Omega-3 Fatty Acids: mechanisms of benefit and therapeutic effects in pediatric and adult NAFLD

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Abbreviations: NAFLD, Non-alcoholic fatty liver disease; NCDs, non-communicable diseases; HCC, hepatocellular carcinoma; UNOS, United Network Organ Sharing; CVD, cardiovascular disease; CKD, chronic kidney disease; FFA, free fatty acids; IR, insulin resistance; SNP, single-nucleotide polymorphisms; TGs, triglycerides; RCT, randomized clinical trial.

Glossary: PNPLA3, patatin-like phospholipase domain-containing protein-3; VLDL, very low density lipoprotein-triglyceride; IRS, insulin receptor substrates; SREBP1c, sterol regulatory element-binding protein 1c; ChREBP, carbohydrate response element binding protein; JNK, Jun N-terminal kinase; TGF, transforming growth factor; TNF, tumor necrosis factor; HSC, hepatic stellate cells; TLR, Toll-like receptor; NF-κB, nuclear factor kappa beta, IL, interleukin; ROS, reactive oxygen species; EPA, eicosapentaenoic acid; DHA, docosahexaenoic acid; AA, arachidonic acid; FADS, Fatty acid desaturase; Xu5P, xylulose 5-phosphate; PPAR, peroxisome proliferator-activated receptor; PUFA, polyunsaturated fatty acid; CMKLR, chemokine-like receptor; GPR, G-protein coupled receptor; EPA-E, ethyl eicosapentaenoate; DPA, docosapentaenoic acid; NAS, NAFLD activity score.

Keywords: Non-alcoholic fatty liver disease, non-alcoholic steatohepatitis, omega-3, DHA, EPA

Abstract

Non-alcoholic fatty liver disease (NAFLD) is currently considered the most common liver disease in industrialized countries and it is estimated that it will become the most frequent indication for liver transplantation in the next decade. NAFLD may be associated with moderate (i.e. steatosis) to severe (i.e. steatohepatitis and fibrosis) liver damage and affects all age groups. Furthermore, subjects with NAFLD may be at greater risk of other obesity-related complications later in life, and people with obesity and obesity-related complications (e.g. metabolic syndrome, type 2 diabetes and cardiovascular disease) are at increased risk of developing NAFLD. To date there is no licensed treatment for NAFLD and therapy has been mainly centered on weight loss and increased physical activity. Unfortunately, it is often difficult for patients to adhere to the advised lifestyle changes. Therefore, based on the known pathogenesis of NAFLD, several clinical trials with different nutritional supplementation and prescribed drugs have been undertaken or are currently underway. Experimental evidence has emerged about the health benefits of omega-3 fatty acids, a group of polyunsaturated fatty acids that are important for a number of health-related functions. Omega-3 fatty acids are present in some foods (oils, nuts and seeds) that contain also omega-6 fatty acids, and the best sources of exclusively omega-3 fatty acids are oily fish, krill oil and algae.

In this review, we provide a brief overview of the pathogenesis of NAFLD and we discuss the molecular and clinical evidence of benefits of different omega-3 fatty acid preparations in NAFLD.

Introduction

The increasing trend towards overweight and obesity in the general population observed in the past 20 years has increased the risk of developing related conditions such as non-alcoholic fatty liver disease (NAFLD). For this reason, NAFLD has become a very common chronic non-communicable disease (NCD) in the 21st century. Children, adults and the elderly are all vulnerable to the risk factors that contribute to the increased prevalence of NAFLD (1,2). These risk factors mainly include unhealthy diets and physical inactivity, which in turn are also the leading cause of dyslipidemia, insulin resistance and hypertension that are common shared risk factors for other NCDs often overlapping with NAFLD. Therefore, NAFLD should be considered a multisystem disease affecting several extra-hepatic organs that involves multiple metabolic and endocrine pathways (3).

NAFLD encompasses a histological spectrum of liver disease ranging from simple steatosis to non-alcoholic steatohepatitis (NASH) and cirrhosis. While the former has a lower potential for progression to end stage liver disease, NASH with varying degrees of fibrosis can progress to cirrhosis and hepatocellular carcinoma (HCC) (4,5). NASH was the second most frequent indication for liver transplantation amongst adults in the United Network Organ Sharing (UNOS) registry during the years 2004-2013 (6). NAFLD is projected to become the most common indication for liver transplantation in the next decade.

Collectively, these findings highlight the need for a global, multidimensional approach to target NAFLD-associated multiple health-related risks. The findings also emphasise that therapeutic approaches for ameliorating liver disease in NAFLD should also be evaluated for their impact on NAFLD-related extra-hepatic complications.

Due to several misconceptions about NAFLD, progress in developing pharmacological treatments has been slow, and treatment has been primarily aimed at producing weight loss

and increases in physical activity or exercise (7). However, most patients with NAFLD display a long history of unsuccessful attempts at dietary and lifestyle change. Therefore, with the myriad of pathogenetic mechanisms that have been described in the recent years in this common chronic disease, it is crucial to develop inexpensive, effective and safe treatments. Several clinical trials with different drugs, reviewed in (7), have been initiated and are currently completed (7). Although the beneficial effect of omega-3 fatty acid treatment in NAFLD is controversial, this nutritional approach is safe and inexpensive and in our opinion should not be dismissed without further research.

In this review, we provide a brief overview of the pathogenesis of NAFLD and we discuss the molecular and clinical evidence of benefits of different omega-3 fatty acid preparations in NAFLD.

NAFLD definition and epidemiology

Adults and children

NAFLD is defined by a hepatic fat infiltration involving >5% hepatocytes, in the absence of excessive alcohol intake (defined by 20 g ethanol/d for men and 10 g/d for women), chronic viral infection or any other competing cause of liver disease (8).

The term NAFLD incorporates different hepatic abnormalities that can be revealed by tissue biopsy. The histological phenotype in NAFLD includes intrahepatic accumulation of fat alone (simple steatosis), steatosis in association with various degrees of hepatocellular ballooning, Mallory-Denk bodies and necrotic inflammation (NASH) and NASH with or without varying degrees of fibrosis (9). The histological pattern of pediatric NAFLD has distinct characteristics when compared with that of adults, but whether these differences are due to different mechanisms in the pathogenesis, or represent two phenotypes, is still unclear (10).

Although NAFLD is generally a slowly progressive disease, liver-related risk begins early in life: children are reported with NAFLD as early as 2 years and with cirrhosis and HCC as early as age 7, indicating that early recognition and treatment of NASH is warranted to prevent liver-related complications both in childhood and later in life (4,5,11-13). Consistently, current AGA/ACG/AASLD guidelines acknowledge that onset of NAFLD in childhood may greatly increase risk for severe liver-related complications later in life (8).

NAFLD is the most common chronic liver disease in the industrialized world affecting up to 30% of adults and up to 10% of pediatric population (1,2). The prevalence (of NAFLD) approaches 70%-90% in people with obesity and diabetes (1,2), illustrating that the burden of disease caused by this liver condition is considerable across the globe.

Beside liver-related complications, NAFLD is also an emerging risk factor for type 2 diabetes and cardiovascular disease (CVD) (14). In an analysis of 15 population-based and 9 community-based prospective studies, the presence of NAFLD conferred a 2-fold increased risk of incident CVD and of incident type 2 diabetes mellitus, even after adjusting for metabolic syndrome and other traditional confounders (4). As for liver-related complications, NAFLD-related cardio-metabolic risk starts to increase early in life, and the presence and severity of fatty liver have been independently associated with early markers of cardio-metabolic risk, including left ventricular dysfunction, carotid artery intima-media thickness and insulin resistance (15-17). Recently, alterations NAFLD have been also independently associated with subclinical myocardial remodeling and dysfunction in adults, providing further insight into a possible link between NAFLD and heart failure (18).

Lastly, NAFLD has been associated with an increased risk of chronic kidney disease (CKD) and with severity of liver histology, thus predicting advanced stages of CKD independently of traditional risk factors (19). Even in this case, the analysis of the UNOS database during the years 2002-2011 placed NASH-related cirrhosis as an increasing indication for simultaneous liver-kidney transplantation and, of concern, demonstrated a reduced 5-year liver graft, kidney graft, and patient survival in NASH patients in comparison to transplantation for other causes of cirrhosis (20).

NAFLD pathogenesis

Since 1998, when Day and James proposed the ‘‘two hits’’ theory in NASH, the responsible pathogenetic mechanisms have been revisited many times with an evolving improved understanding of the disease (21,22). NAFLD, in fact, is now recognized as a complex multifactorial disease that requires the concurrence of various factors and consequent activation of several cellular response and molecular pathways that involve the liver as the ‘principal actor’ and some other organs as affecting and affected ‘bit players’ (14,23). The development of NAFLD is often triggered by various combinations of unhealthy diets, overweight and genetic predisposition, while the appearance and progression of liver injury towards NASH and fibrosis has been linked to multiple processes involving free fatty acid (FFA) accumulation, insulin resistance (IR), imbalance of adipocytokine levels, oxidative stress, autophagy, apoptosis and gut-derived and host-mediated immune responses (24-26).

Genetic susceptibility

As simple steatosis in NAFLD is observed when hepatic *de novo* FFA synthesis or import exceed the catabolism and export respectively, single-nucleotide polymorphisms (SNPs) in genes that regulate synthesis, storage and export of triglycerides (TGs) may strongly influence the susceptibility to disease (14). A genetic variant (I148M) of the patatin-like phospholipase domain-containing protein-3 (PNPLA3), which exhibits a disrupted enzymatic activity in lipid export, has been found associated with hepatic steatosis and elevated serum levels of ALT in a genome-wide study showing that this variant was most frequent in Hispanic individuals, the ethnic group with the highest occurrence of NAFLD (27). The relevance of this SNP in conferring human susceptibility to NASH and fibrosis progression has been shown in several studies as illustrated by an Italian study in 253 patients with NAFLD (28). Furthermore, it has been reported that the association between the I148M

variant of PNPLA3 and serum levels of liver enzymes was associated with the size of the abdominal fat depot and high dietary carbohydrate and sugar consumption (29,30).

Other studies have shown that some SNPs within genes involved in insulin sensitivity, inflammation and fibrosis may also affect both the mechanism and the extent of steatosis and its progression to NASH. The role of these SNPs in NAFLD has been reviewed by Anstee & Day (24).

Mechanisms inducing hepatocellular steatosis

Hepatic steatosis is a typical trait within the spectrum of liver disease that occurs with NAFLD, appearing as over-accumulation of intra-cellular TGs (FAs esterified to glycerol). Intrahepatic TG accumulation is closely associated with IR and alterations of FFA metabolism within liver and in other peripheral tissues (e.g. skeletal muscle and adipose tissue). Four main mechanisms appear to be responsible for intra-hepatic TG accumulation (**Figure 1**): (i) increased intake of dietary fats; (ii) increased influx of FFAs from enhanced lipolysis in subcutaneous or visceral adipose tissue, or high hepatic *de novo* lipogenesis; (iii) inadequate fatty acid oxidation; or (iv) impaired hepatic export of lipids within very low density lipoprotein-triglyceride (VLDL) (31). Thus, hepatocellular steatosis/TG accumulation occurs when lipid influx and *de novo* synthesis exceeds the rate of lipid utilization by β-oxidation or re-esterification of FFAs to TGs that may be stored as lipid droplets, or packaged and exported as VLDL (32). Liver fat is highly correlated with all the components of the metabolic syndrome; and amongst these components, IR represents the main hallmark of the pathophysiological process (33). The insulin signaling cascade, mediated by tyrosine kinase receptors, leads to phosphorylation of several substrates including insulin receptor substrates (IRS). Insulin stimulation of IRS-1 and -2 triggers activation of intracellular PI3K (phosphoinositide 3-kinase) and AKT/PKB (protein kinase B) pathways, which are involved

in the metabolic effects of insulin. AKT/PKB activation results in translocation of the glucose transporter, GLUT4, to the plasma membrane, facilitating glucose uptake and the expression of key lipogenic genes, with a simultaneous decrease in gluconeogenic gene expression. Furthermore, insulin has a potent action to suppress adipose tissue lipolysis. However, IR in adipose tissue impairs the insulin mediated suppression of hormone-sensitive lipase resulting in peripheral adipose lipolysis and net efflux of FFAs to the liver (34-36). Hyperinsulinemia and hyperglycemia also up-regulate the level of hepatic lipogenic transcription factors including sterol regulatory element-binding protein 1c (SREBP1c) and carbohydrate response element binding protein (ChREBP), thus promoting hepatic synthesis of FFAs, while the resulting decrease of apolipoprotein B-100 affects the normal hepatic lipid export in VLDL (37). SREBP-1c is one of the three transcription factors (SREBP-1a, SREBP-1c, and SREBP-2) involved in the regulation of *de novo* lipogenesis and is expressed in liver, adipose tissue and skeletal muscle. ChREBP is a transcription factor that can activate genes involved in glycolysis and lipogenesis to promote TG synthesis, e.g. glycerol-phosphate acyltransferase, acetyl-CoA carboxylase, and fatty acids synthase (38).

