterial Components
LPS (Endotoxin)
Small Intestine, Cecum & Colon
1st Hit: Steatosis
2nd Hit: Inflammation. Oxidative Stress & Insulin Resistance
3rd Hit: Cell Death & Fibrosis
Glucose
ALT/AST
VLDL
Cholesterol
Triglyceride
Fasting Hyperglycemia Dyslipidemia

Figure 3

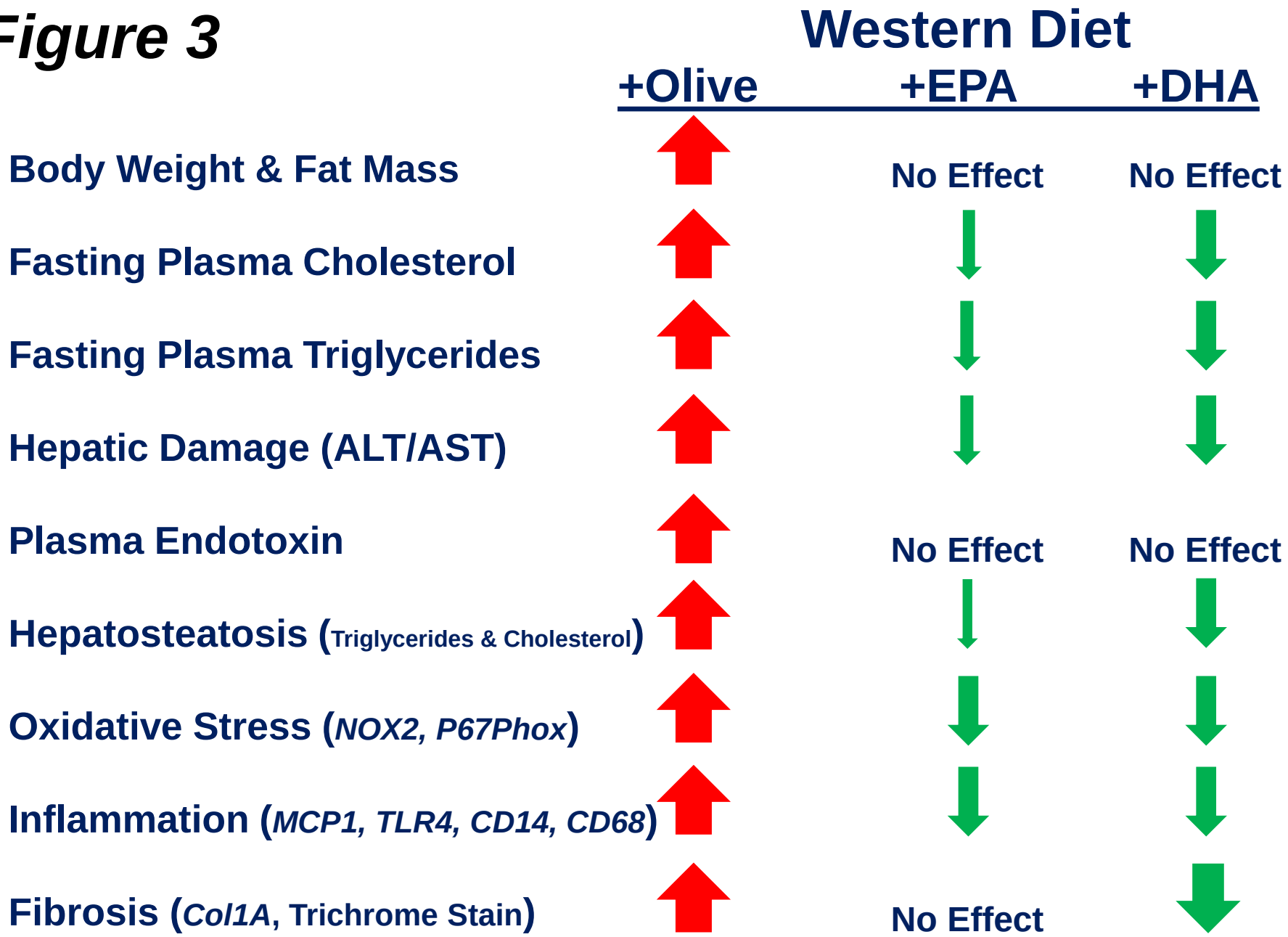
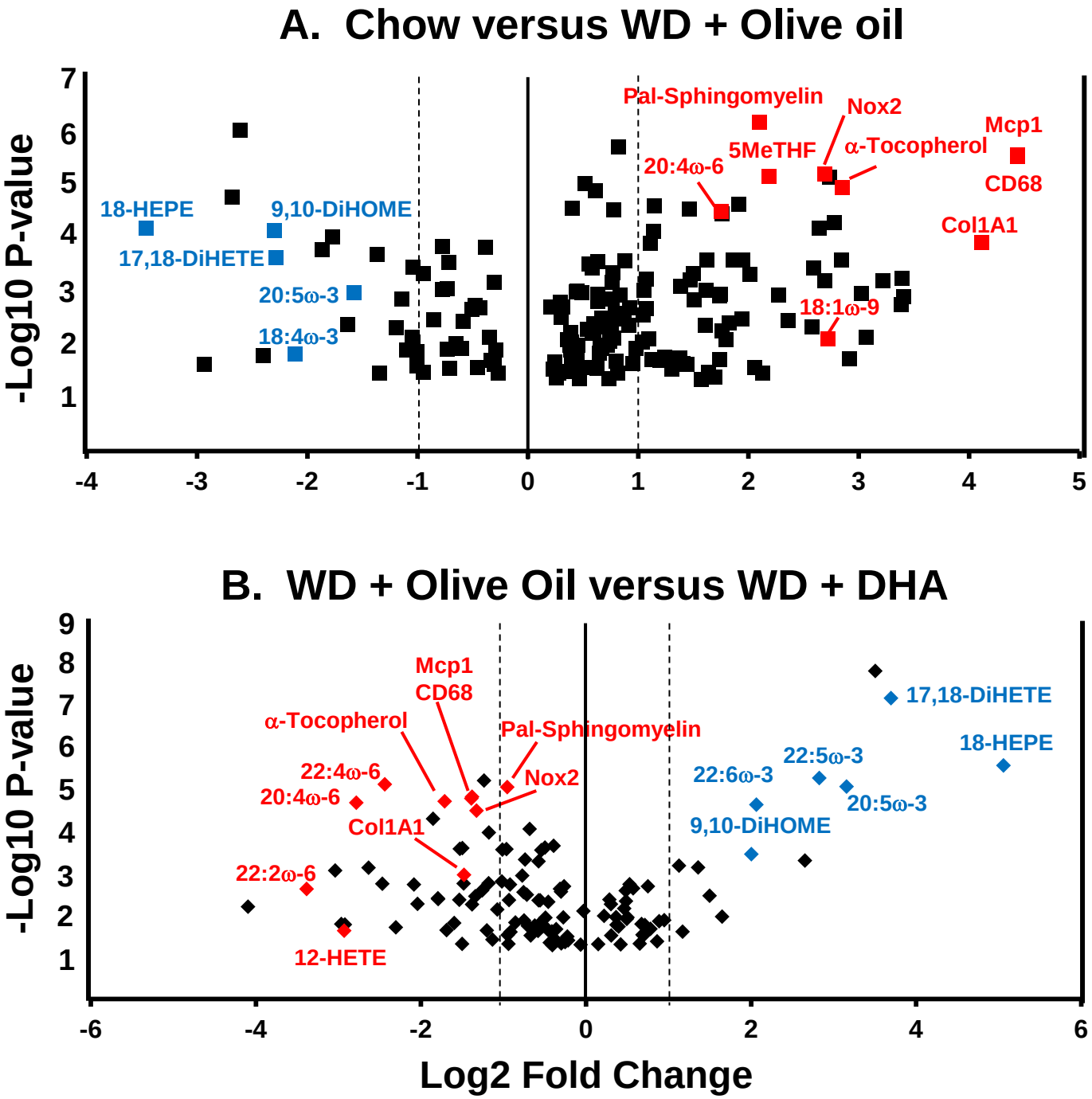

