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REVIEW ARTICLE

Targeting n-3 Polyunsaturated Fatty Acids in Non-alcoholic Fatty Liver Disease

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Abstract: ***Background*:** Non-alcoholic fatty liver disease (NAFLD) is characterized by abnormal hepatic accumulation of triacylglycerides in the absence of alcohol consumption, in association with oxidative stress (OS), a pro-inflammatory state and insulin resistance (IR), which are attenuated by n-3 long-chain polyunsaturated fatty acids (FAs) $_{C20\text{-}C22}$ (LCPUFAs) supplementation. Main causes of NAFLD comprise high caloric intake and a sedentary lifestyle, with high intakes of saturated FAs.

***Methods*:** The review includes several searches considering the effects of n-3 LCPUFAs in NAFLD *in vivo* and *in vitro* models, using the PubMed database from the National Library of Medicine-National Institutes of Health.

***Result*:** The LCPUFAs eicosapentaenoic acid (C20:5 n-3, EPA) and docosahexaenoic acid (C22:6 n-3, DHA) have a positive effect in diminishing liver steatosis, OS, and the levels of aspartate aminotransferase, alanine aminotransferase and pro-inflammatory cytokines, with improvement of insulin sensitivity and adiponectin levels. The molecular pathways described for n-3 LCPUFAs in cellular and animal models and humans include peroxisome proliferator–activated receptor-α activation favouring FA oxidation, diminution of lipogenesis due to sterol responsive element binding protein-1c downregulation and inflammation resolution. Besides, nuclear factor erythroid-2-related factor-2 activation is elicited by n-3 LCPUFA-derived oxidation products producing direct and indirect antioxidant responses, with concomitant anti-fibrogenic action.

***Conclusion*:** The discussed effects of n-3 LCPUFA supplementation support its use in NAFLD, although having a limited value in NASH, a contention that may involve n-3 LCPUFA oxygenated derivatives. Clinical trials establishing optimal dosages, intervention times, type of patients and possible synergies with other natural products are needed in future studies.

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1. INTRODUCTION

Non-alcoholic fatty liver disease (NAFLD) is a multifactorial metabolic disorder considered as the hepatic manifestation of metabolic syndrome, which is characterized by (i) abnormal accumulation of triacylglycerides (TAGs) (more than 5% of the total weight) in the absence of alcohol consumption [1]; (ii) progression into nonalcoholic steatohepatitis (NASH), fibrosis, cirrhosis and hepatocellular carcinoma [2]; and (iii) development of insulin resistance (IR), oxidative stress (OS) and inflammation as concomitant factors. This is

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concordant with the increasing incidence of NAFLD which is directly linked to the high prevalence of obesity and type 2 diabetes worldwide [3]. Under these conditions, the depletion of n-3 long chain polyunsaturated fatty acids $_{C20\text{-}C22}$ (n-3 LCPUFAs) in patients with NAFLD can contribute to the development of fatty liver due to an imbalance in the ability to regulate lipid metabolism [4].

The n-3 PUFAs correspond to a family of organic molecules that have important physiological and biochemical roles in the organism throughout the life cycle [5]. In this regard, α-linolenic acid (18:3 n-3, ALA) is the first FA of the n-3 family that is considered essential for humans and other mammals, since these organisms do not have the necessary enzyme to incorporate C = C double bonds at position 15 of the chain [6]. In addition, this FA is the metabolic precursor of the other n-3 PUFAs, a process in which ALA is desaturated and elongated to become of greater length and desaturation takes place until reaching 24 carbon atoms and 6 double bonds [7] in docosahexaenoic acid (C22:6 n-3, DHA) [8] (Fig. **1**). The synthesis of n-3 LCPUFAs from ALA is a complex metabolic process, which involves the participation of desaturases (FADSs) and elongases (ELOVLs) (Fig. **1**), in addition to an important interaction with other nutrients (vitamins and minerals), hormones and the intracellular redox state [9]. Both FADS1 and FADS2 share common regulatory features including insulin dependency of their expression, suppression by LCPUFAs [9] and diminution of their activity by a redox sensitive mechanism associated with obesity-induced liver OS [10,11] or iron-induced OS [12]. The liver is one of the most active organs accounting for n-3 LCPUFA biosynthesis, exceeding that of brain and heart, thus playing a critical role in providing these FAs to extrahepatic tissues via secretion in very low density lipoproteins [13,14].