Interestingly, several recent studies have highlighted how the Jun N-terminal kinase, JNK1, signaling pathway may play a major role in the pathogenesis of both IR and NAFLD. JNK1 is activated by circulating FFAs, cytokines and endoplasmic reticulum stress, and may be induced by a high-fat diet in wild-type mice. In turn, activated JNK1 may induce IR, directly *via* phosphorylation of IRS-1 at an inhibitory site and indirectly through induction of pro-inflammatory cytokines in target cells such as macrophages. Healthy mice fed a high-fat diet have elevated levels of activated JNK1 in the liver, adipose tissue and skeletal muscle, together with IR (37,39,40).

Mechanisms leading to NASH and fibrosis

The progression of NAFLD to its more worrisome necro-inflammatory complications occurring in NASH, involves the contribution of multiple mechanisms (**Figure 2**). Increased FFA fluxes in the liver and in other tissues (that influence NAFLD), trigger lipotoxicity *via* multiple pathways. Increased FFA fluxes cause increased oxidative stress, mitochondrial dysfunction, overproduction of cytokines (e.g. tumor necrosis factor-α, TNF-α) and growth factors (e.g. tumor growth factor- β , TGF-β), inhibition of adiponectin in adipocytes, activation of hepatic stellate cells (HSCs) and activation of the endotoxin-mediated innate immune response (41,42). Intrahepatic lipid accumulation may induce direct liver damage by activation of different intracellular pathways, including the Bax and Toll-like receptor 4 (TLR4) pathways, resulting in up-regulation of several pro-inflammatory cytokines and apoptotic signals and a subsequent local necro-inflammatory response (43,44). Both human and animal models show that the close association between chronic hepatic inflammation and hepatic steatosis is mainly mediated by activation of pro-inflammatory transcription factors such as nuclear factor kappa beta (NF-κB). The nuclear translocation of NF-κB may lead to elevated hepatic expression of inflammatory cytokines such as TNF-α, interleukin-6 (IL-6) and IL-1β, and activation of Kupffer cells. FFAs may directly activate the Ikk-b/NF-κB pathway in hepatocytes (45). Feldstein et al, demonstrated that simply incubating HepG2 cells in FFA-containing medium increased lysosomal permeability and triggered synthesis of TNF-α (46). TNF-α is a pleiotropic cytokine that activates signaling mechanisms that may culminate in hepatocyte apoptosis, activation of HSCs, and hepatic accumulation of inflammatory cells. In addition to its pro-inflammatory effects, TNF-α promotes IR. Elevated levels of TNF-α were detected in obese patients with IR and also in patients with NASH in whom TNF-α levels correlated with histological severity (47). Finally, the excessive rate of lipid oxidation due to increased hepatic FFA load may promote an overproduction of reactive

oxygen species (ROS) with subsequent generation of toxic lipid peroxides leading to oxidative stress, activation of inflammatory pathways, and mitochondrial damage (48). For this reason, oxidative stress is considered one of the key second "hits" that plays an important role in the progression from steatosis to NASH and cirrhosis. Furthermore, electron microscopy of hepatocytes from patients with NAFLD shows the presence of significant mitochondrial structural abnormalities which may explain the alteration in mitochondrial respiratory chain activity in patients with NASH (49). It has been suggested that mitochondrial abnormalities may be the consequence of two events. From one perspective, FFA β-oxidation may take place within an environment of preexisting defects in mitochondrial oxidative phosphorylation. From another perspective, it is possible that impaired mitochondrial function may simply be a consequence of increased lipid peroxidation, since lipid peroxidation products alter both mitochondrial DNA and respiration. Finally, mitochondrial dysfunction increases TNF-α expression resulting in additional inhibition of mitochondrial electron transport and release of ROS that in turn stimulates lipid peroxidation and consequent hepatic injury (50).

Omega-3 fatty acid and lipid metabolism in NAFLD: effects in the liver and other relevant tissues

Omega-3 and omega-6 polyunsaturated fatty acids are classified based on the location of the last double bond relative to the terminal methyl end of the molecule. Both fatty acids are obtained through the diet and metabolized in the liver (51). Omega-3 and omega-6 fatty acids are important components of cell membranes modulating microdomain composition, membrane fluidity, flexibility and permeability. Moreover, they also influence membrane receptor signaling, protein activity, lipid metabolism and are involved in the regulation of gene expression (52,53). The metabolic pathway for the biosynthesis of omega-3 and omega-6 fatty acids involves several common enzymatic reactions including desaturation, elongation and oxidation (**Figure 3**). In the liver, dietary α-linolenic acid [ALA (18:3ω-3)] and linoleic acid [LA (18:2ω-6)] are metabolised by two key enzymes Δ6 and Δ5 desaturase to form eicosapentaenoic acid (EPA; 20:5ω-3) and docosahexaenoic acid (DHA; 22:6ω-3) or arachidonic acid (AA; 20:4ω-6), respectively. The first reaction involving Δ6-desaturase is a rate-limiting step and therefore the excess of one polyunsaturated fatty acid (e.g. ω-6 LA) can significantly affect the conversion of the alternative fatty acid (e.g. ω-3 ALA). Thus, the optimal ratio of omega-6 to omega-3 fatty acids should be 1-4:1 as importantly both Δ6 and Δ5 desaturases have greater affinity for omega-3 fatty acids (51,54). In the industrialized diet there is markedly higher consumption of omega-6 fatty acids compared with omega-3 fatty acids, with consequent increased production of ω-6 AA from ω-6 LA, and likely impaired production of ω-3 EPA and ω-3 DHA from ω-3 ALA. In healthy individuals, the conversion rate of ALA to EPA is less than 5% to 10% and to DHA is only 2 to 5% (55).

Several factors can influence the activities of Δ5 and Δ6-desaturases, including diet, obesity, insulin resistance, oxidative stress, and liver disease. Araya et al. showed that Δ5 and Δ6-

desaturase activities were lower in obese NAFLD patients, compared with non-obese non-NAFLD subjects. Fatty acid desaturase 1 and 2 (FADS1 and FADS2) are the two genes that encode the enzymes Δ5 and Δ6 desaturase, respectively (56). Recent evidence indicates that the rs174547 allele variant in the FADS gene cluster is associated with decreased desaturase activity affecting plasma levels of both omega-3 and omega-6 fatty acids (57).

Omega-3 fatty acids have beneficial effects in the regulation of hepatic lipid metabolism, adipose tissue function, and inflammation, reducing hepatic TG accumulation (54). In the liver, TGs are packaged into VLDLs and then transported to peripheral tissues. Newly formed VLDL contains only apolipoprotein B-100 that is a ligand for activation of the LDL-R (58) and omega-3 fatty acids lower TG concentration by suppressing hepatic VLDL apoB production. DHA in particular, inhibits the secretion of apoB100 by promoting its autophagic degradation (59). Omega-3 fatty acids down-regulate gene expression of several transcriptional factors involved in lipogenesis and TG accumulation in NAFLD. At the transcriptional level, omega-3 fatty acids can down-regulate gene expression of SREBP-1c by inhibiting activation of the liver X receptor (LXR), thereby reducing lipogenesis (58,60). Interestingly, Jump et al. have shown that DHA has a specific role in down-regulating gene expression of SREBP-1c, through the 26S proteasomal degradation of nuclear SREBP-1c (61). High concentrations of blood glucose may also promote TG accumulation in the liver by increasing xylulose 5-phosphate (Xu5P) production (62). Xu5P activates a protein phosphatase that stimulates the translocation of ChREBP from the cytosol to the nucleus activating glycolytic and lipogenic gene expression that in turn promotes TG accumulation in the liver.

Omega-3 fatty acids are activators of peroxisome proliferator-activated receptors (PPARs) that act as nuclear transcription factors and regulate the expression of genes involved in lipid,

carbohydrate, and protein metabolism (63,64). There are three PPARs: 1) PPARα is mainly expressed in the liver and it is involved in fatty acid β-oxidation, 2) PPARβ/δ is expressed in adipose tissue and induces β-oxidation, and 3) PPARγ is expressed in adipose tissue and inflammatory cells and promotes TG storage in adipocytes. Omega-3 fatty acids activate PPARα and PPARβ/δ promoting mitochondrial and peroxisomal fatty acid oxidation, decreasing intrahepatic TG accumulation (53). Moreover, the activation of PPARγ by omega-3 fatty acids decreases the production of pro-inflammatory cytokines such as TNF-α and IL-6 that in turn reduces hepatic inflammation (54).

Omega-3 fatty acids and resolution of inflammation in NAFLD: evidence from in vitro, animal studies and man

Adipose tissue produces a range of pro-inflammatory cytokines (e.g. TNF-α, IL-6 and IL-8) as well as chemokines and acute phase proteins, and production of these is increased in obesity, especially visceral obesity (65-67). Consequently obese subjects have elevated concentrations of inflammatory cytokines and chemokines, and reduced concentrations of anti-inflammatory adiponectin, in their bloodstream (68-71). These mediators may elicit metabolic and inflammatory changes in target tissues including the liver (72). In addition, exposure of the liver to enhanced levels of FFAs and generation of lipid-derived second messenger molecules such as diacylglycerols, activate hepatic inflammation through NF-κB and other inflammatory signaling pathways (72,73). Hepatic oxidative stress and inflammation may act together to induce hepatocyte damage in people with NAFLD. Another aspect of hepatic inflammation in NAFLD may be exposure to bacterial endotoxin (also known as lipopolysaccharide) as a result of gut "leakiness" (**Figure 2**). Endotoxin is a potent activator of the NF-κB pathway and the inflammatory response in all liver-resident cells. The control of the inflammatory response may be an important strategy for limiting the severity and progression of NAFLD.

The omega-3 fatty acids EPA and DHA have a number of important roles in regulating inflammatory processes and in reducing damaging inflammation (reviewed in detail in 74,75). When intake of EPA and DHA is increased, the contribution to these fatty acids within phospholipids of the membranes of cells involved in inflammation is increased. Normally these cells contain a high proportion of the omega-6 , polyunsaturated fatty acid (PUFA) AA, which is the precursor for the synthesis of eicosanoids produced by the action of cyclooxygenases (prostaglandins, thromboxanes) and lipoxygenases (leukotrienes). AA-

derived eicosanoids such as prostaglandin E_2, leukotriene B_4 and the 4-series cysteinyl leukotrienes are known to play central roles in inflammatory processes. Eicosanoids may affect hepatocyte metabolism directly (76), including promoting de novo lipogenesis and fat accumulation (77,78), or they can modulate the regulation of hepatocyte metabolism by hormones (79,80) or cytokines (81,82).

Incorporation of EPA and DHA into inflammatory cells is largely at the expense of AA, thus decreasing the availability of the usual substrate for eicosanoid synthesis. In accordance with this, increased intake of EPA and DHA has been shown to decrease pro-inflammatory eicosanoid production by inflammatory cells (74,75). In addition, EPA is a substrate for cyclooxygenases and lipoxygenases, giving rise to eicosanoids which differ in structure from those produced from arachidonic acid (they have an additional double bond) and often with lower biological potency (74,75). Novel families of lipid mediators, termed resolvins, protectins and maresins, produced from EPA and DHA have been discovered (83,84). The key function of these mediators appears to be in the resolution of inflammation and so they have been termed “specialized pro-resolving lipid mediators”. Recent studies have begun to address the possible roles of resolvins and protectins in the liver and in NAFLD. Resolvin D1 was able to attenuate hypoxia-induced expression of cyclooxygenase-2, IL-1β, IL-6, and C-C chemokine receptor type 7 in liver slices taken from mice with diet-induced obesity (85). This effect was not seen in liver slices depleted of macrophages, suggesting inflammatory macrophages as the target for resolvin D1. Treating diet-induced obese mice with resolvin D1 increased adiponectin expression, reduced liver macrophage infiltration, skewed macrophages from an M1- to an M2-like anti-inflammatory phenotype, induced a specific hepatic miRNA signature, and reduced inflammatory adipokine expression (85). An earlier study had identified a possible protective role for the resolvin E1 receptor chemokine-like

receptor 1 (CMKLR1) in NAFLD (82). CMKLR1 was detected in primary human hepatocytes, Kupffer cells, bile-duct cells and hepatic stellate cells, but was decreased in human and rodent fatty liver and in fibrotic liver of mice. Adiponectin strongly upregulated CMKLR1 in primary human hepatocytes and in liver tissue while hepatic CMKLR1 was suppressed in the liver of adiponectin deficient mice (86). A recent study showed that pretreatment with resolvin D1 attenuated endoplasmic reticulum stress-induced apoptosis and decreased caspase 3 activity in HepG2 cells (87). These studies suggest that EPA and DHA derived pro-resolution mediators may have a role in reversing the metabolic and inflammatory disturbances seen in NAFLD and they support a role for cell and tissue enrichment in the precursor n-3 PUFAs.