Western Diet
+Olive
+EPA
+DHA
Body Weight & Fat Mass
No Effect
No Effect
Fasting Plasma Cholesterol
Fasting Plasma Triglycerides
Hepatic Damage (ALT/AST)
Plasma Endotoxin
No Effect
No Effect
Hepatosteatosis (Triglycerides & Cholesterol)
Oxidative Stress (NOX2, P67Phox)
Inflammation (MCP1, TLR4, CD14, CD68)
Fibrosis (Col1A, Trichrome Stain)
No Effect

Figure 4


A. Chow versus WD + Olive oil
-Log10 P-value
Pal-Sphingomyelin
Nox2
Mcp1
5MeTHF
α-Tocopherol
20:4ω-6
CD68
Col1A1
18-HEPE
9,10-DiHOME
17,18-DiHETE
20:5ω-3
18:1ω-9
18:4ω-3
B. WD + Olive Oil versus WD + DHA
-Log10 P-value
Mcp1
CD68
17,18-DiHETE
α-Tocopherol
Pal-Sphingomyelin
18-HEPE
22:5ω-3
22:4ω-6
Nox2
22:6ω-3
20:4ω-6
20:5ω-3
Col1A1
9,10-DiHOME
22:2ω-6
12-HETE
Log2 Fold Change