The n-3 LCPUFA-dependent regulation of lipid metabolism is exerted at two levels, namely, the modulation of signal transduction through manipulation of the membrane fatty acids (FAs) composition and the modification of gene transcription [4]. In the latter context, NAFLD underlies a negative regulation in the activity of transcription factor peroxisome proliferator-activated receptor-α (PPAR-α) that lessens FA oxidation, with a simultaneous positive regulation in the activity of the lipogenic transcription factor sterol regulatory element binding protein-1c (SREBP-1c), which is contributed by PPAR-γ upregulation [15,16]. These disturbances enhance the hepatic *de novo* lipogenesis/FA oxidation ratio (Fig. **2**), with concomitant inhibition of FA export from the liver to other organs [17-19]. The substantial decrease in the hepatic levels of n-3 LCPUFAs that is observed in obese humans [4,19] and in animals subjected to high-fat diet (HFD) inducing liver steatosis [20] is related to a reduction in the biosynthesis of these FAs from metabolic precursors due to diminution in the activity of Δ-5 (FA desaturase

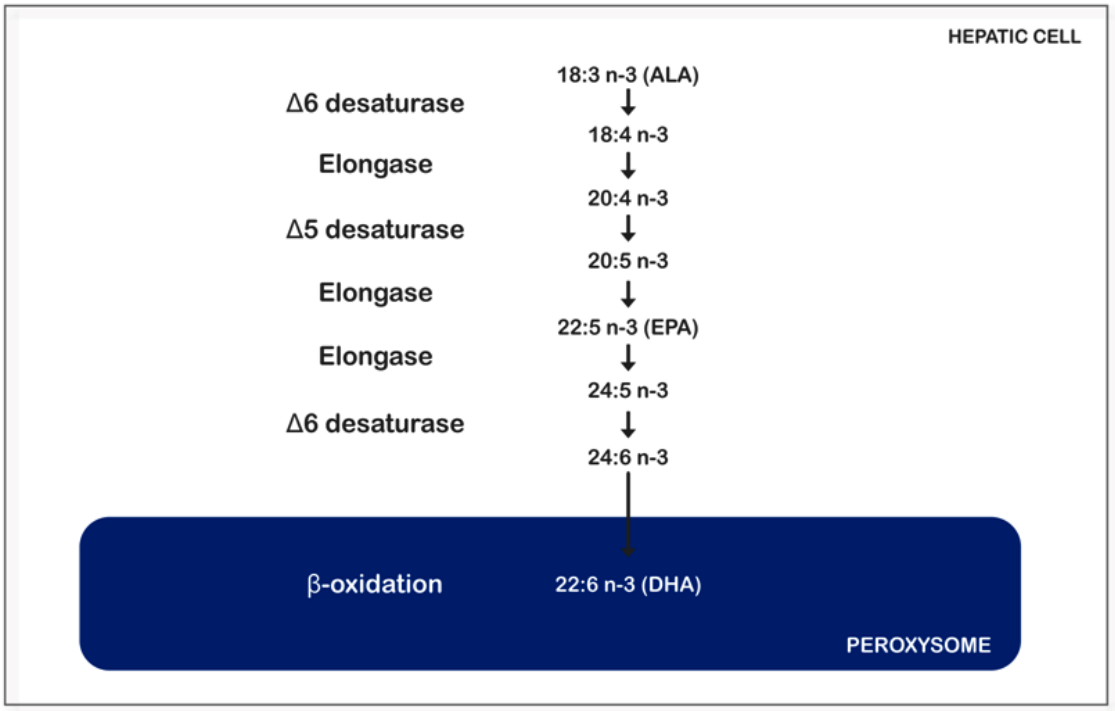

Fig. (1). Biosynthesis of n-3 polyunsaturated fatty acids.

