Non-alcoholic fatty liver disease and its treatment with n-3 polyunsaturated fatty acids

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List of abbreviations:

ALA – alpha-linolenic acid; ALT – alanine aminotransferase; Apo – apolipoprotein; AST – aspartate aminotransferase; BMI – body mass index; ChREBP – carbohydrate response element binding protein; CMKLR1 – chemokine-like receptor 1; COX – cyclooxygenase; CPT-1 – carnitine palmitoyltransferase 1; DAG – diacylglycerol; DHA – docosahexaenoic acid; DPA – docosapentaenoic acid; EE – ethyl esters; EPA – eicosapentaenoic acid; FAS – fatty acid synthase; FATP – fatty acid transport protein; FXR – farnesoid X receptor; GCK – glucokinase; GGT – gamma-glutamyl transpeptidase; HNF-4 α – hepatocyte nuclear factor 4; IkB – inhibitory subunit of NF κ B; IL – interleukin; LOX – lipoxygenase; L-PK – L-pyruvate kinase; LXR – liver X receptor; NAFLD – non-alcoholic fatty liver disease; NAS – NAFLD activity score; NASH – non-alcoholic steatohepatitis; MRI – magnetic resonance imaging; NEFA – non-esterified fatty acids; NF κ B – nuclear factor κ B; PDH – pyruvate dehydrogenase; PFK – phosphofructokinase; PKC – protein kinase C; PNPLA3 – patatin-like phospholipase domain-containing 3; PPAR – peroxisome proliferator activated receptor; PUFAs – polyunsaturated fatty acids; SNPs – single nucleotide polymorphism; SREBP – sterol regulatory element binding protein; TAG – triacylglycerol; TLR – toll-like receptor; TNF- α – tumour necrosis factor α .

Abstract

Background and aims: Non-alcoholic fatty liver disease (NAFLD) is a common liver diseases in Western countries. Metabolic disorders which are increasing in prevalence, such as dyslipidaemias, obesity and type 2 diabetes, are closely related to NAFLD. Insulin resistance is a prominent risk factor for NAFLD. Marine omega-3 (n-3) polyunsaturated fatty acids (PUFAs) are able to decrease plasma triacylglycerol and diets rich in marine n-3 PUFAs are associated with a lower cardiovascular risk. Furthermore, marine n-3 PUFAs are precursors of pro-resolving and anti-inflammatory mediators. They can modulate lipid metabolism by enhancing fatty acid β -oxidation and decreasing *de novo* lipogenesis. Therefore, they may play an important role in prevention and therapy of NAFLD. Methods: This review aims to gather the currently information about marine n-3 PUFAs as a therapeutic approach in NAFLD. Actions of marine n-3 PUFAs on hepatic fat metabolism are reported, as well as studies addressing the effects of marine n-3 PUFAs in human subjects with NAFLD. Results: A total seventeen published human studies investigating the effects of n-3 PUFAs on markers of NAFLD were found and twelve of these reported a decrease in liver fat and/or other markers of NAFLD after supplementation with n-3 PUFAs. The failure of n-3 PUFAs to decrease markers of NAFLD in five studies may be due to short duration, poor compliance, patient specific factors and the sensitivity of the methods used. Conclusions: Marine n-3 PUFAs are likely to be an important tool for NAFLD treatment, although further studies are required to confirm this.

Keywords: NAFLD; omega-3 fatty acids; fish oil; algal oil; insulin resistance; metabolic syndrome.

Backgrounds and Aims

Non-alcoholic fatty liver disease (NAFLD) is defined as a condition in which liver triacylglycerol (TAG) concentration surpasses 5% of wet liver weight in patients without excessive alcohol intake (i.e., alcohol abuse characterized by a consumption of more than 10 g of ethanol per day). NAFLD occurs as the result of an imbalance of hepatic TAG synthesis and export [1, 2]. Liver biopsy is considered the gold-standard diagnostic tool, although its use is limited by risk of morbidity and mortality, sampling error and cost. As hepatic biopsy is not always possible, the definition of NAFLD also involves evidence of hepatic fat accumulation verified by imaging for example by ultrasonography or magnetic resonance spectroscopy [3].

The American Association for the Study of Liver Diseases proposed a NAFLD classification correlating histological characteristics with long-term prognosis: simple steatosis (class 1); steatosis with lobular inflammation (class 2); presence of ballooned hepatocytes (class 3); presence of either Mallory's hyaline or fibrosis (class 4) [2]. Classes 3 and 4 are described as non-alcoholic steatohepatitis (NASH) [2]. Another score was suggested by the NASH Clinical Research Network. This is the NAFLD activity score (NAS) which is characterized by the weighted sum of steatosis (0 to 3), lobular inflammation (0 to 3), and hepatocyte ballooning (0 to 2). The NAS ranges from 0 to 8. A $NAS < 3$ corresponds to "not NASH"; 3 to 4 is classified as "borderline NASH"; and ≥ 5 means a "definitive NASH" diagnosis [4]. The most common NAFLD presentation is asymptomatic with elevation of serum transaminases, particularly alanine aminotransferase (ALT) higher than aspartate aminotransferase (AST) [5].

The classical theory of NAFLD progression comprises lipid accumulation as the first "hit" and an increase in inflammation and the second "hit" as an increase in oxidative stress and lipid peroxidation [6]. The first hit is associated with insulin resistance and increases susceptibility to the second hit, which results in NAFLD progression to NASH (Figure 1). In one cohort study NAFLD progressed to NASH in 47% of subjects and from NASH to more severe hepatic diseases in 25-50% of these subjects (i.e. in 12 to 24% of all subjects) [7]. The early stages of the disease can be reversed (Figure 1). The progression to cirrhosis is related to a poor prognosis and, in one study, of the subjects who progressed to that stage, 50% required liver transplantation, 7% developed hepatocellular carcinoma, and 20% died [8].

Type-2 diabetes has a strong association with NAFLD [9]. Hepatic inflammation and in adipose tissue and alterations in fat metabolism seem to have a causal relation to insulin resistance, dyslipidaemia and cardiovascular risk [10]. Some single-nucleotide polymorphisms (SNPs) have been shown to be related to NAFLD severity and NASH, such as SNPs in the gene encoding patatin-like phospholipase domain-containing 3 (PNPLA3) [11].

Lifestyle modifications are the first treatment option for NAFLD and body weight loss is the most important goal for the majority of patients with NAFLD [12]. However dietary modification

may have a significant role in NAFLD treatment independent of weight loss [5]. Bariatric surgery also seems to be highly effective in treating NAFLD; a systematic review showed an improvement in steatosis in 91% of subjects who underwent bariatric surgery [13]. Marine omega-3 (n-3) polyunsaturated fatty acids (PUFAs) decrease plasma TAG concentrations, regulate hepatic fatty acid and TAG metabolism and have anti-inflammatory properties [14]. These properties may be useful in treatment of NAFLD [15].

Methods

Considering both the increasing importance of NAFLD and the metabolic effects of marine n-3 PUFAs, this review will describe the processes underlying NAFLD, the relevant properties of marine n-3 PUFAs, and the studies in which marine n-3 PUFAs have been used to treat NAFLD. This is a narrative review in which studies showing metabolic pathways and mechanism of action of marine omega-3 were included. Clinical trials addressing the effects of omega-3 on hepatic fat were obtained from PubMed and SciELO websites. The search terms were: NAFLD, NASH, non- alcoholic fatty liver disease, non- alcoholic steatohepatitis, steatosis, liver fat, hepatic fat, fatty liver, omega-3 fatty acids, DHA, EPA, docosahexaenoic acid, eicosapentaenoic acid, fish oil, algal oil.

Results

Risk factors and NAFLD prevalence

NAFLD and NASH reported prevalence is influenced by the method used to diagnose and the population studied. In Western populations, the prevalence of NAFLD is estimated to be between 15 and 30% [16, 17], but this may be an underestimate [18]. In Germany, 4160 subjects were included in a population-based cohort study. They were evaluated by ultrasonography and 30.4% were diagnosed with NAFLD [19]. A study from Scotland reported a NAFLD prevalence of 46.2% in subjects with type-2 diabetes [20]. Hepatic histology in 498 post-mortem livers showed a prevalence of NAFLD of 31% in Greece [21]. In Romania, 3005 hospitalized patients were evaluated by ultrasonography and 20% of them were identified to have NAFLD [22]. A study in Sudan reported a NAFLD prevalence of 20% in 100 asymptomatic subjects [23]. In Korea, a study of potential liver donors found a NAFLD prevalence of 51% [24]. Subjects with NAFLD were characterized in Brazil and 45% were overweight, 44% had type-2 diabetes and 41% had metabolic syndrome [25].