In addition to their effects on lipid mediators, EPA and DHA influence several other aspects of inflammatory processes including leukocyte migration and the production of inflammatory cytokines including TNF-α, IL-1 and IL-6 by monocytes, macrophages and endothelial cells (74,75) and by Kupffer cells (88). These effects also seem to involve incorporation of EPA and DHA into the membranes of inflammatory cells from where they influence cell signaling, transcription factor activation and gene expression (74,75). A key target for omega-3 PUFAs is the pro-inflammatory transcription factor NF-κB which is involved in up-regulation of the genes encoding a number of proteins involved in inflammation including many cytokines, adhesion molecules and cyclooxygenase-2. N-3 fatty acids have been shown to decrease activation of NF-κB in various cell types and this involved decreased phosphorylation of its inhibitory subunit (74,75). Wong et al. (89) demonstrated that DHA inhibited the ability of inflammatory stimuli to initiate recruitment of signaling proteins into raft regions of macrophage membranes, suggesting a membrane-mediated effect of omega-3 PUFAs in down-regulating NF-κB activation.

A second mechanism by which omega-3 PUFAs might influence NF-κB activation involves the anti-inflammatory transcription factor PPAR-γ. One of the actions of PPAR-γ is to physically interfere with the translocation of NF-κB to the nucleus. PPAR-γ can be activated by n-3 fatty acids and their derivatives and DHA induced PPAR-γ in dendritic cells, an effect associated with inhibition of NF-κB activation and reduced production of the pro-inflammatory cytokines TNF-α and IL-6 (63). Furthermore, DHA induced a number of known PPAR-γ target genes in dendritic cells (90), suggesting that this is an important anti-inflammatory mechanism of action of DHA and perhaps also of EPA. Lipid mediators produced from EPA and DHA can also bind and regulate PPAR-γ, this being an important mechanism by which n-3 fatty acids affect inflammatory processes. For example, the EPA derivatives prostaglandin D_3 and 15-deoxy-prostaglandin D_3 activate PPAR-γ in adipocytes, a process linked to the induction of the anti-inflammatory adipokine adiponectin (91). Thus, activation of PPAR-γ may itself be one of the anti-inflammatory mechanisms of action of n-3 fatty acids and this may also link to the inhibition of NF-κB activation described above.

Fairly recently a further mechanism by which n-3 fatty acids can affect NF-κB activation has been revealed. G-protein coupled receptor (GPR)120 is expressed on adipocytes and macrophages (92). One function of GPR120 is that it can bind long chain fatty acids and is involved in anti-inflammatory signaling. Both EPA and DHA enhanced GPR120-mediated gene activation. More detailed examination of the effects of DHA revealed that its ability to inhibit macrophage inflammation (i.e. such as inhibition of IκB kinase phosphorylation, of IκB phosphorylation and degradation, and of TNF, IL-6 and MCP-1 production) was abolished in GPR120 knockdown cells (92). These findings suggest that the inhibitory effect of DHA (and probably also of EPA) on NF-κB might occur via GPR120 which induces signaling that interferes with the pathway that activates NF-κB.

Omega-3 fatty acids as a treatment for NAFLD in man

Clinical evidence in the adult population

The effects of omega-3 fatty acid treatment in NAFLD have recently been summarized in a meta-analysis and systematic review (93). Parker and colleagues reviewed the use of omega-3 fatty acid supplementation in adults with NAFLD. Included studies employed trials involving oral administration of omega-3 fatty acids in male and/or female adults (⩾18 years), including both NAFLD and NASH cohorts where this was specified. In this review, the median duration of treatment with omega-3 fatty acids was 6 months (range: 8 weeks to 12 months) and the median dose of PUFAs was 4 g/day (range: 0.8–13.7 g/day). Six studies specified the dosage of EPA and DHA (range: 0.375–4.626 g of EPA per day; 0.24–2.24 g of DHA per day) and the authors found no reports of adverse effects of omega-3 PUFA supplementation in the studies reviewed.

Studies were excluded if they addressed alcoholic, drug-induced, total parenteral nutrition-induced, viral or genetic causes of liver injury. Outcome measures used were: liver fatness quantified by needle biopsy and histological assessment, proton magnetic resonance spectroscopy (^{1}H MRS), or inferred by ultrasonography; and plasma alanine aminotransferase (ALT) and aspartate aminotransferase. The authors performed three analyses to compare the effect of (i) PUFA *vs*. control on liver fat change, (ii) PUFA *vs*. control on ALT change, and (iii) PUFA *vs*. control on AST change. Sub-analyses were also performed *a priori* for studies, which used randomized clinical trial (RCT) design. Only four of the included studies were RCTs and not all these four studies included objective assessments of change in NAFLD severity obtained by either imaging modalities or biopsy (94-97).

One problem in assessing the effects of omega-3 fatty acid treatment in NAFLD has been the use of imprecise, low sensitivity measures to assess effects of treatment on disease severity.

For example, Capanni et al. tested the effects of 1 g per day of EPA in 42 patients with NAFLD for a period of 12 months. Using the semi-quantitative technique of ultrasound to assess improvements in liver fat, following treatment there was an improvement in liver fat quantity in the treatment group compared to the control group (97). Additionally, Spadaro et al. compared the effects of 2 g per day of omega-3 fatty acids combined with AHA diet recommendations versus the effects of AHA diet recommendations alone, in patients with NAFLD. After 6 months, there was an improvement of liver fat content measured by ultrasound, as well as decreased serum TG levels, decreased alanine aminotransferase and TNF-α levels, and an improvement in insulin sensitivity in the omega-3 fatty acid group (98). Also Zhu et al. studied the effects of 6 g per day of EPA plus docosapentaenoic acid (DPA; 22:5ω-3) and DHA in subjects with NAFLD and hypertriglyceridemia; 144 patients were randomly assigned to treatment or placebo. The duration of the intervention was 6 months and there was an improvement in liver fat content also measured by liver ultrasound in the treatment group compared with placebo group (96). Thus several studies to date that have assessed the effects of omega-3 fatty acids in NAFLD have used imprecise non-specific tests (such as liver ultrasound) to assess change in NAFLD severity with treatment. Additionally, several of the studies to date have been non-randomized studies or not placebo controlled (**Table 1**).

In contrast, very recently Sanyal et al. have tested the effects of two different doses of the ethyl ester of eicosapentaenoic acid (EPA-E) EPANDEL for 12 months in a high quality trial of 243 patients with NAFLD, and used histological end-points to determine whether there was a benefit of omega-3 fatty acid treatment (99). EPADEL is highly purified EPA ethyl ester (EPA-E; ethyl eicosapentaenoate) and has been on the market in Japan for approximately 20 years for the treatment of hyperlipidemia. The study was performed at 37

sites in North America and included subjects with NASH and NAFLD activity scores ≥ 4, with minimum scores of 1 for steatosis and inflammation, along with either ballooning or at least stage 1a fibrosis. A total of 243 subjects were randomly assigned to groups given placebo (n = 75), low-dosage EPA-E (1800 mg/d; n = 82), or high-dosage EPA-E (2700 mg/d; n = 86) for 12 months. The primary efficacy end point was NAFLD activity score (NAS) ≤ 3, without worsening of fibrosis, or a decrease in NAS by ≥ 2 with contribution from >1 parameter, without worsening of fibrosis. Similar proportions of subjects in each group met the primary end point (40%, 37%, and 35.9% for placebo, low-dosage, and high-dosage EPA-E, respectively). EPA-E had no significant effects on steatosis, inflammation, ballooning, or fibrosis scores. Most surprisingly, in this study, high dose EPA-E had only a small beneficial effect in lowering serum TG concentrations by 6.5 mg/dL (0.07 mmol/L) from a baseline concentration of 153 mg/dL (1.74 mmol/L). Although there was no significant difference in the between groups TG-lowering effect of EPA-E treatment, a paired comparison between the high dose EPA-E group and the placebo group showed a significant difference (-6.5 mg/dL high dose EPA-E vs an increase of 12 mg/dL (0.14 mmol/L) in the placebo group; P = 0.03). As to why there was a very minimal effect of EPA-E treatment to reduce serum TG is uncertain and although the results of this well conducted and important phase 2 trial, showed that EPA-E had no significant effect on the histologic features of NASH, it is important to note that EPA only (and not DHA) was tested, and whether a higher dose of EPA would have produced any benefit is unclear.

In the liver EPA can be further elongated to form DPA by the enzyme fatty acid elongase-2 and 5. DPA can also be retroconverted to EPA by peroxisomal acyl-CoA oxidase and one cycle of β-oxidation while Δ6-desaturase converts DPA to DHA (100,101). There are several factors that can influence the activity of Δ6-desaturases, including diet, insulin concentration,

oxidative stress and liver disease. Notably, decreased activity of both Δ5-desaturase and Δ6-desaturase has been shown in the liver of obese NAFLD patients (56). High omega-6 fatty acid intake in the diet also alters the activity of Δ5-desaturase and Δ6-desaturase, increasing the production of AA and decreasing that of EPA. It has been suggested that an alteration in the omega-6 fatty acid to omega-3 fatty acid ratio and the consequent increase of AA-derived eicosanoids may be important in the pathogenesis of NAFLD (102). In hepatocytes, DPA has additional effects: interfering with PPARα in the regulation of β-oxidation and suppression of lipogenic genes in cultured liver cells. Nevertheless, it is thought that the effects of DPA in the liver are not as pronounced as those of EPA and DHA which are known to have greater affinity for PPARα and a more significant impact on the regulation of lipogenesis (103).

Interesting new data suggest a role for impaired hepatic fatty acid desaturation and an unbalanced omega-6 to omega-3 ratio in the pathogenesis of NASH. In a study integrating gene expression profiling of liver biopsies from patients with NASH together with translational studies in mouse hepatocytes, it has been shown that increased expression of Δ5 and Δ6 desaturases (at both mRNAs and protein levels) occurred in the livers of obese mice fed a high fat diet (104). Gas chromatography analyses confirmed impaired fluxes towards the omega-3 fatty acids resulting in an increased omega-6 to omega-3 ratio and a reduced omega-3 index in both the human and mouse livers. Restoration of hepatic omega-3 content in a transgenic mouse model expressing *Caenorhabditis elegans fat-1* gene (which specifically encodes an omega-3 desaturase capable of generating omega-3 fatty acids from omega-6 fatty acids) reduced hepatic insulin resistance, steatosis, macrophage infiltration, necro-inflammation and lipid peroxidation, accompanied by attenuated expression of genes involved in inflammation, fatty acid uptake and lipogenesis. Additionally, this mouse was resistant to the effects of a high fat diet and did not develop hepatic steatosis or significant

hepatic insulin resistance on the diet, emphasising that increasing endogenous omega-3 fatty acids may be important to ameliorate liver disease in NASH.

A comprehensive analysis of plasma lipids and eicosanoid metabolites quantified by mass spectrometry has been performed in 25 people with NAFLD and 50 people with NASH and the data compared with 50 lean normal control subjects (105). The key findings of this study were that there was markedly increased total plasma monounsaturated fatty acids palmitoleic (16:1 n-7) and oleic (18:1 n-9) acids in patients with both simple steatosis and NASH. The levels of palmitoleic acid, oleic acid, and the palmitoleic acid to palmitic acid (16:0) ratio were significantly increased in NAFLD across multiple lipid classes. Interestingly, the DHA to DPA ratio was significantly decreased within phosphatidylcholine, and phosphatidylethanolamine pools, and this finding was most marked in subjects with NASH. However, whether all long chain omega-3 PUFAs have the same effect in NAFLD is uncertain.