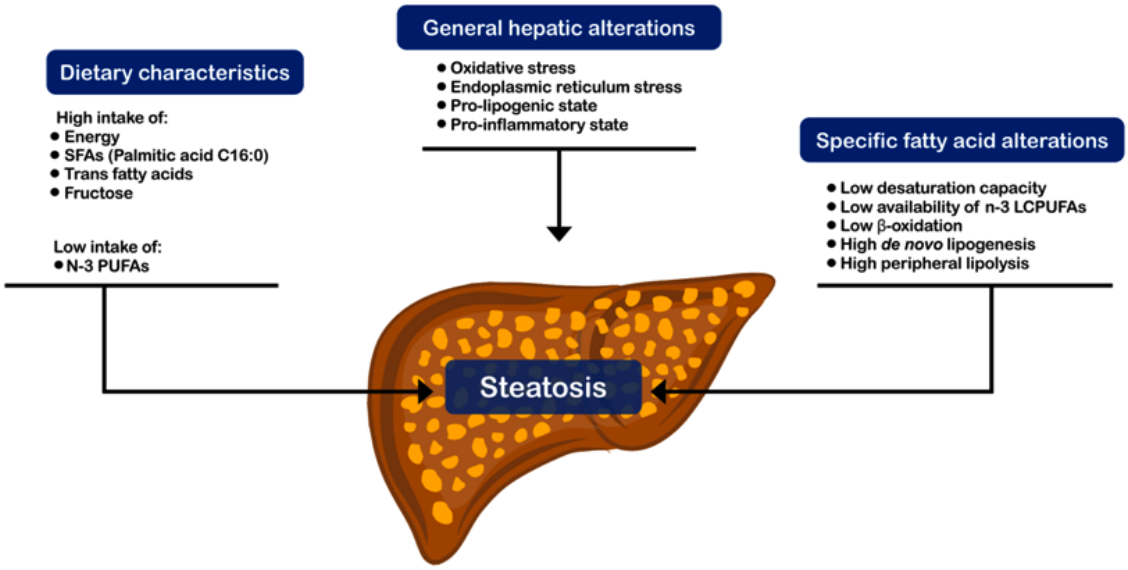

Fig. (2). Dietary characteristics, pathological mechanisms and lipid metabolism alterations leading to hepatic steatosis. *Abbreviations:* n-3 LCPUFAs, n-3 long-chain polyunsaturated fatty acids; SFAs, saturated fatty acids.

1; FADS1) and Δ-6 (FADS2) desaturases and to an increment in FA peroxidation and/or the metabolic use of n-3 LCPUFAs (Fig. **2**) [4]. On the other hand, supplementation with n-3 LCPUFAs as potent ligands of PPAR-α promotes β-FA oxidation and thereby supports weight reduction and fat depots decrease in the liver, thus diminishing liver steatosis (Fig. **3**) [21]. The depletion of liver n-3 LCPUFAs with the concomitant reduction in activated PPAR-α levels may play a role in enhancing the DNA binding capacity of the pro-inflammatory transcription factors nuclear factor-κB (NF-κB) and activator protein 1 (AP-1), which represent the most important mechanism for the progression of steatosis to NASH [22]. Furthermore, n-3 LCPUFAs have anti-inflammatory effects associated with the production of their eicosanoids derivatives such as resolvins and protectins leading to acute and chronic inflammation resolution, with parallel activation of both anti-inflammatory PPAR-α, downregulating NF-κB and AP-1, and antioxidant nuclear factor-erythroid 2 related factor 2 (Nrf2) hindering OS as an inflammation trigger (Fig. **3**) [20,23,24]. Therefore, in this review, we summarize different aspects of NAFLD as well as the health properties of n-3 LCPUFAs as a target for the treatment and the prevention of this disease.