The Dallas Heart Study found a higher prevalence of hepatic steatosis in Hispanic subjects than in Caucasians who in turn had higher prevalence than African-Americans [26]. Another study aimed to understand this lower prevalence of NAFLD in African-Americans in a population-based study that estimated NAFLD and NASH prevalence in 2170 subjects by dual-energy x-ray

absorptiometry. Lower intraperitoneal fat accumulation was found in African-American subjects and this was associated with a protection from NAFLD, although no differences were found in insulin resistance between Hispanic and African-American subjects [27]. The I148M allele of the PNPLA3 gene is a genetic contributor to NAFLD and this allele is most prevalent in Hispanics, Caucasians came after and then African-Americans [28, 29]. Conversely, the PNPLA3 rs6006460[T] allele was common in African-Americans and was associated with lower hepatic fat [29]. Thus, Hispanics may have a genetic predisposition to fatty liver, while African Americans may have a genetic protection against it.

The Third National Health and Nutrition Examination (NHANES III, 1988-1994) in the US estimated NAFLD prevalence in non-institutionalized healthy subjects by using ultrasonography. Hepatic steatosis was found in 21% of subjects and among these 90% were considered to have NAFLD (19% of the study population). A higher prevalence was found in Mexican Americans compared to non-Hispanic whites and African Americans and among men compared to women [30]. The differences in prevalence among ethnic groups are consistent with the studies described earlier. The sex influence on NAFLD is not fully understood. However, male sex is related to higher presence of NASH and fibrosis and greater risk of mortality in subjects with NAFLD [18, 31].

NAFLD is strongly associated with obesity. A study in obese subjects (defined by a body mass index $> 35 \text{ kg/m}^2$) who had liver biopsies whilst undergoing bariatric surgery reported 96% prevalence of NAFLD; of these 26 subjects had NASH (25% of the study population) [32]. This research also confirmed the association of central body fat distribution, abnormal glucose metabolism and hypertension with NASH [32]. Type-2 diabetes is also one of the most important risk factors for NAFLD. In people with type-2 diabetes, the estimated prevalence of NAFLD in hospital-based studies is 45% to 75% and 30% to 70% in population-based studies [33] and in obese people with type-2 diabetes it can reach 56% [34]. In view of this, it is suggested that serum ALT and AST should be investigated in all people with type-2 diabetes [7].

A prospective study evaluated the prevalence of NAFLD and NASH in 328 subjects by ultrasonography and in 134 biopsies for those subjects diagnosed with NAFLD. The prevalence of NAFLD was 46% and Hispanic subjects had the highest prevalence (58%), shadowed by Caucasians (44%) and African-Americans (35%) [35]. Another study evaluated the prevalence of insulin resistance in young, lean, healthy and sedentary subjects of different ethnicities. Asian-Indian men showed higher prevalence of insulin resistance compared to Eastern-Asians, Caucasians, African Americans and Hispanics [36]. Furthermore, Asian-Indians had an increase in hepatic TAG content and plasma interleukin (IL)-6 concentration compared to Caucasians suggesting that Asian-Indian men could be predisposed to develop insulin resistance and hepatic steatosis even with a normal body mass index [36]. Consistent with this, a NAFLD prevalence of 8.7% was found in a rural, predominantly lean, Indian population, suggesting an important role for risk factors other than obesity [37]. Age is positively associated to NAFLD and its progression (fibrosis and cirrhosis) [38].

However this association may be linked to the duration of the disease and also older subjects have more risk factors for NAFLD, such as obesity, type-2 diabetes, hypertension and dyslipidaemia. Furthermore, advanced age increases the risk of complications such as severe fibrosis and hepatocellular carcinoma [18].

Dietary intake has been characterised in some studies of subjects with NAFLD [39-42]. These studies identified a diet distinguished by high intakes of simple carbohydrates, saturated fat and protein from meat and low intakes of marine n-3 PUFAs and micronutrients [39, 40, 43]. A cross-sectional study of a sub-sample of 375 subjects in Israel reported that a high consumption of simple carbohydrates (sugars) from soft drinks and of proteins from all types of meat were a risk factor for developing NAFLD and that a high consumption of fish with high concentration of marine n-3 PUFAs had a protective effect [40]. Also, a higher intake of omega-6 (n-6) fatty acids and a higher dietary n-6/n-3 fatty acid ratio were reported for NAFLD subjects [43].

Marine n-3 PUFAs - sources and intakes

N-3 PUFA definition and classification

N-3 PUFAs have a double bond between carbon 3 and 4 of the hydrocarbon (acyl) chain counting from the methyl end [14]. There are several members of the n-3 fatty acid family, varying in carbon chain length and amount of double bonds. The very long chain highly unsaturated n-3 PUFAs, eicosapentaenoic acid (EPA; 20:5n-3), docosapentaenoic acid (DPA; 22:5n-3) and docosahexaenoic acid (DHA; 22:6n-3) are functionally the most important members of the n-3 fatty acid family [14]. EPA, DPA and DHA are found in a variety of foods, but the richest source is seafood, particularly fatty fish, such as mackerel, pilchards, sardines, salmon, trout, tuna and herring (Table 1). Hence, here we refer to EPA, DPA and DHA as marine n-3 PUFAs. Lean fish such as cod, haddock and plaice and crustaceans and shellfish also contain marine n-3 PUFAs, as do fish oil supplements, cod liver oil, algal oils, krill oil and pharmaceutical grade preparations. Some of the sources of marine n-3 PUFAs and the amount per adult portion size are listed in the Table 1 according to the British Nutrition Foundation food composition data [44]. Eggs and meat have modest amounts of EPA, DPA and DHA [14].

****Table 1****

N-3 PUFA elongation and desaturation

Alpha-linolenic acid (ALA; 18:3n-3) is the precursor of the long chain n-3 PUFAs. It is an essential fatty acid since it cannot be synthesized in animals including humans [45]. Likewise, linoleic acid (18:2n-6) is an essential n-6 fatty acid. Both ALA and linoleic acid must be acquired from diet.

They are both synthesized in plants and consequently are found in seeds, nuts, and seed oils. There is a pathway for conversion of ALA to EPA and on to DPA and DHA [14]. However, this conversion, especially to the end product DHA, is poor [46] and is known to be lower in men than women [47]. This process of ALA conversion to EPA involves desaturation, elongation and another desaturation using delta-6 desaturase, elongase, and delta-5 desaturase, respectively [14]. These same enzymes are also responsible for the analogous conversions in the n-6 fatty acid family from linoleic acid to arachidonic acid (20:4n-6). Despite the greater affinity of delta-6 desaturase for ALA than linoleic acid, the n-6 family shows higher conversion rates due to higher amount of linoleic acid in cellular pools [46]. Therefore, the higher intake of n-6 fatty acids in Western diets seems to result in a low conversion of ALA to bioactive long chain n-3 PUFAs. DPA is formed by elongation of EPA while DHA is formed from DPA by a complex pathway involving several enzymes [46]. Figure 2 illustrates the metabolism of essential fatty acids.