It is possible that DHA and EPA have different effects in NAFLD. In contrast to the study by Puri et al. described above, Scorletti et al. have shown in 103 patients that there was a benefit of 4 g of omega-3 fatty acid treatment/day to decrease liver fat percentage in patients in whom there was good tissue enrichment with DHA (106,107). In this randomized, double blind placebo-controlled trial patients with NAFLD were randomized to DHA+EPA as ethyl esters (n=51), or to olive oil placebo (n=52)]. Liver fat percentage was quantified by magnetic resonance spectroscopy in three liver zones and liver fibrosis was measured using two validated scores (108,109). Adherence to the intervention (Omacor group) and contamination (with DHA and EPA) (placebo group) was assessed by measuring erythrocyte DHA and EPA enrichment (gas chromatography). In the fully adjusted regression model there was a not statistically significant trend towards improvement in liver fat% with

DHA+EPA treatment but there was evidence of contamination with omega-3 fatty acid consumption in the placebo group. Further regression analysis showed that DHA enrichment was independently associated with a decrease in liver fat% (for each 1% enrichment, β=-1.70 (95%CI -2.9,-0.5); p=0.007). No improvement in the fibrosis scores occurred. Additionally, in a further analysis of the treatment effects in this cohort, although the numbers of subjects with the PNPLA3 148MM genotype was small, there was a suggestion that there was no effect of DHA enrichment to decrease liver fat in patients with this specific PNPLA3 genotype (110). These data provide intriguing evidence for a DHA treatment-PNPLA3 genotype effect, to modify the impact of the treatment with omega-3 fatty acids to decrease liver fat in NAFLD.

Recently, Argo et al. have assessed the effects of n-3 polyunsaturated fatty acids vs. placebo while adjusting for the impact of age and weight change on the histological and metabolic effects in NASH patients (ClinicalTrials.gov: NCT00681408) (111). The omega-3 fatty acid dose of 3 g/day is the safe dose recommended by the FDA and is the American Heart Association's upper dose for triglyceride reduction (112). Each 1000 mg capsule contained 70% total omega-3 fatty acids in form of TGs: 35% EPA, 25% DHA, 10% other omega-3 fatty acids, and a little amount of lemon oil. The placebo therapy consisted of identical appearing capsules, containing predominantly soybean oil but also small amounts of fish and lemon oils (only 8% omega-3 fatty acid) to protect blinding. Forty-one subjects with non-cirrhotic NASH were enrolled, and 34 completed the study. 17 received omega-3 fatty acid and 17 received placebo daily for 1 year, with typical counselling on caloric intake and physical activity for all subjects. Whilst omega-3- and placebo-treated groups showed no significant difference for the primary end point of NAS reduction ≥2points without fibrosis progression, among subjects with increased or stable weight, those treated with omega-3 fatty

acids showed a larger decrease in liver fat content by magnetic resonance imaging than placebo-treated subjects. Omega-3 fatty acid treatment also showed significant fat reduction on the paired analysis of image-assisted fat morphometry regardless of weight loss or gain.

Since CVD is very common in people with NAFLD (17, 113) and people with NAFLD are at increased risk of CVD (114, 115), the optimal dose of omega-3 PUFA is likely to be much higher than 0.83 g/day (93). In a large-scale intervention trial of secondary prevention after myocardial infarction, GISSI-Prevenzione investigators identified a substantial reduction in all-cause and cardiovascular mortality with 1 g per day of n-3 PUFAs as ethyl esters (116). However, omega-3 PUFA supplementation may be beneficial for decreasing CVD in NAFLD (117), at a dose of 2 to 4 g per day (118) and the results of numerous clinical trials suggest that omega-3 PUFAs could act as beneficial pleiotropic agents to prevent CVD (117). That said, the mode of administration of the omega-3 fatty acid remains is uncertain. For example, a FFA form of omega-3 PUFA has been found to produce a four-fold higher area under the plasma omega-3 PUFA curve than prescription omega-3-acid ethyl esters in patients on a low-fat diet (119).

Clinical evidence in children population

To date, only two clinical trials have reported data about the outcome of disease in children with NAFLD under treatment with omega-3 fatty acids (**Table 1**). The first study, by Nobili et al., is a double-blind RCT that evaluated in 60 children with biopsy-proven NAFLD the efficacy of 250 mg/day or 500 mg/day DHA versus placebo after 6 months of therapy (120). The authors found that DHA supplementation to the diet and exercise ameliorated the body mass index, the insulin sensitivity index, serum ALT and TG levels, and steatosis at ultrasound. Interestingly, both doses of DHA appeared to be equally effective in reducing

lives steatosis. During a long-term follow-up (12, 18 and 24 months) patients maintained the improvement of metabolic and biochemical parameters, but more importantly there was persistence of the liver fat decrease evaluated via ultrasound and liver biopsy (121, 122). In particular, the follow-up study demonstrated that DHA may improve liver steatosis, hepatocellular ballooning and inflammation even if it is ineffective on fibrosis (123). One postulated mechanism for the effect of DHA on NAFLD-related liver damage is a reduction of ductular reaction and hepatic progenitor cell activation, that was previously found activated in pediatric NAFLD, and down-sizing of pro-inflammatory liver resident macrophages by a mechanism mediated by GPR120 (123, 124).

A second multicenter, double-blind, RCT in children was performed by Janczyk et al. (125). In this study 76 children with hyperechogenicity of the liver on ultrasound or liver histology consistent with NAFLD were treated for 24 weeks with 450-1300 mg omega-3 fatty acids daily (DHA:EPA=3:2) or with an equal dose of sunflower oil containing omega-6 fatty acids. At the follow-up visit after 6 months of a low-calorie diet and physical exercise after discontinuation of study treatment, the data on 64 children that completed the study revealed that supplementation with DHA and EPA reduced the levels of ALT and lipids and improved liver steatosis at ultrasound and insulin resistance to the same extent as placebo (126). Moreover, the authors reported a minimal significant reducing effect on aspartate aminotransferase and gammaglutamyl transpeptidase and an increase in circulating adiponectin.

As stated by Alkhouri et al. (127) there are several potential explanations for discrepant results obtained with omega-3 supplementation in children with NAFLD. Specifically, the biological activities of the different omega-3 fatty acids vary according to their structure and each compound could exert different effects in NAFLD. Although the reason for a

discrepancy in findings between the effects of DHA and EPA in NAFLD is uncertain, there is considerable evidence that EPA and DHA have different biological roles in humans (128). The variable response to omega-3 fatty acid treatment could also be influenced by the broad spectrum of liver disease in NAFLD. Furthermore, the pathogenesis of NAFLD, and perhaps the response to treatment, is also influenced by different genetic and epigenetic factors, and these factors may profoundly influence the efficacy of specific treatments (129). In support of this assertion, that is supported by the data discussed earlier from adults with NAFLD in the WELCOME study (110), Nobili et al. (130) found that in children with NAFLD, the presence of the PNPLA3 I148M gene polymorphism, may reduce the efficacy of DHA on fatty liver.

Future directions

The growing obesity epidemic is believed to be a key player involved in the increasing prevalence of NAFLD**.** The first line of NAFLD treatment to date has been lifestyle interventions such as changes in dietary habits and in physical activity. However, due to poor patient compliance to lifestyle interventions, effective nutritional supplementations and/or targeted drugs are urgently needed to counteract the epidemic of NAFLD that is affecting the health of the public, and is likely to affect the economies of many nations. In this setting, dietary supplementation is very attractive because supplements of omega-3 fatty acids appear to be simple, safe and inexpensive treatments to recover NAFLD-related liver damage. Despite the strong basic science evidence, only a few studies, conducted in a limited number of patients, have tested the effects of high dose omega-3 fatty acids in high quality randomised double blind placebo controlled trials. A few of these studies have suggested beneficial effects of omega-3 fatty acids (specifically DHA) but very few studies have examined genotype-treatment interactions or effects, whereby the effects of omega-3

treatment to ameliorate liver disease, may be increased, attenuated or even abolished. Nevertheless, we suggest that there is now convincing evidence that high dose omega-3 fatty acid treatment and, in particular DHA, may be useful to decrease liver fat content, particularly in individuals who achieve high levels of omega-3 fatty acid tissue enrichment. The evidence shows no convincing effect of omega-3 fatty acid treatment on liver fibrosis or measures of liver inflammation in NAFLD. Therefore, we suggest further studies are urgently needed to test whether high dose early treatment with DHA ameliorates liver fat, and subsequently retards progression of the liver disease to NASH, and NASH-cirrhosis, as there is now good recent evidence that liver fat per se is not a harmless condition in NAFLD and progresses at the same rate over time as steatohepatitis diagnosed on initial liver biopsy (131). In conclusion, certain omega-3 fatty acids may exert a benefit on the liver and on several metabolic syndrome features in NAFLD, highlighting the therapeutic potential of these molecules in this common condition. Although it is possible that there has been a potential benefit conferred by other fat soluble bioactive molecules in some of the fish oil studies in NAFLD, we suggest that, pending further data on the effects of omega-3 treatment in NAFLD, nutritional strategies for patients with NAFLD, should be based on an adequate intake of omega-3-rich foods.

Disclaimers: All authors except PC Calder have nothing to disclose. Potential conflicts of interest: Dr. Calder advises Pronova BioPharma, Aker Biomarine, Smartfish, Sancilio, Solutex, DSM and Vifor Pharma.

Acknowledgements

ES, PCC and CDB are supported in part by the National Institute for Health Research (UK) Southampton Biomedical Research Centre.

VN and AA are supported by the Italian Ministry of Health funds (Fondi di Ricerca Corrente).

References

1. Masarone M, Federico A, Abenavoli L, Loguercio C, Persico M. Non alcoholic fatty liver: epidemiology and natural history. *Rev Recent Clin Trials* 2014;9:126-33.
2. Nobili V, Alkhouri N, Alisi A, Della Corte C, Fitzpatrick E, Raponi M, Dhawan A. Nonalcoholic fatty liver disease: a challenge for pediatricians. *JAMA Pediatr* 2015;169:170-76.
3. Targher G, Byrne CD. A Perspective on Metabolic Syndrome and Nonalcoholic Fatty Liver Disease. Metab Syndr Relat Disord 2015 Feb 25. [Epub ahead of print]
4. Musso G, Gambino R, Cassader M, Pagano GF. Meta-analysis: natural history of non-alcoholic fatty liver disease (NAFLD) and diagnostic accuracy of non-invasive tests for liver disease severity. *Ann Med* 2011;43: 617-49.
5. Noureddin M, Rinella ME. Nonalcoholic Fatty Liver Disease, Diabetes, Obesity, and Hepatocellular Carcinoma. *Clin Liver Dis* 2015;19: 361-79.
6. Wong RJ, Aguilar M, Cheung R, Perumpail RB, Harrison SA, Younossi ZM, Ahmed A. Nonalcoholic steatohepatitis is the second leading etiology of liver disease among adults awaiting liver transplantation in the United States. *Gastroenterology* 2015;148: 547-55.
7. Ratziu V, Goodman Z, Sanyal A. Current efforts and trends in the treatment of NASH. *J Hepatol* 2015;62:S65-S75.
8. Chalasani N, Younossi Z, Lavine JE, Diehl AM, Brunt EM, Cusi K, Charlton M, Sanyal AJ. The diagnosis and management of non-alcoholic fatty liver disease: Practice guideline by the american association for the study of liver diseases, American College of Gastroenterology, and the American Gastroenterological Association. *Hepatology* 2012;55:2005-23.

9. Yeh MM, Brunt EM. Pathological features of fatty liver disease. *Gastroenterology* 2014;147:754-64.

10. Nobili V, Svegliati-Baroni G, Alisi A, Miele L, Valenti L, Vajro P. A 360-degree overview of paediatric NAFLD: recent insights. *J Hepatol* 2013;58:1218-29.

11. Schwimmer JB, Behling C, Newbury R, Deutsch R, Nievergelt C, Schork NJ. Histopathology of pediatric nonalcoholic fatty liver disease. *Hepatology* 2005;42: 641-49.

12. Nobili V, Alisi A, Grimaldi C, Liccardo D, Francalanci P, Monti L, Castellano A, de Ville de Goyet. Non-alcoholic fatty liver disease and hepatocellular carcinoma in a 7-year-old obese boy: coincidence or comorbidity? *Pediatr Obes* 2014;9:e99-e102

13. Feldstein AE, Charatcharoenwitthaya P, Treeprasertsuk S, Benson JT, Enders FB, Angulo P. The natural history of non-alcoholic fatty liver disease in children: a follow-up study for up to 20 years. *Gut* 2009;58:1538-44.

14. Byrne CD, Targher G. NAFLD: A multisystem disease. *J Hepatol* 2015;62:S47-S64.

15. Fintini D, Chinali M, Cafiero G, Esposito C, Giordano U Turchetta A, Pescosolido S, Pongiglione G, Nobili V. Early left ventricular abnormality/dysfunction in obese children affected by NAFLD. *Nutr Metab Cardiovasc Dis* 2014;24:72-4.