2. MATERIAL AND METHODS

The review includes several searches considering the metabolic and beneficial effects of n-3 LCPUFAs in non-alcoholic fatty liver disease *in vivo* and *in vitro* models, using the PubMed database from the National Library of Medicine-National Institutes of Health. A particular emphasis was placed on the molecular pathways involved in the participation of n-3 LCPUFAs in the observed effects.

3. DIETARY ASPECTS OF NAFLD

Diet and lifestyle can significantly affect the clinical depiction and evolution of the disease [25]. Most patients with NAFLD have excessive body weight and other cardiometabolic risk factors such as hypertension, dyslipidemia and diabetes [25]. Obesity is usually due to an excessive caloric intake and a sedentary lifestyle (Fig. **2**) [26]. However, NAFLD is also present in some non-obese individuals, suggesting that the disease is not simply the result of excessive energy intake and may also be associated with diet quality [26]. Changes in the quality and composition of the diet can directly influence the clinical course of NAFLD beyond the simple caloric restriction. Indeed, diet composition modulated either by its macronutrient or micronutrient components can significantly affect most risk factors associated with fatty liver such as hypertension, serum lipids, and insulin response [25]. The Western diet is characterized by high consumption in refined grains, red and highly processed meat, butter, high-fat dairy products, sweets and desserts, soft drinks and hydrogenated fats, and very low consumption of vegetables and low-fat dairy products. These eating patterns are positively associated with abdominal obesity, inflammation and dyslipidemia considered as risks factors for NASH [27]. Studies of dietary habits of patients with NAFLD reveal that they consume less fish oil, a low intake of fish rich in n-3 LCPUFAs, but twice the