N-3 PUFA intake

The consumption of marine n-3 PUFAs is difficult to determine, partly because of the bimodal pattern of fatty fish consumption, the infrequent consumption of fish, poor dietary assessment tools and inadequate food composition tables. Furthermore, the exact marine n-3 fatty acid content of fish is uncertain and variable due to several factors including season, diet, water temperature, stage in the life cycle, and whether wild or farmed [14, 48]. Intakes of marine n-3 PUFAs among adults who do not consume fatty fish are thought to be in the tens to low hundreds of mg per day [14]. Clearly, such intakes can be greatly increased by eating seafood especially fatty fish or by use of supplements [14]. By comparison, adult intakes of ALA are typically 0.5 to 2 g/d [14]. It is useful to compare these intakes to those that are recommended by different authorities. The European Food Safety Authority (EFSA) indicates an adequate intake (AI) for ALA as 0.5% of total energy for all population groups [49]. In adults, consuming a 2000 cal/day diet this would equate to about 1 g ALA/day. EFSA recommends that adults should consume 0.25 g of EPA + DHA daily with an additional 0.1-0.2 g of DHA daily for pregnant and lactating women [49]. The American Heart Association made different recommendations for healthy people (EPA+DHA 0.5 g/d), for people with coronary artery diseases (EPA+DHA, 1 g/d) and for hypertriglyceridemic subjects (EPA + DHA 3-4 g/d) [50]. FAO/WHO recommended an intake of 0.5-2% of energy as ALA + EPA + DHA and 0.25 g to 2 g per day of EPA + DHA for adults [49, 51]. The precise requirement for marine n-3 PUFAs is not known

Fatty acid metabolism and its regulation: sites of action of marine n-3 PUFAs

Dietary lipids digestion and absorption occur mainly in the small intestine through the combined action of bile acid emulsification and pancreatic lipase catalysed hydrolysis. The products of TAG digestion (monoacylglycerols and free fatty acids) are taken up into enterocytes where they are used for resynthesis of TAGs which are secreted as components of nascent chylomicrons into the

lymphatic system. Soon after food consumption, the chylomicrons reach the bloodstream. Here, interactions with other lipoproteins result in apolipoprotein (apo) exchange: apoA-I and apoA-IV are replaced by apoE and apoC-II. These changes aid the vascular metabolism of the chylomicrons. For example, in adipose tissue apoC-II activates lipoprotein lipase which hydrolyses chylomicron TAGs making the component fatty acids available to adipocytes. As a consequence fatty acids are then stored in adipose tissue as TAGs and the TAG-poor chylomicron remnants remain in the circulation to be cleared by hepatocytes via recognition of apoE by hepatic LDL receptors. Hepatocytes can reuse the components of the uptaken chylomicron remnants (e.g. cholesterol, fatty acids) to resynthesize TAGs and other components of very low density lipoproteins (VLDL) which are subsequently released into the bloodstream [52, 53]. Marine n-3 PUFAs have been reported to lower the concentration of chylomicrons and TAGs after an oral fat tolerance test [54]. Furthermore, circulating apoB-48 and apoB-100 concentrations were reduced by EPA and DHA compared to safflower oil [54]. In the fed state, EPA and DHA increased LPL activity and accelerated chylomicron TAG clearance [54].

Several transcription factors are related to the control of hepatic lipid metabolism and Table 2 lists the potential effects of n-3 PUFAs on these factors. Liver X receptor (LXR) is activated by oxysterols, which are cholesterol metabolites. LXR controls reverse cholesterol transport through expression of ATP-binding cassette transporters A1 and G1, promotes *de novo* fatty acid synthesis by increasing expression of the transcription factors sterol regulatory element binding protein (SREBP)-1c and carbohydrate response element binding protein (ChREBP), and promotes glycolysis via the phosphofructokinase (PFK)-2/fructose-bisphosphatase-2 system [53]. PUFAs are able to regulate SREBP and ChREBP and therefore the genes controlled by these transcription factors [55], many of which encode enzymes involved in fatty acid and TAG synthesis and lipoprotein assembly. In vitro, EPA and DHA decreased the expression of several genes related to *de novo* fatty acid synthesis, including SREBP-1c itself, and DHA also prevented LXR α activation [56]. In obese (*ob/ob*) mice marine n-3 PUFAs ameliorated hepatic steatosis by suppressing SREBP-1 expression [57]. Farnesoid X receptor (FXR) is a transcription factor greatly expressed in the liver, intestine, kidneys and adrenal cortex. It has a central part in bile acid metabolism downregulating synthesis, secretion and reabsorption. FXR is also able to decrease cholesterol and TAG synthesis through down regulation of SREBP-1c, SREBP-2 and LXR [58]. Linoleic acid, arachidonic acid and DHA have been shown to be FXR ligands while stearic and palmitic acids had no FXR binding activity [59]. Hepatocyte nuclear factor 4 (HNF-4 α) is another nuclear receptor modulated by fatty acids [60]. It is expressed in liver, kidneys, intestine and pancreas [60] and regulated several genes related to lipoprotein, iron, and carbohydrate metabolism, cytochrome P450 monooxygenases and bile acid synthesis [61]. The length of fatty acid chain and degree of saturation seem to influence its transcription: saturated fatty acids increase the transcription of HNF-4 α , while PUFAs have the opposite effect [61]. Rat hepatocytes cultured with fish oil rich chylomicron remnant-like particles showed a decrease in HNF-4 α mRNA

and protein and a reduced expression of genes encoding apo B and microsomal transfer protein, which are regulated by HNF-4 α [62].

SREBP-1a, -1c and -2 are key lipogenic transcription factors. SREBP-1c and SREBP-2 are highly expressed in the liver. The SREBP-1c increases expression of genes connected with fatty acid and TAG synthesis while SREBP-2 activates genes for enzymes involved in synthesis of cholesterol [53]. Fish oil decreases SREBP-1 gene expression [63, 64], although the mechanism by which this occurs is not fully elucidated. Plasma and intracellular membrane enrichment with PUFAs leads to cholesterol migration from highly concentrated areas, such as the plasma membrane, to less concentrated membranes, such as endoplasmic reticulum membrane, which impairs SREBP migration from the endoplasmic reticulum to Golgi complex [65]. Furthermore, PUFAs increase the hydrolysis of membrane sphingomyelin to ceramide and phosphocholine, which decreases the membrane sphingomyelin content and consequently impairs free cholesterol solubilisation and increases intracellular cholesterol concentration, inhibiting SREBP-2 [65].

ChREBP increases the expression of L-pyruvate kinase (L-PK), a glycolytic enzyme, and the expression of lipogenic genes, as malic enzyme, ATP-citrate lyase, acetyl-CoA carboxylase, fatty acid synthase, stearoyl-CoA desaturase and fatty acid elongases [53]. Glucose absorbed by hepatocytes after a meal enters the glycogenic pathway. However, when the liver is replete with glycogen, glucose is diverted to fatty acid synthesis. Glucokinase (GCK), PFK, L-PK, and pyruvate dehydrogenase (PDH) kinases control glycolytic flux. Pyruvate, the main product of glycolysis, provides carbon for *de novo* fatty acid synthesis via acetyl-CoA. ChREBP expression is stimulated by glucose and this transcription factor activates hepatic L-PK gene expression. L-PK is the enzyme responsible for converting phosphoenolpyruvate to pyruvate. Pyruvate is metabolized by PDH to generate acetyl-CoA, which is combined with oxaloacetate to form citrate. ATP-citrate lyase splits the citrate exported to cytoplasm back into acetyl-CoA and oxaloacetate. Acetyl-CoA carboxylase (ACC) converts acetyl-CoA to malonyl-CoA in the cytoplasm. Fatty acid synthase (FAS) consumes malonyl-CoA as the carbon donor to generate palmitic acid [53]. Furthermore, FAS might be linked to the synthesis of an endogenous peroxisome proliferator activated receptor (PPAR)- α ligand, 1-palmitoyl-2-oleoyl-sn-glycerol-3-phosphocholine, indicating a contra-regulation of *de novo* synthesis of fatty acids to stimulate β -oxidation [66]. Dentin and colleagues demonstrated effects of PUFAs on ChREBP in vivo and in vitro. Linoleic acid, EPA and DHA were able to downregulate ChREBP gene expression through accelerating ChREBP mRNA decay [67]. FAS was also downregulated and demonstrated to be controlled by ChREBP and by SREBP. L-PK is not under SREBP regulation, but was decreased by ChREBP inhibition [67].

Lipolysis in adipocytes generates non-esterified fatty acids (NEFAs), which enter the bloodstream and can be taken up by hepatocytes and enter the hepatic fatty acid pool. Adipocyte TAG is hydrolysed by adipose tissue TAG lipase to release a NEFA and diacylglycerol (DAG), which is hydrolysed by hormone-sensitive lipase to another NEFA and monoacylglycerol. Monoacylglycerol

lipase generates a third NEFA and glycerol [53]. These processes are promoted by stress hormones like adrenaline as a means of providing NEFAs as an energy source in times of need. Further, insulin inhibits lipolysis in adipose tissue, so that in insulin resistant states the flux of NEFAs from adipocytes is increased [52] resulting in elevated concentrations of NEFAs in the plasma [52]. Hepatocytes take up NEFAs from the bloodstream mostly by CD36, fatty acid transport protein (FATP) 2, FATP4 and FATP5 [53].