16. Pacifico L, Di Martino M, De Merulis A, Bezzi M, Osborn JF, Catalano C, Chiesa C. Left ventricular dysfunction in obese children and adolescents with nonalcoholic fatty liver disease. *Hepatology* 2014;59:461-70.

17. Targher G, Day CP, Bonora E. Risk of cardiovascular disease in patients with nonalcoholic fatty liver disease. *N Engl J Med* 2010;363:1341-50.

18. VanWagner LB, Wilcox JE, Colangelo LA, Lloyd-Jones DM, Carr JJ, Lima JA, Lewis CE, Rinella ME, Shah SJ. Association of nonalcoholic fatty liver disease with

subclinical myocardial remodeling and dysfunction: A population-based study. *Hepatology* 2015 Apr 24. doi: 10.1002/hep.27869.

19. Musso G, Gambino R, Tabibian JH, Ekstedt M, Kechagias S, Hamaguchi M, Hultcrantz R, Hagström H, Yoon SK, Charatcharoenwitthaya P, George J, Barrera F, Hafliðadóttir S, Björnsson ES, Armstrong MJ, Hopkins LJ, Gao X, Francque S, Verrijken A, Yilmaz Y, Lindor KD, Charlton M, Haring R, Lerch MM, Rettig R, Völzke H, Ryu S, Li G, Wong LL, Machado M, Cortez-Pinto H, Yasui K, Cassader M. Association of non-alcoholic fatty liver disease with chronic kidney disease: a systematic review and meta-analysis. *PLoS Med* 2014;11: e1001680.

20. Singal AK, Salameh H, Kuo YF, Wiesner RH. Evolving frequency and outcomes of simultaneous liver kidney transplants based on liver disease etiology. *Transplantation* 2014;98: 216-21.

21. Day CP, James OFW. Steatohepatitis: a tale of two ‘‘hits’’. *Gastroenterology* 1998;114:842-45.

22. Brunt EM. Pathology of nonalcoholic fatty liver disease. *Nat Rev Gastroenterol Hepatol* 2010;7:195-203.

23. de Wit NJ, Afman LA, Mensink M, Müller M. Phenotyping the effect of diet on non-alcoholic fatty liver disease. *J Hepatol* 2012;57:1370-3.

24. Anstee QM, Day CP. The genetics of NAFLD. *Nat Rev Gastroenterol Hepatol* 2013;10:645-55.

25. Mehal WZ. The Gordian Knot of dysbiosis, obesity and NAFLD. *Nat Rev Gastroenterol Hepatol* 2013;10:637-44.

26. Wree A, Broderick L, Canbay A, Hoffman HM, Feldstein AE. From NAFLD to NASH to cirrhosis-new insights into disease mechanisms. *Nat Rev Gastroenterol Hepatol* 2013;10:627-36.

27. Romeo S, Kozlitina J, Xing C, Pertsemlidis A, Cox D, Pennacchio LA, Boerwinkle E, Cohen JC, Hobbs HH. Genetic variation in PNPLA3 confers susceptibility to nonalcoholic fatty liver disease. *Nat Genet* 2008;40:1461-5.

28. Valenti L, Al-Serri A, Daly AK, Galmozzi E, Rametta R, Dongiovanni P, Nobili V, Mozzi E, Roviaro G, Vanni E, Bugianesi E, Maggioni M, Fracanzani AL, Fargion S, Day CP. Homozygosity for the patatinlike phospholipase-3/adiponutrin I148M polymorphism influences liver fibrosis in patients with nonalcoholic fatty liver disease. *Hepatology* 2010;51:1209-17.

29. Giudice EM, Grandone A, Cirillo G, Santoro N, Amato A, Brienza C, Savarese P, Marzuillo P, Perrone L. The association of PNPLA3 variants with liver enzymes in childhood obesity is driven by the interaction with abdominal fat. *PLoS One* 2011;6:e27933.

30. Davis JN, Lê KA, Walker RW, Vikman S, Spruijt-Metz D, Weigensberg MJ, Allayee H, Goran MI. Increased hepatic fat in overweight Hispanic youth influenced by interaction between genetic variation in PNPLA3 and high dietary carbohydrate and sugar consumption. *Am J Clin Nutr* 2010;92:1522-7.

31. Koo SH. Nonalcoholic fatty liver disease: molecular mechanisms for the hepatic steatosis. *Clin Mol Hepatol* 2013;19:210-5.

32. Dowman JK, Tomlinson JW, Newsome PN. Pathogenesis of non-alcoholic fatty liver disease. *QJM* 2010;103:71-83.

33. Bugianesi E, Moscatiello S, Ciaravella MF, Marchesini G. Insulin resistance in nonalcoholic fatty liver disease. *Curr Pharm Des* 2010;16:941-51.

34. Postic C, Girard J. Contribution of de novo fatty acid synthesis to hepatic steatosis and insulin resistance: lessons from genetically engineered mice. *J Clin Invest* 2008;118:829-38.

35. Delarue J, Magnan C. Free fatty acids and insulin resistance. *Curr Opin Clin Nutr Metab Care* 2007;10:142-48.

36. Holt HB, Wild SH, Wood PJ, Zhang J, Darekar AA, Dewbury K, Poole RB, Holt RI, Phillips DI, Byrne CD. Non-esterified fatty acid concentrations are independently associated with hepatic steatosis in obese subjects. *Diabetologia* 2006;49:141-8.

37. Smith BW, Adams LA. Nonalcoholic fatty liver disease and diabetes mellitus: pathogenesis and treatment. *Nat Rev Endocrinol* 2011;7:456-65.

38. Ishii S, Iizuka K, Miller BC, Uyeda K. Carbohydrate response element binding protein directly promotes lipogenic enzyme gene transcription. *Proc Natl Acad Sci* U S A 2004;101:15597-602.

39. Hirosumi J, Tuncman G, Chang L, Görgün CZ, Uysal KT, Maeda K, Karin M, Hotamisligil GS. A central role for JNK in obesity and insulin resistance. *Nature* 2002;420:333-6.

40. Takeuchi O, Akira S. Pattern recognition receptors and inflammation. *Cell* 2010;140:805-20.

41. Marra F, Gastaldelli A, Svegliati Baroni G, Tell G, Tiribelli C. Molecular basis and mechanisms of progression of non-alcoholic steatohepatitis. *Trends Mol Med* 2008;14:72-81.

42. Byrne CD. Dorothy Hodgkin Lecture 2012: non-alcoholic fatty liver disease, insulin resistance and ectopic fat: a new problem in diabetes management. *Diabet Med* 2012;29:1098-107.

43. Jou J, Choi SS, Diehl AM. Mechanisms of disease progression in nonalcoholic fatty liver disease. *Semin Liver* Dis 2008;28:370-9.

44. Malaguarnera M, Di Rosa M, Nicoletti F, Malaguarnera L. Molecular mechanisms involved in NAFLD progression. *J Mol Med* 2009;87:679-95.

45. Yamamoto M, Takeda K. Current views of toll-like receptor signaling pathways. *Gastroenterol Res Pract* 2010;240365.

46. Feldstein AE, Werneburg NW, Canbay A, Guicciardi ME, Bronk SF, Rydzewski R, Burgart LJ, Gores GJ. Free fatty acids promote hepatic lipotoxicity by stimulating TNF-alpha expression via a lysosomal pathway. *Hepatology* 2004;40:185-94.

47. Tilg H. The role of cytokines in non-alcoholic fatty liver disease. *Dig Dis* 2010;28:179-85.

48. Chalasani N, Deeg MA, Crabb DW. Systemic levels of lipid peroxidation and its metabolic and dietary correlates in patients with nonalcoholic steatohepatitis. *Am J Gastroenterol* 2004;99:1497-502.

49. Dey A, Swaminathan K. Hyperglycemia-induced mitochondrial alterations in liver. *Life Sci* 2010;87:197-214.

50. Pérez-Carreras M, Del Hoyo P, Martín MA, Rubio JC, Martín A, Castellano G, Colina F, Arenas J, Solis-Herruzo JA. Defective hepatic mitochondrial respiratory chain in patients with nonalcoholic steatohepatitis. *Hepatology* 2003;38:999-1007.

51. Calder PC. Mechanisms of action of (n-3) fatty acids. *J Nutr* 2012;142:592S-9S.

52. Chapkin RS, Wang N, Fan YY, Lupton JR, Prior IA. Docosahexaenoic acid alters the size and distribution of cell surface microdomains. *Biochim Biophys Acta* 2008;1778:466-71.

53. Stillwell W, Wassall SR. Docosahexaenoic acid: membrane properties of a unique fatty acid. *Chem Phys Lipids* 2003;126:1-27.

54. Scorletti E, Byrne CD. Omega-3 Fatty acids, hepatic lipid metabolism, and nonalcoholic Fatty liver disease. *Annu Rev Nutr* 2013;33:231-48.

55. Davis BC, Kris-Etherton PM. Achieving optimal essential fatty acid status in vegetarians: current knowledge and practical implications. *Am J Clin Nutr* 2003;78(3 Suppl):640S-646S.

56. Araya J, Rodrigo R, Pettinelli P, Araya AV, Poniachik J, Videla LA. Decreased liver fatty acid delta-6 and delta-5 desaturase activity in obese patients. *Obesity* 2010;18:1460-3.

57. Merino DM, Johnston H, Clarke S, Roke K, Nielsen D, Badawi A, El-Sohemy A, Ma DW, Mutch DM. Polymorphisms in FADS1 and FADS2 alter desaturase activity in young Caucasian and Asian adults. *Mol Genet Metab* 2011;103:171-8.

58. Schaeffer L, Gohlke H, Muller M, Heid IM, Palmer LJ, Kompauer I, Demmelmair H, Illig T, Koletzko B, Heinrich J. Common genetic variants of the FADS1 FADS2 gene cluster and their reconstructed haplotypes are associated with the fatty acid composition in phospholipids. *Hum Mol Genet* 2006;15:1745-56.

59. Shearer GC, Savinova OV, Harris WS. Fish oil - How does it reduce plasma triglycerides? *Biochim Biophys Acta* 2012;1821:843-51.

60. Caviglia JM, Gayet C, Ota T, Hernandez-Ono A, Conlon DM, Jiang H, Fisher EA, Ginsberg HN. Different fatty acids inhibit apoB100 secretion by different pathways: unique roles for ER stress, ceramide, and autophagy. *J Lipid Res* 2011;52:1636-51.

61. Takeuchi Y, Yahagi N, Izumida Y, Nishi M, Kubota M, Teraoka Y, Yamamoto T, Matsuzaka T, Nakagawa Y, Sekiya M, Iizuka Y, Ohashi K, Osuga J, Gotoda T, Ishibashi S, Itaka K, Kataoka K, Nagai R, Yamada N, Kadowaki T, Shimano H. Polyunsaturated fatty acids selectively suppress sterol regulatory element-binding protein-1 through proteolytic processing and autoloop regulatory circuit. *J Biol Chem* 2010;285:11681-91.

62. Jump DB, Botolin D, Wang Y, Xu J, Demeure O, Christian B. Docosahexaenoic acid (DHA) and hepatic gene transcription. *Chem Phys Lipids* 2008;153:3-13.

63. Khan SA, Ali A, Khan SA, Zahran SA, Damanhouri G, Azhar E, Qadri I. Unraveling the complex relationship triad between lipids, obesity, and inflammation. *Mediators Inflamm* 2014;2014:502749.

64. Wahli W, Michalik L. PPARs at the crossroads of lipid signaling and inflammation. *Trends Endocrinol Metab* 2012;23:351-63.

65. Hotamisligil GS, Shargill NS, Spiegelman BM. Adipose expression of tumor necrosis factor-alpha: Direct role in obesity-linked insulin resistance. *Science* 1993;259:87-91.

66. Trayhurn P, Wood IS. Adipokines: Inflammation and the pleiotropic role of white adipose tissue. *Br J Nutr* 2004;92:347-55.

67. Calder PC, Ahluwalia N, Brouns F, Buetler T, Clement K, Cunningham K, Esposito K, Jönsson LS, Kolb H, Lansink M, Marcos A, Margioris A, Matusheski N, Nordmann H, O'Brien J, Pugliese G, Rizkalla S, Schalkwijk C, Tuomilehto J, Wärnberg J, Watzl B,

Winklhofer-Roob BM. Dietary factors and low-grade inflammation in relation to overweight and obesity. *Br J Nutr* 2011;106(Suppl 3):S5-S78.