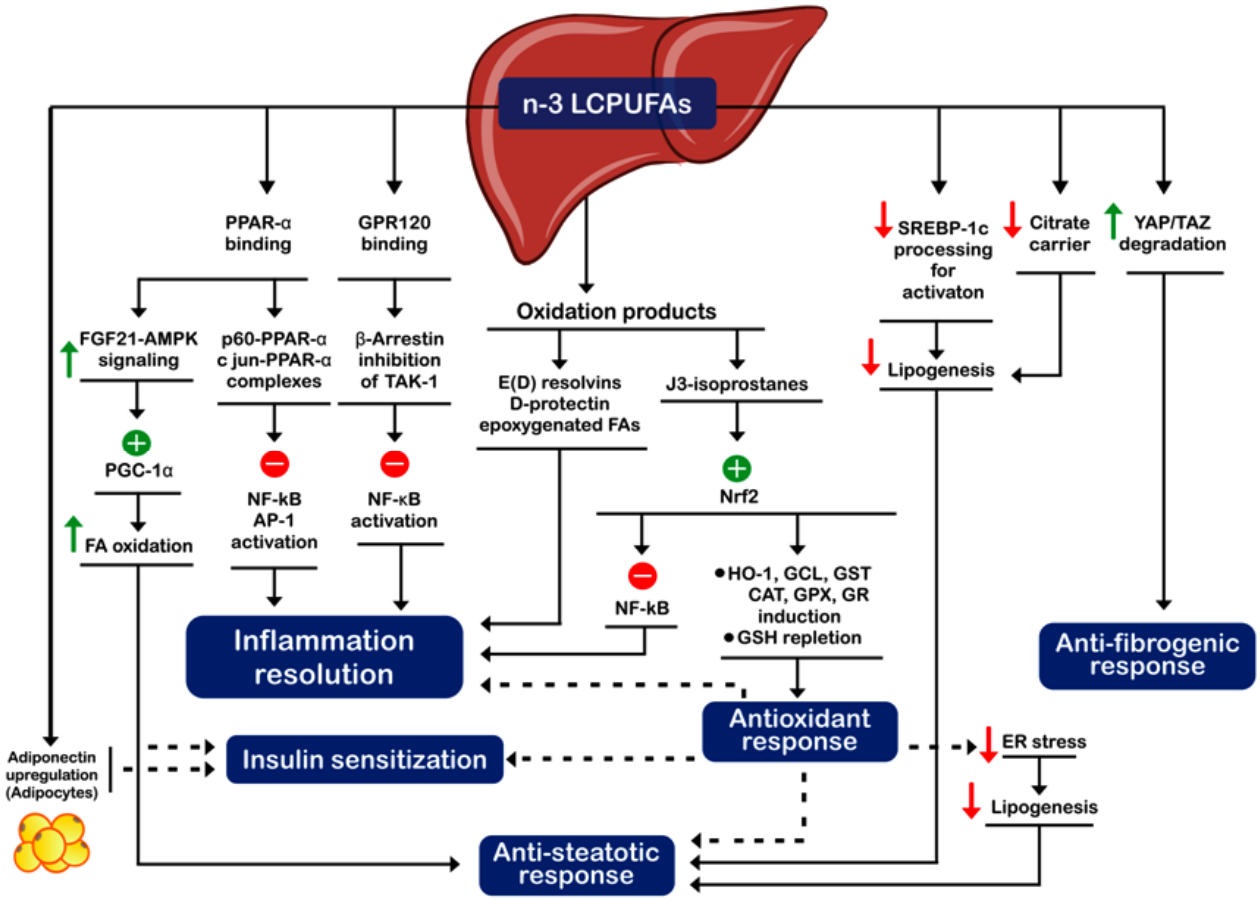

Fig. (3). Protective cellular responses afforded by n-3 long-chain polyunsaturated fatty acids (n-3 LCPUFAs) via direct ( ) and indirect ( ) signaling. *Abbreviations:* AMPK, AMP-activated protein kinase; AP-1, activating protein-1; CAT, catalase; ER, endoplasmic reticulum; FA, fatty acid; FGF21, fibroblast growth factor-21; GLC, glutamate-cysteine ligase; GSH, reduced glutathione; GPX, glutathione peroxidase; GPR120, G protein-coupled receptor-120; GR, glutathione reductase; GST, glutathione-S-transferase; HO-1, heme oxygenase-1; Nrf2, nuclear factor erythroid-2-related factor-2; PGC-1α, peroxisome proliferator-activated receptor-γ coactivator-1α; PPAR-α, peroxisome proliferator-activated receptor-α; NF-κB, nuclear factor κB; SREBP-1c, sterol regulatory element binding protein-1; TAK-1, transforming growth factor-β-activated kinase-1; TAZ, coactivator with PDZ-binding motif; YAP, Yes-associated protein.

amount of soft drinks and 27% more meat compared to the general population [28, 29]. Higher intake of soft drinks and meat is associated with an increased risk of NAFLD, independent of age, sex, body mass index (BMI) and total energy intake [29]. The diet of pediatric patients with NAFLD is characterized by an overconsumption of fructose, soft drinks, red meat, saturated fat and cholesterol and by a low intake of fiber, fish, vitamin E and n-3 LCPUFAs (Fig. **2**) [30]. The latter feature in adult obese patients is accompanied by diminution in liver activity of fatty acid Δ-5 and Δ-6 desaturases (Fig. **2**) [31], an inadequate intake of the n-3 LCPUFAs precursor ALA and an elevated intake of *trans* FAs, such as elaidic acid (*trans* 18:1 n-9), that are potent desaturase inhibitors [4], inducing a drastic decrease in the PUFA desaturation capacity of the liver.

Increased consumption of added sugars is associated with NAFLD, obesity and IR, with fructose playing an emerging role as a potentially harmful component favouring pro-steatotic and pro-inflammatory responses [32]. Fructose is primarily metabolized in the liver, which is characterized by (i) the hepatic uptake via GLUT2 having a greater affinity for fructose (Km = 11 mM) than for glucose (Km = 17 mM); (ii) the initial phosphorylation of fructose to fructose-1P by fructokinase, which is more active than hexokinase or glucokinase for glucose phosphorylation; and (iii) fructose bypasses phosphofructokinase-1 that controls the glycolytic pathway, with rates of fructose processing being uncontrolled, concentration dependent and inducing ATP depletion [32,33]. Under these conditions, liver steatosis is increased in patients with biopsy-proven NAFLD and fructose consumption 2- to 3-fold higher