Upon entering the cell fatty acids are converted into acyl-CoA in the cytosol by acyl-CoA synthetase. Short and medium chain acyl-CoAs can cross membranes and enter organelles like mitochondria where they are readily oxidised [52]. Carnitine palmitoyltransferase 1 (CPT-1) is an enzyme that enables long chain fatty acid translocation to the mitochondrial matrix for β -oxidation. CPT-1 represents the rate-limiting step of β -oxidation and its activity is downregulated by malonyl-CoA [53]. Hence, when malonyl-CoA concentrations are elevated fatty acid β -oxidation is inhibited and fatty acid synthesis is promoted. However, in insulin resistant states NEFA supply to the liver can exceed demand and the fatty acids are incorporated into TAG. This provides a direct causal link between insulin resistance, elevated blood NEFAs, hepatic TAG synthesis and NAFLD. Mitochondrial β -oxidation generates energy from short, medium and long chain fatty acids. The product of β -oxidation is acetyl-CoA which feeds to the tricarboxylic acid cycle or is converted to ketones bodies [52]. The electron transport chain generates ATP as a result of fatty acid oxidation. There is a certain level of inefficiency in the electron transfer chain and in other oxidation reactions, such that reactive oxygen species are generated and lipid peroxidation occurs [52]. Gene expression of enzymes of hepatic fatty acid oxidation is regulated by the transcription factor PPAR- α , which ultimately promotes both mitochondrial and peroxisomal β -oxidation [68]. Marine n-3 PUFAs can upregulate and activate hepatic PPAR- α [69], meaning that they act to partition fatty acids in the direction of β -oxidation and far from TAG synthesis.

****Table 2****

Insulin resistance

As indicated above, the increase in hepatic TAG accumulation can be subsequent to increased lipolysis in adipose tissue, leading to increase in serum NEFA concentrations which are taken up by hepatocytes driving TAG synthesis [70]. An estimate of hepatic TAG origin in patients with NAFLD demonstrated a dominance of preformed NEFAs as the main source (59%), followed by *de novo* lipogenesis (in the fasting state 26% of hepatic TAG and 23% of VLDL TAG were resultant from *de novo* lipogenesis) [71]. It is remarkable that *de novo* lipogenesis made such a contribution to TAG synthesis in patients with NAFLD in the fasting state and that this did not increase in the postprandial

period after a meal with 30% of fat [71]. This suggests that these patients had reached a threshold for *de novo* fatty acid synthesis. Also, the turnover of hepatic TAG was lower in NAFLD patients (20 to 60 days compared to 1-2 days in healthy subjects) and the percentage of hepatic TAG derived from the diet was 15% [71].

As defined by Angulo, from NAFLD to NASH, liver histology shows steatosis, inflammatory cell infiltration, hepatocyte ballooning and necrosis, glycogen nuclei, Mallory bodies, and fibrosis [72]. NAFLD and NASH are classically characterized by the increased amount of hepatic TAG [73]. Nevertheless, these patients also present a lower hepatic content of EPA and DHA [73]. The majority of NAFLD is associated with obesity, which involves adipose tissue inflammation [74]. When more than normal insulin concentrations are required to generate a given metabolic response and/or when normal insulin concentrations are not enough for these responses the condition is called insulin resistance [75]. Individuals with NASH proved by liver biopsy presented an increased pancreatic insulin secretion, severe insulin resistance and similar hepatic insulin removal from the bloodstream compared to healthy subjects [76]. Figure 3 summarises the main metabolic alterations due to insulin resistance and their effect on NAFLD development.

The liver produces glucose during the fasting state via gluconeogenesis or glycogenolysis and this production is suppressed by insulin in the postprandial state so long as the liver is insulin sensitive. Insulin inhibits glucose-6 phosphatase, which converts glucose-6-phosphate into glucose, and phosphoenolpyruvate carboxykinase, responsible for phosphoenolpyruvate formation. Thus in the insulin sensitive state, insulin suppresses hepatic glucose output. Hepatic insulin resistance means the loss of the ability to block hepatic glucose production and output during the postprandial period.

The loss of insulin-mediated glucose uptake into skeletal muscle and skeletal muscle glycogen synthesis, both of which are insulin sensitive processes, means reduced demand for glucose and the sparing of glucose for hepatic *de novo* lipogenesis. Enlarged *de novo* lipogenesis in the liver seems to come ahead of adipose tissue insulin resistance and the higher flux of NEFAs to the liver [77]. Hepatic TAG accumulation does not itself seem to be toxic. Fatty liver is not always accompanied by insulin resistance and despite all of the information available, it is not yet known which comes first, insulin resistance or fatty liver [78]. Subjects with NAFLD and NASH show an increase in hepatic DAG and increased TAG to DAG and n-6 to n-3 fatty acid ratios [73]. DAG and TAG accumulation in the liver can be due to multiple causes as pointed out by Jornayvaz and Schulman: increased delivery of chylomicron remnants, increased release of NEFAs from adipose tissue, postprandial hyperinsulinemia raising hepatic *de novo* lipogenesis, and lower β -oxidation due to decreased mitochondrial function [79]. NAFLD patients also showed a decrease in hepatic phosphatidylcholine and phosphatidylethanolamine [73] while NAFLD and NASH patients had an increase in hepatic free cholesterol and total n-6 fatty acids compared to control individuals [73]. The fatty acid profile of hepatic lipid fractions in subjects with NASH showed a lower than normal content of n-6 and n-3 PUFAs in TAG, a lower than normal amount of arachidonic acid in DAG and PC, an

increased n-6 to n-3 fatty acid ratio in TAG and in the free fatty acid pool, and a lower than normal hepatic content of long chain fatty acids [73].

In mice, a high amount of DAG in hepatocytes seems to be more toxic than high TAG [80]. Inhibition of DAG acyltransferase 2 (DGAT2) in a mouse model of NAFLD decreased hepatic TAG but increased oxidative stress, NEFAs, lobular inflammation and fibrosis [80]. Also, hepatic DAG content could be related to hepatic insulin resistance [79]. Protein kinase C ϵ (PKC) has high affinity for DAG and its activation is implicated in hepatic insulin resistance [79].

Obese individuals undergoing bariatric surgery showed a strong positive association between hepatic DAG amount and a measure of insulin resistance [81]. This correlation was stronger between DAG in lipid droplets compared to membrane DAG. PKC ϵ was the most abundant PKC isoform found in liver and was strongly associated with the DAG content in hepatic lipid droplets [81]. Among the DAG, the ones composed of C18:1-C16:0, C18:1-C18:1, C18:1-C18:2 and C16:0-C18:2 had higher concentration and positive association with insulin resistance. The C20:4-C20:5 DAG was inversely associated with insulin resistance [81]. Endothelial cells incubated with DHA showed a decrease in PKC ϵ activation, cyclooxygenase (COX)-2 mRNA expression, COX-2 protein expression and prostaglandin production [82]. DHA was able to attenuate nuclear factor κ B (NF κ B) activation [82]. PKC ϵ and PKC θ were related to insulin resistance in liver and muscle, respectively [83, 84]. Furthermore, PKC ϵ knockout mice were protected from insulin resistance caused by a high fat diet, despite having increased hepatic fat [85].

Ceramides are sphingolipid-derived constituents of cell membranes. They are generated by three different pathways: *de novo* synthesis, sphingomyelinase pathway, or salvage pathway [86]. *De novo* synthesis of ceramides from palmitoyl-CoA is regulated by cellular redox status and increases in oxidative stress can raise ceramide synthesis [86, 87]. Hepatic ceramide synthesis was increased in obese and insulin resistant individuals and all ceramide species were positively associated with plasma tumor necrosis factor (TNF)- α [88]. Also, a decrease in adiponectin concentration seems to be related to the increase in hepatic ceramide content due to the inhibitory effects of adiponectin on ceramide synthesis [86]. Furthermore, when myricin, a ceramide synthesis inhibitor, was given to *ob/ob* mice fed a high fat diet there was a decrease in body weight, hepatic steatosis and inflammation and improved insulin sensitivity showing a potential role for inhibition of ceramide synthesis in the treatment of obesity comorbidities [89]. Although no clear association between ceramides and human NAFLD has been established, increased hepatic ceramides seem to be one of several alterations generated by insulin resistance and ceramides act as lipid mediators increasing cytokine expression, mitochondrial dysfunction, oxidative stress, and lipoprotein aggregation [86].