68. Müller S, Martin S, Koenig W, Hanifi-Moghaddam P, Rathmann W, Haastert B, Giani G, Illig T, Thorand B, Kolb H. Impaired glucose tolerance is associated with increased serum concentrations of interleukin 6 and co-regulated acute-phase proteins but not TNF-alpha or its receptors. *Diabetologia* 2002;45:805-12.

69. Herder C, Illig T, Rathmann W, Martin S, Haastert B, Müller-Scholze S, Holle R, Thorand B, Koenig W, Wichmann HE, Kolb H, for the KORA Study Group. Inflammation and type 2 diabetes: Results from KORA Augsburg. *Gesundheitswesen* 2005;67(Suppl 1):S115-S121.

70. Herder C, Peltonen M, Koenig W, Kräft I, Müller-Scholze S, Martin S, Lakka T, Ilanne-Parikka P, Eriksson JG, Hämäläinen H, Keinänen-Kiukaanniemi S, Valle TT, Uusitupa M, Lindström J, Kolb H, Tuomilehto J. Systemic immune mediators and lifestyle changes in the prevention of type 2 diabetes: Results from the Finnish Diabetes Prevention Study. *Diabetes* 2006;55:2340-6.

71. de Luca C, Olefsky JM. Inflammation and insulin resistance. *FEBS Letters* 2008;582:97-105.

72. Samuel VT, Liu ZX, Qu X, Elder BD, Bilz S, Befroy D, Romanelli AJ, Shulman GI. Mechanism of hepatic insulin resistance in non-alcoholic fatty liver disease. *J Biol Chem* 2004;279:32345-53.

73. Cai D, Yuan M, Frantz DF, Melendez PA, Hansen L, Lee J, Shoelson SE. Local and systemic insulin resistance resulting from hepatic activation of IKK-beta and NF-kappaB. *Nat Med* 2005;11:183-90.

74. Calder PC. Omega-3 polyunsaturated fatty acids and inflammatory processes: Nutrition or pharmacology? Br *J Clin Pharmacol* 2013;75:645-62.

75. Calder PC. Marine omega-3 fatty acids and inflammatory processes: Effects, mechanisms and clinical relevance. *Biochim Biophys Acta* 2015;1851:469-84.

76. Puschel GP, Jungermann K. Integration of function in the hepatic acinus: intercellular communication in neural and humoral control of liver metabolism. *Prog Liver Dis 1994*;12:19-46.

77. Enomoto N, Ikejima K, Yamashina S, Hirose M, Shimizu H, Kitamura T, Takei Y, Sato And N, Thurman RG. Kupffer cell-derived prostaglandin E(2) is involved in alcohol-induced fat accumulation in rat liver. *Am J Physiol Gastrointest Liver Physiol* 2000;279:G100-G106.

78. Janin Henkel, Katja Frede, Nancy Schanze, Heike Vogel, Annette Schumann, Astrid Spruss, Ina Bergheim and Gerhard P Puschel. Stimulation of fat accumulation in hepatocytes by PGE2-dependent repression of hepatic lipolysis, b-oxidation and VLDL-synthesis. *Lab Invest* 2012;92:1597-606.

79. Hespeling U, Jungermann K, Püschel GP. Feedback-inhibition of glucagon-stimulated glycogenolysis in hepatocyte/Kupffer cell cocultures by glucagon-elicited prostaglandin production in Kupffer cells. *Hepatology* 1995;22:1577-83.

80. Henkel J, Neuschafer-Rube F, Pathe-Neuschafer-Rube A, Püschel GP. Aggravation by prostaglandin E2 of interleukin-6-dependent insulin resistance in hepatocytes. *Hepatology* 2009;50:781-90.

81. Fennekohl A, Lucas M, Püschel GP. Induction by interleukin 6 of G(s)-coupled prostaglandin E(2) receptors in rat hepatocytes mediating a prostaglandin E(2)-

dependent inhibition of the hepatocyte's acute phase response. *Hepatology* 2000;31:1128-34.

82. Perez S, Aspichueta P, Ochoa B, Chico Y. The 2-series prostaglandins suppress VLDL secretion in an inflammatory condition-dependent manner in primary rat hepatocytes. *Biochim Biophys Acta* 2006;1761:160-71.

83. Bannenberg G, Serhan CN. Specialized pro-resolving lipid mediators in the inflammatory response: an update. *Biochim Biophys Acta* 2010;1801:1260-73.

84. Serhan CN, Chiang N, van Dyke TE. Resolving inflammation: dual anti-inflammatory and pro-resolution lipid mediators. *Nat Rev Immunol* 2008;8:349-61.

85. Rius B, Titos E, Morán-Salvador E, López-Vicario C, García-Alonso V, González-Périz A, Arroyo V, Clària J. Resolvin D1 primes the resolution process initiated by calorie restriction in obesity-induced steatohepatitis. *FASEB J* 2014;28:836-48.

86. Wanninger J1, Bauer S, Eisinger K, Weiss TS, Walter R, Hellerbrand C, Schäffler A, Higuchi A, Walsh K, Buechler C. Adiponectin upregulates hepatocyte CMKLR1 which is reduced in human fatty liver. *Mol Cell Endocrinol* 2012;34:248-54.

87. Jung TW, Hwang HJ, Hong HC, Choi HY, Yoo HJ, Baik SH, Choi KM. Resolvin D1 reduces ER stress-induced apoptosis and triglyceride accumulation through JNK pathway in HepG2 cells. *Mol Cell Endocrinol* 2014;391:30-40.

88. Billiar T, Bankey P, Svingen B, Curran RD, West MA, Holman RT, Simmons RL, Cerra FB. Fatty acid uptake and Kupffer cell function: fish oil alters eicosanoid and monokine production to endotoxin stimulation. *Surgery* 1988;104:343-9.

89. Wong SW, Kwon WJ, Choi AM, Kim HP, Nakahira K, Hwang D. Fatty acids modulate Toll-like receptor 4 activation through regulation of receptor dimerization and recruitment

into lipid rafts in a reactive oxygen species-dependent manner. J Biol Chem 2009;284:27384-92.

90. Zapata-Gonzalez F, Rueda F, Petriz J, Domingo P, Villarroya F, Diaz-Delfin J, de Madariaga MA, Domingo JC. Human dendritic cell activities are modulated by the omega-3 fatty acid, docosahexaenoic acid, mainly through PPAR(gamma):RXR heterodimers: comparison with other polyunsaturated fatty acids. *J Leukoc Biol* 2008;84:1172-82.

91. Lefils-Lacourtablaise J, Socorro M, Géloën A, Daira P, Debard C, Loizon E, Guichardant M, Dominguez Z, Vidal H, Lagarde M, Bernoud-Hubac N. The eicosapentaenoic acid metabolite 15-deoxy-δ(12,14)-prostaglandin J3 increases adiponectin secretion by adipocytes partly via a PPARγ-dependent mechanism. *PLoS One* 2013;8:e63997.79-791.

92. Oh DY, Talukdar S, Bae EJ, Imamura T, Morinaga H, Fan WQ, Li P, Lu WJ, Watkins SM, Olefsky JM. GPR120 is an omega-3 fatty acid receptor mediating potent anti-inflammatory and insulin-sensitizing effects. *Cell* 2010;142:687-98.

93. Parker HM, Johnson NA, Burdon CA, Cohn JS, O'Connor HT, George J. Omega-3 supplementation and non-alcoholic fatty liver disease: a systematic review and meta-analysis. *J Hepatol* 2012;56:944-51.

94. Chen R, Guo Q, Zhu WJ, Xie Q, Wang H, Cai W. Therapeutic efficacy of (omega)-3 polyunsaturated fatty acid capsule in treatment of patients with non-alcoholic fatty liver disease. *World Chin J Digestol*. 2008;16:2002-6.

95. Sofi F, Giangrandi I, Cesari F, Corsani I, Abbate R, Gensini GF, et al. Effects of a 1-year dietary intervention with n-3 polyunsaturated fatty acid-enriched olive oil on non-alcoholic fatty liver disease patients: a preliminary study. *Int J Food Sci Nutr* 2010;61:792-802.

96. Zhu FS, Liu S, Chen XM, Huang ZG, Zhang DW. Effects of n-3 polyunsaturated fatty acids from seal oils on nonalcoholic fatty liver disease associated with hyperlipidemia. *World J Gastroenterol* 2008;14:6395-400.

97. Capanni M, Calella F, Biagini MR, Genise S, Raimondi L, Bedogni G, Svegliati-Baroni G, Sofi F, Milani S, Abbate R, Surrenti C, Casini A. Prolonged n-3 polyunsaturated fatty acid supplementation ameliorates hepatic steatosis in patients with non-alcoholic fatty liver disease: a pilot study. *Aliment Pharmacol Ther* 2006;23:1143-51.

98. Spadaro L, Magliocco O, Spampinato D, Piro S, Oliveri C, Alagona C, apa G, Rabuazzo AM, Purrello F. Effects of n-3 polyunsaturated fatty acids in subjects with nonalcoholic fatty liver disease. *Dig Liver Dis* 2008;40:194-9.

99. Sanyal AJ, Abdelmalek MF, Suzuki A, Cummings OW, Chojkier M. No significant effects of ethyl-eicosapentanoic acid on histologic features of nonalcoholic steatohepatitis in a phase 2 trial. *Gastroenterology* 2014;147:377-84.

100. Burdge GC. Metabolism of alpha-linolenic acid in humans. *Prostaglandins Leukot Essent Fatty Acids* 2006;75:161-68.

101. Calder PC, Yaqoob P. Understanding omega-3 polyunsaturated fatty acids. *Postgrad Med* 2009;121:148-57.

102. Molendi-Coste O, Legry V, Leclercq IA. Why and How Meet n-3 PUFA Dietary Recommendations? *Gastroenterol Res Pract* 2011;2011:364040.

103. Kaur G, Cameron-Smith D, Garg M, Sinclair AJ. Docosapentaenoic acid (22:5n-3): a review of its biological effects. *Prog Lipid Res* 2011;50:28-34.

104. López-Vicario C, González-Périz A, Rius B, Morán-Salvador E, García-Alonso V, Lozano JJ, Bataller R, Cofán M, Kang JX, Arroyo V, Clària J, Titos E. Molecular interplay

between Δ5/Δ6 desaturases and long-chain fatty acids in the pathogenesis of non-alcoholic steatohepatitis. *Gut* 2014;63:344-55.

105. Puri P, Wiest MM, Cheung O, Mirshahi F, Sargeant C, Min HK, Contos MJ, Sterling RK, Fuchs M, Zhou H, Watkins SM, Sanyal AJ. The plasma lipidomic signature of nonalcoholic steatohepatitis. *Hepatology* 2009;50:1827-38.

106. Scorletti E, Bhatia L, McCormick KG, Clough GF, Nash K, Calder PC, Byrne CD; WELCOME Trial Investigators. Design and rationale of the WELCOME trial: A randomised, placebo controlled study to test the efficacy of purified long chain omega-3 fatty treatment in non-alcoholic fatty liver disease. *Contemp Clin Trials* 2014;37:301-11.

107. Scorletti E, Bhatia L, McCormick KG, Clough GF, Nash K, Hodson L, Moyses HE, Calder PC, Byrne CD; WELCOME Study. Effects of purified eicosapentaenoic and docosahexaenoic acids in non-alcoholic fatty liver disease: Results from the *WELCOME study. *Hepatology* 2014;60:1211-21.

108. Rosenberg WM, Voelker M, Thiel R, Becka M, Burt A, Schuppan D, Hubscher S, Roskams T, Pinzani M, Arthur MJ; European Liver Fibrosis Group. Serum markers detect the presence of liver fibrosis: a cohort study. *Gastroenterology* 2004;127:1704-13.

109. Angulo P, Hui JM, Marchesini G, Bugianesi E, George J, Farrell GC, Enders F, Saksena S, Burt AD, Bida JP, Lindor K, Sanderson SO, Lenzi M, Adams LA, Kench J, Therneau TM, Day CP. The NAFLD fibrosis score: a noninvasive system that identifies liver fibrosis in patients with NAFLD. *Hepatology* 2007;45:846-54.

110. Scorletti E, West AL, Bhatia L, Hoile SP, McCormick KG, Burdge GC, Lillycrop KA, Clough GF, Calder PC, Byrne CD. Treating liver fat and serum triglyceride levels in NAFLD, effects of PNPLA3 and TM6SF2 genotypes: results from the WELCOME* trial. *J Hepatology* July 2015. In press.