than controls, and showed upregulation of hepatic fructokinase and fatty acid synthase (FAS), with fructokinase protein levels and activity being enhanced in a mouse AML hepatocyte cell line [33]. The above findings support the role of fructose in the pathogenesis of NAFLD, which involves (i) steatosis with elevated *de novo* lipogenesis and n-3 LCPUFA depletion [34]; (ii) enhanced lipogenesis-related to SREBP-1c, carbohydrate regulatory element binding protein (ChREBP), FAS and fatty acid translocate FAT/CD-36 upregulation of mRNA expression, whereas that of PPAR-α and long chain acyl CoA dehydrogenase (LCAD) associated with FA oxidation was downregulated [35]; and (iii) ER stress activation partly contributing to hepatic SREBP-1c activation and lipid accumulation in fructose-evoked NAFLD [36]. Overconsumption of fructose also favoured the establishment of a pro-inflammatory state, as shown by upregulation of TNF-α mRNA expression in epididymal adipose tissue [35], as well as the hepatic inflammasome components NOD-like receptor family, pyrin domain containing 3 (NLRP3), apoptosis-associated speck-like protein (ASC) and caspase-1, with concomitant increase in IL-1β and IL-18 levels in rat liver [37].

4. LIPID INTAKE AND NAFLD

The diet quality plays a key role in the pathogenesis or prevention of NAFLD as shown by the beneficial effects of monounsaturated fatty acids (MUFAs) and LCPUFAs [17]. Diets consumed by patients with NAFLD tend to have higher content of saturated fat and cholesterol and lower content of n-3 LCPUFAs than healthy people's diets [15,38]. The ratio of total fat and hepatic fat showed that the amount of fat consumed correlates directly with changes in fatty liver and serum fasting insulin levels. Low-carbohydrate diets have shown to reduce alanine aminotransferase (ALT) levels, improve insulin sensitivity and reduce hepatic fat, independent of weight loss [39]. The fat content in the liver depends on the synthesis of TAGs and on the mechanism involved in the very-low-density lipoprotein (VLDL) secretion rate. Oleic acid (C18:1 n-9), palmitic acid (C16:0), stearic acid (C18:0) and linoleic acid (LA, C18:2 n-6) have proved to be strong stimulators of TAG synthesis and secretion by hepatocytes, while ALA, γ-linolenic acid (C18:3 n-6), arachidonic acid (AA, C20:4 n-6), DHA and eicosapentaenoic acid (EPA, C20:5, n-3) do not stimulate hepatic lipogenesis [40]. Thus, different types of fat may act differently in NAFLD [41]. A study conducted in rats revealed that a diet rich in saturated fatty acids (SFAs) stimulates mitochondrial OS and contributes to damage hepatocytes. Exposure of liver cells to stearic acid and palmitic acid induces their apoptosis by the activation of caspase 3 and the stimulation of DNA fragmentation. Food records of patients with NASH revealed that they consume more SFAs (14% of total energy intake) than control subjects (10% of total energy intake). Intake of SFAs greater than 10% of total energy stimulates IR, but less than 10% of total energy decreases low-density lipoprotein (LDL*)* cholesterol and TAG levels [39]. Furthermore, diets high in SFAs are associated with IR and NAFLD, thus the evaluation of both the ratio of saturated and unsaturated fat and total liver fat is an important aspect in the pathogenesis of chronic liver disease and metabolic diseases [42,43]. A deficiency of essential FAs is relatively rare, but an insufficient intake of n-3 PUFAs and conversion of ALA to n-3 LCPUFAs is common. Also, the consumption of LA is increased in the industrialized world, while the intake of ALA has decreased. LA and ALA compete as substrates for the enzymatic transformation of LCPUFAs, with higher intake of LA reducing the formation of n-3 LCPUFAs [4]. Furthermore, only a small fraction of ALA is converted to EPA and even less to DHA, thus insufficient intake and/or inadequate conversion of ALA contributes to a poor status of n-3 LCPUFAs [44]. It has been suggested that an adequate n-6/n-3 ratio should be about 3/1, however, considering that modern diet is rich in meals with high n-6 levels, the actual consumption is close to 15/1 [45].