Obese women with fatty liver showed increased expression in subcutaneous adipose tissue of macrophage markers (CD38, monocyte chemoattractant peptide 1 and CCL3) and plasminogen activator inhibitor 1, decreased expression of PPAR- γ and adiponectin, and increased amounts of ceramides, sphingomyelins, ether phospholipids and TAG compared to obese women without fatty

liver [74]. The amount of ceramides, C16:0-ceramide and DAG in subcutaneous adipose tissue showed a positive association with insulin resistance [90]. In this study, the total amount of ceramides in adipose tissue was higher in obese type-2 diabetic and obese non-diabetic subjects compared to lean non-diabetic subjects [90].

Cholesterol metabolism revealed an association with NAFLD progression and NASH in obese subjects submitted to gastric bypass. Serum VLDL and LDL cholesterol concentrations were positively correlated to hepatic inflammation and fibrosis. Also serum VLDL cholesterol was associated to hepatic cholesterol content [91]. Although no clear link between liver TAG and cholesterol synthesis has been demonstrated, Caballero and colleagues reported that NAFLD patients had an enhanced expression of SREBP-2, which regulates cholesterol synthesis, and steroidogenic acute regulatory transfer protein, a polypeptide related in mitochondrial cholesterol transport [92]. Genetically modified obese mice fed a high fat diet developed hyperinsulinemia, diabetes, hypercholesterolemia, and hypoadiponectinemia. In this animal model, hyperinsulinemia induced SREBP-2 expression, which up-regulated LDL receptor, decreased bile acid synthesis and cholesterol and bile acid secretion resulting in accumulation of hepatic cholesterol [93].

The amount of liver free cholesterol increased gradually from individuals with normal hepatic histology to NAFLD and NASH [73]. The increase in hepatic fat leads to increased cholesterol synthesis. NAFLD patients seem to have lower rates of cholesterol absorption and higher rates of cholesterol synthesis compared to control individuals. Hepatic fat showed a positive correlation with markers of cholesterol synthesis and a negative correlation to cholesterol absorption [94]. Hypertrophied adipocytes display an accumulation of intracellular cholesterol and a decrease in plasma membrane free cholesterol concentration; the latter is due to the increase in cell surface area. The imbalance in membrane cholesterol concentration results in SREBP-2 activation and membrane instability, which increases membrane permeability and disturbs the integrity of membrane invaginations with a high concentration of signalling molecules (caveolae). These disturbances are related to a reduction in insulin signalling and GLUT4 translocation and an increase in cytokine secretion. Furthermore, the decrease in membrane cholesterol induces cholesterol synthesis [95, 96].

In NAFLD, hepatic cholesterol metabolism is dysregulated with an increase in hepatic cholesterol synthesis, uptake of cholesterol-rich lipoproteins, alterations in intracellular compartmentalization, changes in cholesterol absorption and secretion, altered intracellular cholesterol esterification and de-esterification and modified nuclear regulators of cholesterol homeostasis [96]. An increase in inflammation is one of the hallmarks of NAFLD progression to NASH. Saturated fatty acids are capable of directly activate toll-like receptor 4 (TLR4) [97, 98]. NEFAs can induce macrophages to increase gene expression of TNF- α and IL-6 through TLR4 signalling [99]. Particularly palmitic acid, a saturated fatty acid, strongly stimulated IL-6 expression in macrophages and pre-treatment with EPA and DHA inhibited the induction of TNF- α mRNA expression by palmitic acid [99]. Decreased hepatic and adipose tissue inflammation prevented insulin

resistance in TLR4 knockout C57BL/6 mice fed a high fat diet. Saturated free fatty acids can produce intracellular inflammatory signalling, but how this generates insulin resistance is still not clear [99]. Lipid peroxidation of membranes and formation of antigens by lipoperoxidation products bound to hepatocyte proteins can generate cell death and fibrosis [100]. Furthermore, the liver is exposed to several gut-derived toxins and small intestine bacterial overgrowth and increase in gut permeability happens in a high proportion of subjects with NAFLD [101].

Inflammation and Omega-3 fatty acids

One of the most prominent actions of marine n-3 PUFAs is their ability to modulate inflammatory responses. N-3 PUFAs can act in several different forms to influence the inflammatory process. Incorporation of PUFAs into membrane phospholipids of inflammatory cells maintains the fluidity and alters lipid raft formation [102, 103]. Likewise membrane derived second messengers, such as DAG or NEFAs, have their action influenced by their fatty acid composition, which can be modified by n-3 PUFAs [103, 104]. Membrane non-esterified PUFAs and oxidized PUFA derivatives can interact with surface or intracellular fatty acid receptors on inflammatory cells. Moreover, PUFAs are able to indirectly influence inflammation through changes in complex lipids, lipoproteins, metabolites and hormones [103].

Eicosanoids are generated from PUFAs with 20 carbons. They have a crucial role in inflammation as both mediators and regulators of inflammatory processes. Arachidonic acid has a high concentration in membrane phospholipids in cells involved in inflammation. As a result, arachidonic acid is the main precursor for synthesis of eicosanoid mediators. These include 2-series prostaglandins such as prostaglandin E₂ and D₂ formed in the COX pathway and 4-series leukotrienes such as leukotriene B₄ and E₄ formed in the 5-lipoxygenase (LOX) pathway [105, 106]. Eicosanoids have roles in the liver and might directly affect hepatocyte metabolism [107] or they can control the regulation of hepatocyte metabolism by hormones [108, 109] or cytokines [110, 111]. Indeed, prostaglandin E₂ has been demonstrated to promote de novo lipogenesis and fat accumulation in hepatocytes [112, 113].

Endocannabinoids are also a type of eicosanoid derived from membrane phospholipids. Arachidonoyl ethanolamide and 2-arachidonoylglycerol are the two major endocannabinoids involving arachidonic acid [114, 115]. They act through CB1 and CB2 receptors and have both pro- and anti-inflammatory effects. Marine n-3 PUFAs can reduce arachidonic acid derived prostaglandins and leukotrienes [116, 117] and arachidonic acid containing endocannabinoids [118, 119].

EPA is likewise used by COX and LOX enzymes, but the 3-series prostaglandins and 5-series leukotrienes produced from EPA are less biologically active than the ones derived from arachidonic acid [103]. Docosahexaenoyl ethanolamide and eicosapentaenoyl ethanolamide are endocannabinoids that include marine n-3 PUFAs; these are also ligands for CB1 and CB2 receptors and have strong

anti-inflammatory actions [118, 119]. Furthermore, EPA and DHA can be used by COX and LOX enzymes to generate anti-inflammatory and inflammation resolving compounds including resolvins, protectins and maresins [120-123]. Among these, the pro-resolving actions of resolvin E1, resolvin D1 and protectin D1 are well described and through these effects they act to limit tissue damage [103, 124]. Pro-resolving mediators inhibit transendothelial migration of neutrophils and decrease inflammatory cytokine production; for example resolvin D1 impairs IL-1 β production and protectin D1 decreases IL-1 β and TNF- α production [119, 124, 125]. Figure 4 illustrates the main classes of lipid mediators derived from arachidonic acid, EPA and DHA.

Recent studies have begun to report the possible roles of resolvins and protectins in the liver and in NAFLD. Resolvin D1 was able to attenuate hypoxia-induced expression of COX-2, IL-1 β , IL-6, and C-C chemokine receptor type 7 in liver slices taken from mice with diet-induced obesity [126]. 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 [126]. An earlier study had identified a possible protective role for the resolvin E1 receptor chemokine-like receptor 1 (CMKLR1) in NAFLD [127]. CMKLR1 was identified in liver stellate cells, primary human hepatocytes, Kupffer cells and bile-duct cells, but was decreased in human and rodent fatty liver and in mice fibrotic liver. Adiponectin strongly upregulated CMKLR1 in primary human hepatocytes and in liver tissue while hepatic CMKLR1 was suppressed in the liver of adiponectin deficient mice [127]. A recent study showed that pretreatment with resolvin D1 attenuated ER stress-induced apoptosis and decreased caspase 3 activity in HepG2 cells [128]. Furthermore, resolvin D1 significantly decreased tunicamycin-induced triglyceride accumulation. These studies suggest that EPA and DHA derived pro-resolving mediators could 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 marine n-3 PUFAs.