111. Argo CK, Patrie JT, Lackner C, Henry TD, de Lange EE, Weltman AL, Shah NL, Al-Osaimi AM, Pramoonjago P, Jayakumar S, Binder LP, Simmons-Egolf WD, Burks SG, Bao Y, Taylor AG, Rodriguez J, Caldwell SH. Effects of n-3 fish oil on metabolic and histological parameters in NASH: A double-blind, randomized, placebo-controlled trial. *J Hepatol* 2015;62:190-7.

112. Kris-Etherton PM, Harris WS, Appel LJ, for the Nutrition Committee. Fish Consumption, Fish Oil, Omega-3 Fatty Acids, and Cardiovascular Disease. *Circulation* 2002;106:2747-57.

113. Bhatia LS, Curzen NP, Calder PC, Byrne CD. Non-alcoholic fatty liver disease: a new and important cardiovascular risk factor? *Eur Heart J* 2012;33:1190-200.

114. Sung KC, Wild SH, Kwag HJ, Byrne CD. Fatty Liver, Insulin Resistance, and Features of Metabolic Syndrome: Relationships with coronary artery calcium in 10,153 people. *Diabetes Care* 2012;35:2359-64.

115. Sung KC, Wild SH, Byrne CD. Development of new fatty liver, or resolution of existing fatty liver, over five years of follow-up, and risk of incident hypertension. *J Hepatol* 2014;60:1040-5.

116. [No authors listed]. Dietary supplementation with n-3 polyunsaturated fatty acids and vitamin E after myocardial infarction: results of the GISSI-Prevenzione trial. Gruppo Italiano per lo Studio della Sopravvivenza nell'Infarto miocardico. *Lancet* 1999;354:447-55.

117. Saravanan P, Davidson NC, Schmidt EB, Calder PC. Cardiovascular effects of marine omega-3 fatty acids. *Lancet* 2010;376:540-50.

118. McKenney JM, Sica D. Role of prescription omega-3 fatty acids in the treatment of hypertriglyceridemia. *Pharmacotherapy* 2007;27:715-28.

119. Davidson MH, Kling D, Maki KC. Novel developments in omega-3 fatty acid-based strategies. *Curr Opin Lipidol* 2011;22:437-44**.**

120. Nobili V, Bedogni G, Alisi A, Pietrobattista A, Risé P, Galli C, Agostoni C. Docosahexaenoic acid supplementation decreases liver fat content in children with non-alcoholic fatty liver disease: double-blind randomised controlled clinical trial. *Arch Dis Child* 2011;96:350-3.

121. Nobili V, Alisi A, Della Corte C, Risé P, Galli C, Agostoni C, Bedogni G. Docosahexaenoic acid for the treatment of fatty liver: randomised controlled trial in children. *Nutr Metab Cardiovasc Dis* 2013;23:1066-70.

122. Alisi A, Feldstein AE, Villani A, Raponi M, Nobili V. Pediatric nonalcoholic fatty liver disease: a multidisciplinary approach. *Nat Rev Gastroenterol Hepatol* 2012;9:152-61.

123. Nobili V, Carpino G, Alisi A, Franchitto A, Alpini G, De Vito R, Onori P, Alvaro D, Gaudio E. Hepatic progenitor cells activation, fibrosis, and adipokines production in pediatric nonalcoholic fatty liver disease. *Hepatology* 2012;56:2142-53.

124. Nobili V, Carpino G, Alisi A, De Vito R, Franchitto A, Alpini G, Onori P, Gaudio E. Role of docosahexaenoic acid treatment in improving liver histology in pediatric nonalcoholic fatty liver disease. *PLoS One* 2014;9:e88005.

125. Janczyk W, Socha P, Lebensztejn D, Wierzbicka A, Mazur A, Neuhoff-Murawska J, Matusik P. Omega-3 fatty acids for treatment of non-alcoholic fatty liver disease: design and rationale of randomized controlled trial. *BMC Pediatr* 2013;13:85.

126. Janczyk W, Lebensztejn D, Wierzbicka-Rucińska A, Mazur A, Neuhoff-Murawska J, Matusik P, Socha P. Omega-3 Fatty Acids Therapy in Children with Nonalcoholic Fatty Liver Disease: A Randomized Controlled Trial. *J Pediatr* 2015 Mar 11. pii: S0022-3476(15)00103-1. [Epub ahead of print]

127. Alkhouri N. The Use of Omega-3 Fatty Acids in Nonalcoholic Fatty Liver Disease: The End of the Road or a New Beginning? *J Pediatr* 2015 Apr 4. pii: S0022-3476(15)00250-4. doi: 10.1016/j.jpeds.2015.03.003. [Epub ahead of print]

128. Woodman RJ, Mori TA, Burke V, Puddey IB, Barden A, Watts GF, Beilin LJ. Effects of purified eicosapentaenoic acid and docosahexaenoic acid on platelet, fibrinolytic and vascular function in hypertensive type 2 diabetic patients. *Atherosclerosis* 2003;166: 85-93.

129. Alisi A, Cianfarani S, Manco M, Agostoni C, Nobili V. Non-alcoholic fatty liver disease and metabolic syndrome in adolescents: pathogenetic role of genetic background and intrauterine environment. *Ann Med* 2012;44:29-40.

130. Nobili V, Bedogni G, Donati B, Alisi A, Valenti L. The I148M variant of PNPLA3 reduces the response to docosahexaenoic acid in children with non-alcoholic fatty liver disease. *J Med Food* 2013;16:957-60.

131. McPherson S, Hardy T, Henderson E, Burt AD, Day CP, Anstee QM. Evidence of NAFLD progression from steatosis to fibrosing-steatohepatitis using paired biopsies: Implications for prognosis and clinical management. *J Hepatol* 2015;62:1148-55.

Figure legends

Figure 1. Overview of hepatic fatty acid metabolism during NAFLD.

Intra-hepatic TG accumulation occurs by four key pathways: (i) increased intake of dietary fats; (iia) increased influx of FFAs from enhanced lipolysis in subcutaneous or visceral adipose tissue, or (iib) high hepatic *de novo* lipogenesis (DNL) from non-lipid dietary precursors (e.g. carbohydrates); (iii) inadequate fatty acid (FA) oxidation; or (iv) impaired hepatic export of triglycerides (TGs) within very low density lipoprotein-triglyceride (VLDL).

Figure 2. Main mechanisms involved in NAFLD pathogenesis.

Two main predisposing factors contribute to NAFLD pathogenesis: a particular genetic background that confers to a subject a susceptibility to disease and an unhealthy lifestyle (inadequate diets and lacks of physical exercise). NAFLD is a multi-phenotype disease that may evolve from a benign form (steatosis) to a more severe histological pattern (NASH, non-alcoholic steatohepatitis). The development of steatosis and its progression to NASH involves gut and adipose tissue, besides the liver. These organs contribute in different ways to produce metabolic and molecular derangements that cause the activation of the liver response that determines steatosis or further triggers of NASH and fibrosis.

Figure 3. Synthesis and metabolites of omega-6 and omega-3 fatty acids.

In the liver, dietary α-linolenic acid is metabolised to stearidonic acid by Δ6-desaturase; this first reaction is a rate-limiting step and competes with the conversion of linoleic acid to arachidonic acid in omega-6 fatty acid metabolism. In omega-3 fatty acid metabolism, Δ6-desaturase participates twice, once in the first step in which α-linolenic acid is desaturated to

stearidonic acid and subsequently in the conversion of DPA to DHA; on the other hand, Δ6-desaturase activity is utilised for omega-6 fatty acid metabolism only once. Δ5-desaturase and Δ6-desaturase compete to metabolise these two polyunsaturated fatty acids. Omega-6 acids produce 2- and 4-series prostaglandins, thromboxanes and leukotrienes, whereas omega-3 fatty acids produce 3- and 5-series prostaglandins and leukotrienes and also resolvins and protectins. DHA and EPA can form new endocannabinoid products docosahexaenoyl ethanolamide (DHEA) and eicosapentaenoyl ethanolamide (EPEA).

Table 1: Summary of randomized placebo controlled trials using non-invasive imaging (ultrasound-excepted) or liver histology to assess effects of omega-3 polyunsaturated fatty acid treatment on NAFLD.

Ref No	Diagnosis of NAFLD	Population	Intervention	Comparator	Follow-up	Primary outcomes to assess improvement in NAFLD severity	Conclusion
95	Increase in ALT ≥ 6 months Ultrasound diagnosis of fatty liver	n=11	Omega-3 fatty acids 0.83 g/day as dietary supplementation to olive oil	Placebo	12 months	Improvement of liver echo-texture and of the Doppler Perfusion Index (DPI)	A significant reduction in ALT, AST, GGT, serum TG, increase in HDL-C with a significant benefit on the liver ultrasonographic pattern (regression of bright echo-texture and increase of DPI)
99	Liver biopsy	n=243	Omega-3 fatty acids, EPA-E 1800 mg/day	Placebo	12 months	NAFLD progression: • Proportion of responders (NAS ≤3 with fibrosis unchanged and a ≥2 decrease in NAS with fibrosis unchanged) • Proportion meeting criteria (NAS ≤3 with fibrosis unchanged) • Proportion meeting criteria (≥2 decrease in NAS with fibrosis unchanged) NAFLD progression; NAS; Body weight; AST ALT	No improvement or worsening of NAS score No improvements in LFTs 4% improvement in serum TG No change in body weight
105 106	Liver biopsy or non-invasive imaging	n=103	Purified omega-3 polyunsaturated fatty acid	Olive oil 4g/day	15-18 months	• Change in liver fat percentage identified by MRS spectroscopy (mean difference of change in liver	ITT analyses: Non significant trend toward improvement in liver fat % 10% improvement in liver fat % (placebo) 35% improvement in liver fat % with

	evidence of fatty liver + features of MetS with exclusion of other causes of liver fat		4g/day (Omacor/Lovaza)			fat percentage in three regions of the liver) • Change in liver fibrosis biomarkers	Omacor Secondary pre-specified analyses: DHA enrichment associated with decrease in liver fat % (p=0.007) (adjusting for all confounders)
111	NASH diagnosed by liver biopsy and exclusion of other causes of steatohepatitis	n=41	Omega-3 fish oil 3g/day Nordic Natural	Soybean oil but also small amounts of fish and lemon oils (only 8% n-3) to protect blinding	12 months	Primary outcome was a decrease of at least 2 points in the NAS at the end-of the study as compared to the baseline liver biopsy (without fibrosis progression).	Omega-3 treatment did not show more histologic fat reduction by NAS scoring, but the fish oil group did show a greater fat reduction in the subgroup of subjects with a baseline histologic steatosis score >2 (p = 0.009). Among subjects who either gained or had stable weight, n-3 treated subjects showed a larger difference in liver fat content by MRI than placebo treated subjects (p=0.014 for the 2nd quartile and p=0.003 for the 3rd quartile, for change in weight (n-3 group).
120 **121** **123**	NAFLD diagnosed by liver biopsy and exclusion of other causes of liver disease	n=60	250 mg/day or 500 mg/day DHA	Placebo	24 months	Improvements at : • ALT levels • Liver ultrasound • Liver biopsy	The treatment with DHA caused a significant reduction of BMI and in ALT and TG levels and improvement of liver ultrasound compared to control group. DHA also ameliorated liver histology in terms of steatosis, inflammation and ballooning.
125 **126**	NAFLD diagnosed by ultrasound or liver biopsy and exclusion of other causes of liver disease	n=64	450-1300 mg omega-3 fatty acids daily (DHA:EPA= 3:2)	Placebo	24 weeks	Observed improvements (ALT and liver steatosis at ultrasound) in the treatment arm were similar to those observed in the placebo group.	Supplementation with DHA + EPA was not superior to placebo in achieving the primary outcome. A significant improvement of secondary outcomes (aspartate aminotransferase and gammaglutamyl transpeptidase and adiponectin levels) was reported.