In NAFLD patients, a study based on biopsies showed that the n-6/n-3 ratio is significantly correlated with the TAG content of the liver [28], with a significant correlation between the n-6/n-3 PUFA ratio and the amount of hepatic TAG being established, as a marker of the severity of steatosis [46]. Low intake of dietary sources of n-3 PUFAs is suggested to be associated with NAFLD. In agreement with these observations, rats and mice exhibiting depletion of n-3 PUFAs for two generations show several features of metabolic syndrome including hepatic steatosis [47,48], and a study in 35 pediatric patients with NAFLD reported a low intake of n-3 fatty acids and a significant negative correlation between intake of EPA plus DHA and ALT [49]. There is a paucity of research focused on the intake of n-3 FAs in pediatric patients with NAFLD, although St-Jules *et al.* found that the minority of pediatric patients consumed the recommended eight ounces of fish per week (22/223 (10%)) and 200 mg of n-3 LCPUFAs per day (12/223 (5%)), features that are associated with increased portal and lobular inflammation after controlling for potentially confounding variables [50]. In contrast, the results regarding cholesterol in-

take are controversial, with some reports confirming that there are no significant differences in cholesterol intake among patients with NAFLD and control patients [40], whereas an increased cholesterol intake is associated with induction of *de novo* synthesis of FA in hepatocytes, thus contributing to the development of NAFLD [51]. Dietary supplementation with krill oil (rich in EPA and DHA) or pine nut oil (containing the unusual n-6 PUFA, pinolenic acid) contributes to the prevention and/or the treatment of hepatic steatosis, via diminution in the levels of the hepatic lipogenic factors citrate carrier (CIC), acetyl-CoA carboxylase (ACC) and FAS [52]. In contrast, HFD or the dietary administration of conjugated linoleic acid (trans-10, cis-12-conjugated linoleic acid, CLA) induces hepatic steatosis. The average consumption of CLA in the United States is estimated at less than 0.5 g/day, however, the actual intakes by individuals consuming processed oils may be several folds greater than this, because the concentration of all CLA isomers in partially hydrogenated oil is 9.8% of the total fatty acids. Thus, the amounts of CLA consumed alone or with other partially hydrogenated *trans* FAs and processed foods can reach levels even beyond what is needed to induce IR and NAFLD [53]. Supplementation of animal diets with CLA alters tissue lipids and FA composition and increases lipid peroxidation, inflammation, incidence of NAFLD, IR and diabetes [53]. CLA increases the liver concentrations of oleic acid and the n-6/n-3 PUFA ratio, suggesting that the worsening of the inadequacy of n-3 PUFAs by CLA exacerbates the development of fatty liver and IR and that these conditions aggravated by CLA could be prevented by a concomitant increase in the intake of n-3 PUFAs, specifically ALA [53]. Lastly, it has been demonstrated that the intake of n-3 PUFAs can modify the composition of the intestinal microbiota, favoring the production of short-chain fatty acids, and a possible modulation of the pro-inflammatory state in intestine and a pro-lipogenic status in the liver [54], however, these aspects require more research.

5. MOLECULAR ASPECTS OF NAFLD

In recent years, IR and OS are considered as the major determinants of the onset of NAFLD and the progression of steatosis to steatohepatitis [55], although being closely related to other conditions characteristic of the metabolic syndrome such as central obesity, hypertension and hyperlipidemia [56,57]. IR promotes lipolysis in peripheral adipose tissue, increasing the flow of free FAs (FFAs) to the liver and the hepatic production of TAGs. Studies in humans have shown that peripheral lipolysis, systemic levels of FAs and hepatic *de novo* lipogenesis are increased in subjects with NAFLD [58], with hyperinsulinemia upholding the transcriptional regulation of genes that in turn stimulate hepatic *de novo* lipogenesis. Besides, high levels of glucose and insulin support the synthesis of FAs and inhibition of β-oxidation in the liver, thus enhancing the transformation of FFAs into TAGs [59]. Obese patients with NAFLD showed increased OS-related parameters compared to healthy subjects, including (i) reduced antioxidant potential; (ii) high free radical activity; (iii) greater formation of the Kupffer cell-dependent superoxide radical ($O_2^{\bullet}$-) and lipid peroxidation response; and (iv) reduction of the systemic antioxidant capacity of plasma [60], a redox unbalance known to trigger endoplasmic reticulum (ER) stress through protein oxidation and unfolding [61].