In addition to modulation of the lipid mediator milieu (prostaglandins, leukotrienes, resolvins, protectins etc.) marine n-3 PUFAs can decrease chemotaxis of human neutrophils and monocytes [129] and can lower adhesion molecule expression on human endothelial cells [130]. Some of the anti-inflammatory effects of marine n-3 PUFAs are due to decreased activation of the prototypical pro-inflammatory transcription factor NF κ B, which has key roles in regulating expression of genes encoding many inflammatory cytokines, adhesion molecules and COX-2 [103].

Cytokines are small proteins secreted by a great variety of cell lines which are responsible for modulation of the inflammatory response. The effects of marine n-3 PUFAs in production of inflammatory cytokines seems to be strongly dependent on their dose [103]. C57BL/6 mice were fed on a diet having fish oil and were then exposed to LP-BM5 murine leukemia virus to mimic HIV

infection. Compared to corn oil fed mice, those fed fish oil had improvements in immune function and a decrease of pro-inflammatory cytokines which was linked to the decreased production of active metabolites of arachidonic acid [131]. Many other in vitro and animal studies show that marine n-3 PUFAs can lower inflammatory cytokine production [119]. A study in healthy human subjects supplemented with different doses of EPA + DHA and antioxidants showed a 'U-shaped' dose response association between marine n-3 PUFAs and EPA incorporation in phospholipids and decrease in TNF- α and IL-6 production by peripheral blood mononuclear cells, showing a greater response to 1 g/d of EPA + DHA compared to 0.3 g and 2 g/d supplementation [132]. TNF- α is elevated in NAFLD and seems to participate of the disease progression to NASH [7]. In contrast, adiponectin is decreased in insulin resistance, obesity, diabetes and NAFLD [133]. Adiponectin has anti-lipogenic and anti-inflammatory actions; therefore the increase in TNF- α and decrease in adiponectin favours liver fat accumulation and inflammation [7, 133].

NF κ B is a trimer in the cytosol that contains an inhibitory subunit called the inhibitory subunit of NF κ B (I κ B). Phosphorylation of I κ B allows NF κ B translocation to the nucleus, binding to DNA and regulation of gene expression [134]. Several extracellular inflammatory stimuli can trigger NF κ B activation, often acting through toll-like receptor 4. Marine n-3 PUFAs are able to reduce I κ B phosphorylation and consequential activation of NF κ B in macrophages [135]. NAFLD subjects present an increase in oxidative stress, toll-like receptor expression, cytokines and adipokines, such as TNF- α , IL-6, leptin and resistin, and a decrease in adiponectin [136]. Insulin resistance is closely related to NAFLD and its progression, and additionally insulin resistance is responsible for the increase of NEFA supply to liver [136]. NEFA and cholesterol accumulation in mitochondria are associated with an increase in oxidative stress and TNF- α expression [137]. Also, high-fat and high carbohydrate diets can alter gut "leakiness" leading to lipopolysaccharide translocation causing endotoxemia, which contributes to inflammation [137]. NAFLD subjects presented enhanced gut permeability and small intestinal bacterial overgrowth compared to healthy subjects [138].

Treatment of NAFLD

There is no specific pharmaceutical treatment for NAFLD and NASH. Lifestyle modification is the first approach indicated for subjects with NAFLD. Exercise and weight loss are able to ameliorate insulin resistance and reduce the amount of liver fat. A 10% reduction in body weight was able to decrease the hepatic TAG content in about 50% of overweight male and female subjects [139]. Another study found improvement in liver histology with >7% of body weight loss [140]. Physical exercise seems to be able to reduce liver fat independent of weight loss, although weight loss is still essential for NAFLD treatment. Physical exercise combined with energy restriction has conclusive benefits in NAFLD [141]. Furthermore, exercise can result in weight loss without energy restriction in a dose-response manner [142]. Below 150 min of aerobic exercise per week generates little weight

loss while > 150 minutes per week is able to generate modest weight loss (~2-3 kg) and 225-420 minutes per week of aerobic exercise can result moderate weight loss (~5-7.5 kg) [142, 143].

Bariatric surgery has shown positive results in NAFLD as reported from a meta-analysis [13]. A reduction in steatosis was found in 91.6% of subjects, improvement or resolution of steatohepatitis in 81.3% of subjects, and improvement or resolution of fibrosis in 65.5% of subjects who underwent bariatric surgery [13]. However, the evolution of NAFLD after bariatric surgery seems to be closely related to insulin resistance, since subjects with severe insulin resistance presented lower reduction in liver fat after surgery than those who were less insulin resistant [144].

Nine months of Orlistat™ combined with a diet of 1400 kcal per day and vitamin E (800 IU per day) generated an 8% weight loss in subjects with NAFLD, but the weight loss was not different from what was seen in the placebo group, which received vitamin E and the same low calorie diet and lost 6% of weight [145]. Neither group exhibited differences in liver histology, but when groups were analysed together it was possible to verify that > 9% of weight loss caused a reduction in serum aminotransferases, insulin resistance, steatosis, ballooning and NAS [145]. Thiazolidinediones seem to improve insulin sensitivity, decrease plasma glucose, haemoglobin A1c, steatosis and inflammation, but have no effects on fibrosis [146]. However, weight gain and lower extremity edema are frequently reported side effects of thiazolidinediones [146]. Safety and efficacy of thiazolidinedione (pioglitazone and rosiglitazone) should be evaluated in larger randomized controlled trials, as suggested by Musso and colleagues [146]. Metformin administrated together with lifestyle modification was able to decrease insulin resistance and plasma glucose and increase weight loss associated with improved steatosis, ballooning, and inflammation [147]. However, paediatric subjects with NAFLD did not benefit from 2 years metformin treatment when compared to a vitamin E supplemented group and the placebo group [147]. Vitamin E has shown favourable effects improving steatosis, ballooning, and inflammation compared to pioglitazone and placebo groups, but results are highly variable and doses above 400 IU may be related to a rise in all cause-mortality [148]. Ursodeoxycholic acid, a FXR ligand, was not able to treat NAFLD and high doses need further investigation to ensure safety [146]. Marine n-3 PUFAs have shown positive effects on NAFLD treatment and will be discussed in the next section.

Algal and marine omega-3 polyunsaturated fatty acids for NAFLD treatment

In the past few years several clinical studies have evaluated whether algal or marine n-3 PUFAs have a role in treatment of NAFLD. A total of 17 published reports were found that investigated the effects of n-3 PUFAs on liver fat in human subjects with NAFLD. Fourteen of these studies used mixtures of EPA and DHA, 2 studies used purified ethyl esters of EPA (EPA-EE) and one study used DHA in the absence of EPA. Table 3 summarizes the results from these studies.

Table 3

In a study including patients with hyperlipidaemia and NAFLD, the effects of fish oil (15 ml daily providing 1.58 g/d DHA and 2.25 g/d EPA for 24 weeks) were compared with those of Orlistat and Atorvastatin [149]. After the treatment, ultrasound revealed normal liver patterns in 35% of patients receiving fish oil, 61% of patients receiving Atorvastatin and 86% of patients receiving Orlistat. Fish oil treatment also decreased serum triglycerides (34%), total cholesterol (11%), AST (61%), ALT (39%) and GGT (22%) as compared to the corresponding values at study entry. A significant decrease (13%) in BMI was found only in the Orlistat group [149]. In another study with fish oil (1 g/d for 12 months, EPA:DHA being 0.9:1.5), Capanni et al. reported that liver steatosis as determined by ultrasound (Doppler perfusion index) improved by more than 60% after treatment with fish oil however did not change in the control group when compared to the pre-supplement values [150]. Fish oil also decreased serum concentrations of ALT (73%), GGT (40%) and TAG (59%), which did not change in the control group [150].

Two studies evaluated the effects of caloric restriction with and without fish oil supplementation. One of these studies assessed the effects of fish oil (2 g/d for 6 mo, exact amounts of EPA and DHA not specified) in combination with 30% caloric restriction and the caloric restriction alone [151]. At the end of the study, BMI was decreased by 2.9% in the caloric restriction group and by 6.3% in the group receiving fish oil and caloric restriction when compared to the pre-treatment values. As determined by ultrasound complete, partial or no regression of steatosis was seen in 33, 50 and 17% subjects in the fish oil group and in 0, 28, and 70%, respectively, in control group. The results suggest that caloric restriction alone decreased liver fat an effect which was enhanced by fish oil. However, only the marine n-3 PUFAs group showed a reduction of serum ALT (30%), GGT (29%), TAG (25%), and TNF- α (19%) and HOMA-IR (25%) [151]. A subsequent study also imposed 25-30% caloric restriction along with seal oil (6 g/d for 24 wk) or placebo oil (unspecified, 6 g/d) to subjects with NAFLD diagnosed by ultrasound [152]. In the end of the treatment period complete fat regression was found in 19.7% of the subjects in the seal oil group, and 7.3% of subjects in the caloric restriction alone group; corresponding overall fat regressions were 53.0% and 35.3%, respectively. Serum ALT and TAG also decreased in both groups but the decrease was significantly bigger in the seal oil than in the placebo oil group. It is worth noting that seal oil contains more DPA than fish oils [153]. Results of these two studies involving caloric restriction and n-3 PUFA supplementation are generally in agreement.