Figure 1

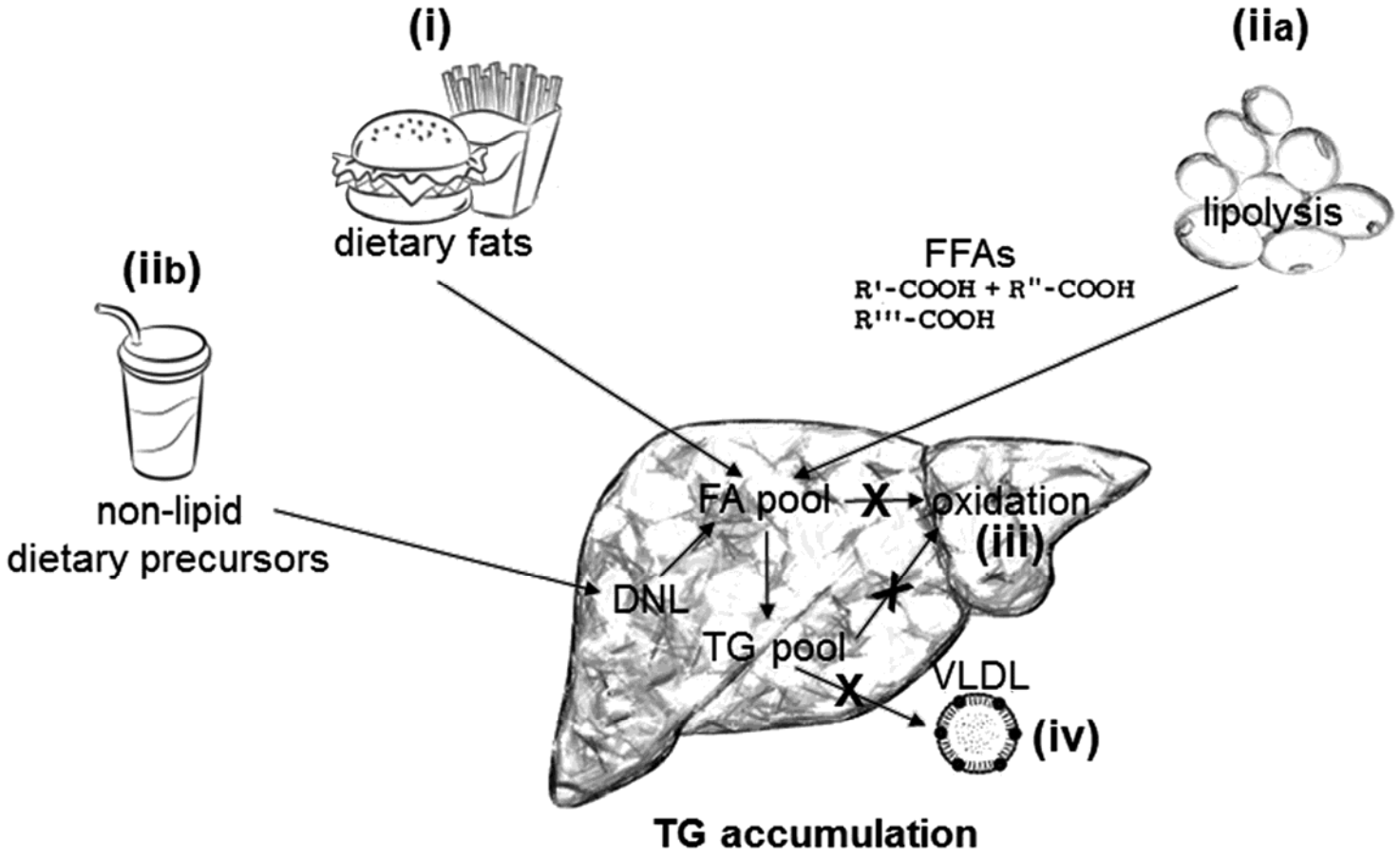

(i)
dietary fats
(iia)
lipolysis
FFAs
R'-COOH + R''-COOH
R'''-COOH
(iib)
non-lipid
dietary precursors
FA pool
oxidation
(iii)
DNL
TG pool
VLDL
(iv)
TG accumulation

Figure 2

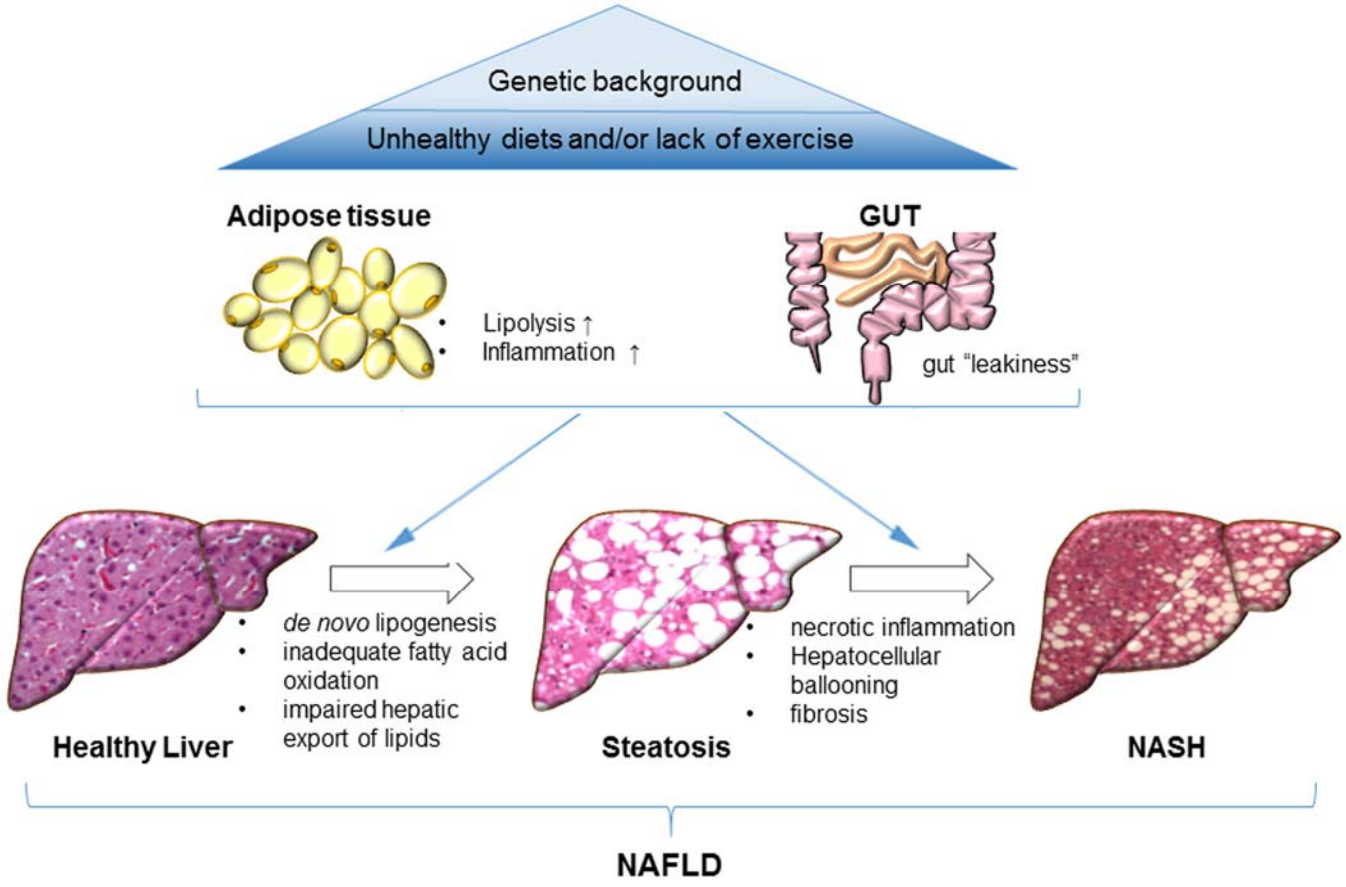
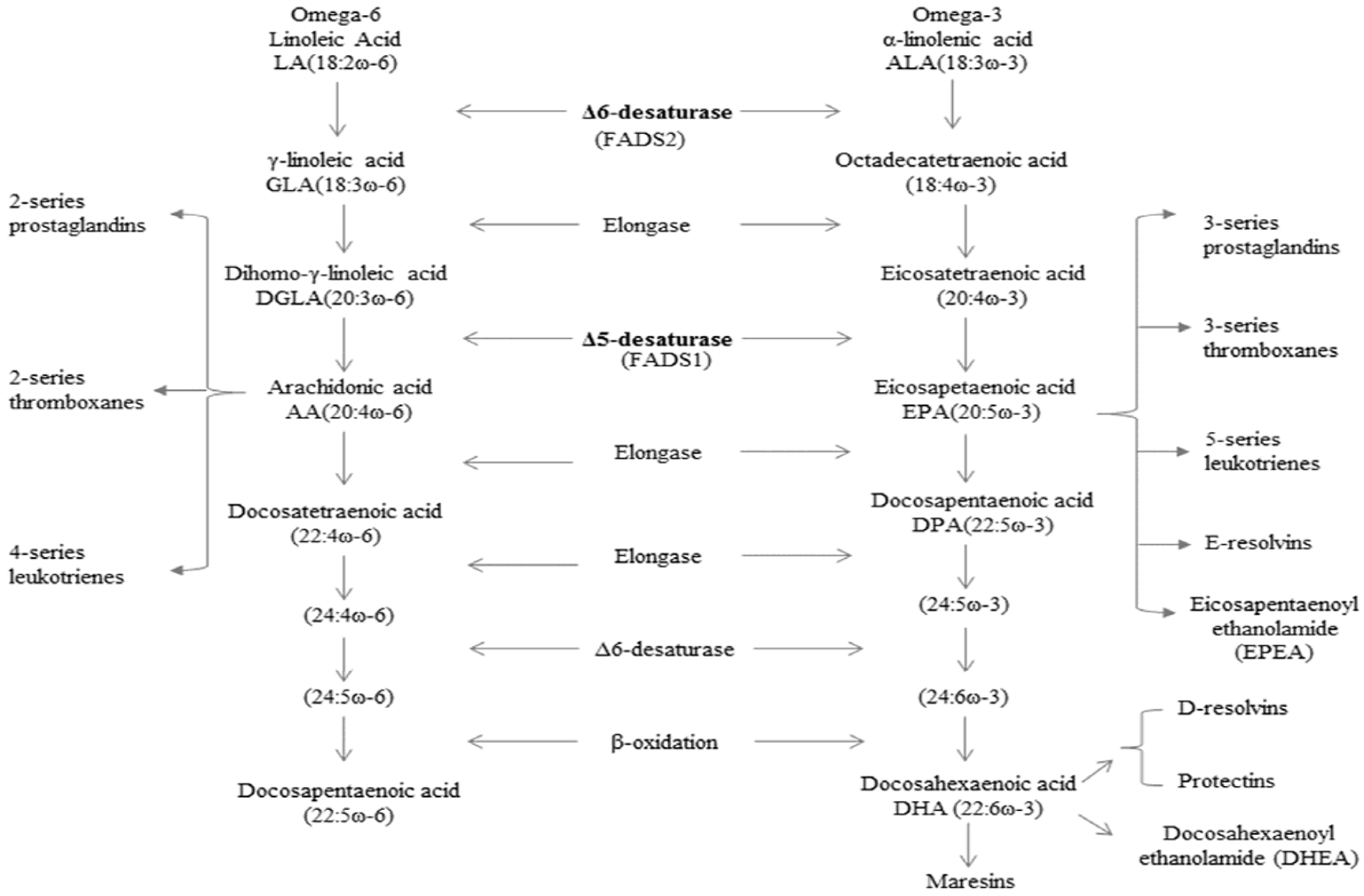

Genetic background
Unhealthy diets and/or lack of exercise
Adipose tissue
Lipolysis ↑
Inflammation ↑
GUT
gut "leakiness"
de novo lipogenesis
inadequate fatty acid oxidation
impaired hepatic export of lipids
Healthy Liver
necrotic inflammation
Hepatocellular ballooning
fibrosis
Steatosis
NASH
NAFLD

Figure 3


Omega-6
Linoleic Acid
LA(18:2ω-6)
Omega-3
α-linolenic acid
ALA(18:3ω-3)
Δ6-desaturase
(FADS2)
γ-linoleic acid
GLA(18:3ω-6)
Octadecatetraenoic acid
(18:4ω-3)
2-series
prostaglandins
Elongase
3-series
prostaglandins
Dihomo-γ-linoleic acid
DGLA(20:3ω-6)
Eicosatetraenoic acid
(20:4ω-3)
Δ5-desaturase
(FADS1)
3-series
thromboxanes
2-series
thromboxanes
Arachidonic acid
AA(20:4ω-6)
Eicosapetaenoic acid
EPA(20:5ω-3)
Elongase
5-series
leukotrienes
Docosatetraenoic acid
(22:4ω-6)
Docosapentaenoic acid
DPA(22:5ω-3)
4-series
leukotrienes
Elongase
E-resolvins
(24:4ω-6)
(24:5ω-3)
Eicosapentaenoyl
ethanolamide
(EPEA)
Δ6-desaturase
(24:5ω-6)
(24:6ω-3)
D-resolvins
β-oxidation
Protectins
Docosapentaenoic acid
(22:5ω-6)
Docosahexaenoic acid
DHA (22:6ω-3)
Docosahexaenoyl
ethanolamide (DHEA)
Maresins