Another fish oil supplementation study determined liver fat using MRS which provides a more quantitative estimation of liver fat than ultrasound [154]. This was a sequential study with 4 wk of placebo oil (mixture of eladic, linoleic and palmitic oil, 9 g/d) followed by 8 wk of fish oil (9 g/d, providing EPA 4.63 g/d and DHA 2.15 g/d). Sixteen overweight and obese (mean BMI 36.2 kg/m², range 27-61 kg/m²) subjects were involved mean pre-study liver fat of 10.6% (range 2.4-26.9%). Fish

oil supplementation significantly lowered plasma TAGs but did not alter liver fat. Small number, heterogeneous population, and short duration of supplementation may be the basis for lack of change in liver fat by a relatively high dose of fish oil [154].

Effects of purified ethyl ester of EPA (EPA-EE 2.7 g/d for 12 m) on markers of NAFLD and NASH were investigated in 23 liver biopsy proven NASH patients, although repeat biopsy was performed in only 7 subjects [155]. During EPA-EE supplementation, patients were allowed to continue their medications and any dietary restrictions they had been following for the previous 12 months. At the end of EPA-EE supplementation, liver steatosis diagnosed by ultrasound was decreased in 12 patients (52%, mean steatosis grades before and after EPA-EE were 2.1 and 1.6, respectively). EPA-EE supplementation also significantly decreased serum concentrations of ALT, AST, NEFAs, sTNF-R1, sTNF-R2, ferritin, and thioredoxin, and increased serum EPA content and EPA/AA ratio. Results of second liver biopsy in 7 subjects showed that EPA-EE decreased steatosis (29%), fibrosis (59%), lobular inflammation (48%), ballooning (44%) and NAS (39%). The results of this small and heterogeneous study are fairly convincing that EPA-EE improves markers for both NAFLD and NASH. However, these results are at variance from another large, multicentre study that supplemented two concentrations of EPA-EE (1.8 g and 2.7 g/d for 12 mo) and performed liver biopsies before and after the supplementation in all subjects [156]. In the latter study, neither dose of EPA-EE had an effect on steatosis, inflammation, ballooning or fibrosis scores. The only beneficial effect of EPA-EE in this study was a 4.3 % reduction in plasma TAGs by EPA-EE at 2.7 g/d when compared with the corresponding values before the supplement. It is not possible to identify the exact cause for these inconsistencies, but several factors including differences in the subject characteristics, compliance with EPA-EE consumption, changes in diet and life style may have contributed to the different results.

Since insulin resistance is often found in patients with polycystic ovary syndrome (PCOS) and NAFLD, and females with PCOS frequently develop NAFLD [157]. Cussons and collaborators evaluated the effects of fish oil on indicators of NAFLD in 25 obese women with PCOS in a randomized cross over study [157]. Fish oil (4 g/d, providing EPA 1.08 g/d and DHA 2.24 g/d) and the olive oil (4 g/d) were each given for 8 wk with a washout period of 8 wk in between. Liver fat as determined by MRS was significantly decreased by fish oil compared to placebo in 12 women (mean 18.2 vs 14.8%). The other 13 women did not have NAFLD and hence the liver fat did not differ between the two treatments (2.4 vs 2.7%). If the data from all women were pooled, the mean liver fat after fish oil and placebo treatments was 8.2 and 10.4%, respectively. The reduction in liver fat within 8 wk of fish oil supplementation might be due to higher proportion of DHA used compared to that of EPA and the high sensitivity of MRS to detect changes in liver fat.

The effects of DHA supplementation in the absence of EPA on markers of NAFLD and NASH were assessed in a randomized controlled trial with obese children [158-160]. Sixty children were divided into three groups of 20 each and were given DHA 250 and 500 mg/day or placebo (germ

oil providing linoleic acid 290 mg/d) for 24 mo combined with diet and exercise. Liver fat using ultrasound and biochemical markers for NAFLD were determined every 6 mo and an additional liver biopsy was carried out in the group receiving DHA 250 mg/d after 18 mo of treatment. Liver fat and serum TAGs were significantly decreased and ISI increased while ALT did not change in both DHA groups after 6 mo of DHA supplementation. There was no further decrease in liver fat at 12, 18, and 24 mo when compared to 6 mo of DHA supplementation. Liver biopsy after 18 mo of DHA 250 mg/d revealed decreases in hepatic steatosis 70%, ballooning 70%, NAS 30% and PNHS 91%. Authors of this study also investigated the potential mechanisms by which DHA may reduce NAFLD and NASH. Both DHA concentrations increased hepatic expression of GPR120, a macrophage lipid-sensing receptor. DHA also modulated the macrophage response decreasing toll-like receptor and the TNF- α signalling pathway [160]. In hepatocytes and macrophages, DHA decreased the nuclear translocation of serine-311-phosphorylated NF- κ B and GPR120-positive M ϕ /Kupffer cell pool showing that DHA could decrease the inflammatory macrophage pool and lead to an anti-inflammatory macrophage polarization [160].

Subjects with well-controlled type 2 diabetes and biopsy proven NASH were part of a prospective double-blinded, randomized, placebo-controlled trial. EPA and DHA (2.16 g and 1.44 g per day, respectively) and corn oil as placebo were given to 18 and 19 patients, respectively. After 48 wk of supplementation, a second liver biopsy was performed on all study participants. Marine n-3 PUFAs did not enhance hepatic histology or biochemical parameters, but liver fat and NAS were decreased in the corn oil group. In addition to the lack of effect on liver fat, n-3 PUFAs increased HOMA-IR. Besides the small number of participants, this study did not monitor tissue incorporation of marine n-3 PUFAs or report any other measure of compliance. Hence poor compliance cannot be excluded [161]. On the other hand, recent work showed an inverse correlation among liver fat percentage and erythrocyte DHA enrichment [162]. This randomized double-blinded placebo trial provided 1.84 g of EPA and 1.52 g of DHA per day (as ethyl esters) for a minimum of 15 and maximum of 18 mo to 51 individuals or olive oil as a placebo to 52 individuals. Each 1% of DHA enrichment of RBC fatty acids was associated with a 3.3 % decrease in hepatic fat as determined by MRS. A 6% DHA enrichment was correlated with a 20% reduction in hepatic fat. In contrast to the inverse correlation between RBC DHA and liver fat, RBC EPA concentration did not show a similar association with liver fat. These data advocate that DHA might be more effective than EPA in decreasing liver fat. Furthermore, the PNPLA 3 148M/M genotype was associated with higher liver fat and lower DHA tissue enrichment in the end of the study [163].

Argo et al. investigated the effects of fish oil supplementation and increased aerobic exercise along with decreased caloric intake in subjects with liver biopsy proven NASH in a randomized, double blind and placebo control study [164]. This study included 34 subjects, 17 of which received fish oil (3 g/d, providing EPA 1.05 g, DHA 0.75 g, and 0.24 g other n-3 PUFAs) and the other 17 received soybean oil as placebo for 12 months. In addition to the liver biopsy to determine NAS score

before and after the treatment, liver fat was also determined by MRI. N-3 PUFAs significantly decreased liver fat and markers of liver injury but not NAS. Body weight was reduced in subjects who received n-3 PUFAs, but the decrease in hepatic fat was independent of weight loss. However, the improvement in markers of cell injury was only found in subjects who lost weight and received fish oil. Furthermore, in subjects who lost weight, the effects of weight loss and n-3 PUFAs was synergistic for reduction of liver fat. In this study n-3 PUFAs did not change insulin sensitivity and serum ALT concentration.

The effects of algal oil containing DHA and EPA were studied on markers of fatty liver in overweight/obese children with NAFLD determined by ultrasound in a randomized, double-blinded placebo controlled trial [165]. The amount of algal oil was based on subject's weight: < 40 kg received 0.45 g of algal oil/per day (267 mg of DHA and 177.5 mg of EPA daily); 40-60 kg received 0.9 g/per day (534 mg of DHA and 355 mg of EPA daily) and > 60 kg received 1.3 g/per day (800 mg of DHA and 532.5 mg of EPA daily) for 24 weeks. Sunflower was used as placebo in amounts similar to those of algal oil. The n-3 PUFA group comprised 30 and placebo group 34 children. Both groups also received nutritional counselling as part of their treatment. After 24 wk, there was no difference between the groups for the number of subjects with decrease in serum ALT, liver hyperechoenicity, insulin resistance and serum lipid levels. However, the n-3 PUFA group showed significant decreases in serum AST and GGT, markers of liver damage, and increased serum adiponectin compared to placebo group.

Another study also evaluated algae oil supplementation in children. In this double-blinded, parallel-group, randomized, placebo controlled trial, 250 mg/d algal oil (39% DHA $\approx$ 97.5 mg/d) was compared to 290 mg/d germ oil (mainly linoleic acid) in a 6 mo intervention. Children around 11 years old also received recommendations of a low-caloric diet (25-30 kcal/kg/d) and daily exercise (60 min/day, 5 times/week). The study included 25 participants in the DHA group and 26 participants in the placebo group. DHA supplementation decreased liver fat by 53.4% and the hepatic fat fraction from 14% to 6.5% assessed by magnetic resonance imaging (MRI). The reduction in hepatic fat was bigger than in placebo group (22.6%), in which no differences were observed in the hepatic fat fraction throughout the 6 mo trial. Furthermore, DHA supplementation reduced abdominal visceral adipose tissue and epicardial adipose tissue compared to placebo group [166].

Obese adolescents with NAFLD aged 13-14 years old were randomly allocated into two groups: PUFA group (n=56), receiving 1 g/d of PUFA containing 380 mg of EPA and 200 mg of DHA (as described in the supplement's manufacture website) and placebo group (n=52), which received placebo (no description found in the article). All children received lifestyle counselling comprising a diet with 25-30 kcal/kg/d for weight loss and scheduled physical activity (one hour, three times per week) in addition to the encouragement of self-initiated physical activities. They were followed for one year and NAFLD was evaluated by ultrasonography. After 12 mo, both groups had a decrease in weight, BMI and hepatic steatosis. The improvements were more prominent in the PUFA

group, which had a higher increase in plasma HDL cholesterol, lower TAG, ALT, AST, insulin and HOMA index and liver fat compared to placebo group. These results showed an additional effect of n-3 supplementation in children that underwent a lifestyle change [167].

In another study, n-3 fatty acids supplementation was evaluated in a prospective, randomized, controlled, unblinded trial. Subjects were separated in two groups: PUFA group (n=39) receiving 50 ml 1:1 ratio EPA:DHA added into daily diet and control group (n=30) receiving normal saline for 6 mo. Liver biopsies were done in the beginning and in the end of the study. After 6 mo treatment, PUFA group showed improvement in the steatosis grade, necro-inflammatory grade, fibrosis stage and ballooning score compared to control group. Both groups had an increase in physical activity and a decrease in BMI and percentage of smokers. PUFA group also showed a decrease in the serum levels of ALT, AST, TAG, total cholesterol, protein C reactive, malondialdehyde, type IV collagen and pro-collagen type I pro-peptide [168].

Nogueira et al. conducted a double-blinded, randomized, controlled trial in which n-3 PUFA was provided as a mix of flaxseed oil and fish oil (0.945g n-3 per day, 64% ALA, 16% EPA and 21% DHA) and compared to mineral oil (2 ml per day). Liver biopsies were performed in the beginning and after 6 mo supplementation. N-3 group (n=27) and placebo group (n=23) showed an increase in plasma n-3 fatty acids and a decrease in arachidonic acid. No differences between groups and among basal and after 6 months supplementations were observed in liver histology, except from lobular inflammation that was improved in the placebo group. However, both groups showed positive association between individual increase in plasma n-3 fatty acids and the percentage of patients with improvements in NAFLD markers. Subjects in the placebo group may have increased their n-3 intake, which is supported by the enhancement in plasma n-3 fatty acids. This off-protocol n-3 ingestion may have masked the possible benefits of n-3 in the n-3 group [169].

In summary, out of the seventeen published reports, eight studies used fish oil, one seal oil, two purified EPA-EE, two a mixture of purified EPA-EE and DHA-EE; one a mixture of algal EPA and DHA, two algal DHA in the absence of EPA and one a mix of flaxseed oil and fish oil.. Study duration ranged from 2 to 24 mo, and the daily amount of n-3 PUFAs used ranged from 250 mg DHA to 6.8 g of a mixture of EPA and DHA. Five studies also involved dietary restriction and exercise along with n-3 PUFA supplementation. Results from these 5 studies suggest that n-3 PUFAs improve NAFLD independent of weight loss. Seven studies with fish oil, 1 with seal oil, 1 with EPA-EE, 2 with algal DHA demonstrated a decrease in markers of NAFLD. Two studies with fish oil, 1 with EPA-EE, 2 with a mixture of algal EPA and DHA and 1 with a mix of flaxseed oil and fish oil did not find any changes in liver fat or other markers of inflammation. The failure of n-3 PUFAs to decrease markers of NAFLD in these studies may be due to short duration, poor compliance, patient specific factors and the sensitivity of the methods used. One of the studies showed that the decrease in liver fat was associated with RBC enrichment with DHA but not EPA. The 2 fish oil studies that did not find

reduction in liver fat with n-3 PUFAs used almost twice the amount of EPA compared to DHA. These findings advocate that DHA is more effective than EPA in the reduction of NAFLD.

Conclusions

NAFLD is growing public health concern due to a rapid growth in its worldwide incidence. The balance between hepatic fatty acid synthesis and utilization and between hepatic uptake and export seem to be dysregulated in NAFLD. Obesity is one of the main risk factors for NAFLD establishment and dietary habits are therefore closely related to its pathophysiology. Furthermore, increased hepatic inflammation and insulin resistance are related to NAFLD progression to NASH, which can lead to more severe and irreversible liver disorders. Marine n-3 PUFAs are able to modulate fatty acid metabolism decreasing *de novo* fatty acid synthesis and increasing hepatic fatty acid β -oxidation. They also have anti-inflammatory effects. Supplementation with marine n-3 PUFAs has been shown to decrease hepatic fat content in several studies and seems to be an alternative treatment for NAFLD subjects, especially when combined with dietary restriction. Marine n-3 PUFAs are safe, well tolerated and have few side effects. A minimum of 1.52 g of DHA and 1.08 g of EPA per day offered for at least 6 months seems to be required to have an effect. A higher percentage of DHA in relation to EPA also appears to be favourable. Adequately sized and properly controlled randomized clinical trials for longer periods are still required to fully evaluate the benefits of marine n-3 PUFAs in NAFLD. Safety of high doses should also be evaluated.

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Figure legends

Figure 1. The progression of NAFLD showing the first and second hits.

Figure 2. Elongation and desaturation of essential fatty acids. N-6 and n-3 PUFAs are elongated and desaturated by the same enzymes.

Figure 3. Insulin resistance and its metabolic effects. The increase in hepatic TAG, cholesterol and DAG is consequent to the decrease in fatty acid β -oxidation and increase in de novo fatty acid and TAG synthesis. These alterations lead to an increase in hepatic oxidative stress and inflammation. Insulin resistance is considered one of the main reasons for hepatic TAG accumulation, which is considered the first “hit” to NAFLD development. Insulin resistant adipose tissue increases lipolysis and is more inflamed, resulting in higher levels of circulation NEFAs, which can be taken up by the liver and esterified into TAG, and of inflammatory mediators. In addition, insulin resistance decreases the glucose uptake and the glycogen synthesis in muscle cells, causing less glucose utilization and raising the circulating glucose levels. Glucose can be taken up by liver by an insulin-independent transporter and be converted to pyruvate. Pyruvate is a precursor of acetyl-CoA and malonyl-CoA, which can be converted into fatty acids through de novo lipogenesis reactions.

Figure 4. Pro-inflammatory, anti-inflammatory and pro-resolving mediators derived from arachidonic acid, EPA and DHA. COX – cyclooxygenases; LOX – lipoxygenases; PGs – prostaglandins; LTs – leukotrienes.