The pathogenesis of NAFLD has a complex molecular basis, with significant participation of gene transcription factors involved in lipid metabolism, inflammatory processes and antioxidant responses. As mentioned before, PPARs are transcription factors that regulate FA metabolism thus influencing metabolic pathways essential for energy metabolism such as adipocyte differentiation, inflammation and lipoprotein metabolism [15,62-64]. Specifically, PPAR-α, plays a role in lipid metabolism by favoring the expression of numerous genes involved in FA absorption, transport, oxidation and ketogenesis, besides reducing the expression of pro-inflammatory genes (Fig. **3**) [62]. This transcription factor is expressed in tissues with a high FA oxidation activity, such as liver, brown adipose tissue, kidney, small intestine, heart, skeletal muscle and nervous system cells, which is activated by n-3 LCPUFAs (EPA and DHA) [21]. The effects of PPAR-α activation are associated with both an increase in the catabolism of FAs and a diminution in the inflammatory response [65]. In this respect, activation of PPAR-α by DHA inhibits the expression of cytokine-induced molecules vascular cell adhesion molecule 1 (VCAM-1) as well as plasma levels of interleukin 6 (IL-6), which is associated with NF-κB downregulation [66]. The latter nuclear transcription factor is activated by OS and pro-inflammatory cytokines such as tumor necrosis factor-alpha (TNF-α), IL-1 and IL-6, and it is essential in many cellular processes particularly in those associated with inflammation, immunity, cell proliferation and apoptosis [67]. The activation of hepatic NF-κB is observed in NAFLD [68] where the drastic depletion in the levels of n-3 LCPUFAs in the liver [4] may produce a significant decrease in the activity of PPAR-α, a situation which will trigger pro-lipogenic [69] and pro-inflammatory conditions [70], a

molecular alteration being of major importance in the development and progression of NAFLD [16]. Another important PPAR in NAFLD is PPAR-γ, which regulates the expression of genes involved in adipocyte lipogenic pathways by promoting FA uptake and adipocyte differentiation, resulting in increased intracellular content of TAGs [15]. In NAFLD, there is an increase in the expression of PPAR-γ in the liver [16], which has a causal role in hepatic steatosis by mechanisms involving FA uptake, binding and transport that favours *de novo* lipogenesis [16,71]. In addition to PPAR-α and PPAR-γ, PPAR-β/δ activation may also be beneficial in NAFLD due to upregulation of forkhead box-containing protein O subfamily 1 (FOXO-1) a transcription factor that diminishes the expression of key hepatic gluconeogenic enzymes and glucose generation, however, PPAR-β/δ agonists are not available for use in the cinical setting [64]. Moreover, the activity of the transcription factor Nrf2, which is involved in the expression of antioxidant enzymes, is downregulated in NAFLD [72]. Nrf2-dependent expression of antioxidant enzymes includes heme oxygenase-1 (HO-1), glutamate cysteine ligase (GCL) [73], glutathione peroxidase (GPX), glutathione reductase (GR), glutathione S-transferase (GSTs) and catalase [74], besides that of detoxification enzymes and ABC transporters [73]. It has been reported that the deletion of its gene promotes the rapid development and progression of the disease, whereas the early activation of Nrf2 may avoid effects such as lipid accumulation and lipid peroxidation (Fig. **3**) [75]. Furthermore, Nrf2 activation produces a simultaneous suppression of NF-κB, thereby reducing the inflammatory response (Fig. **3**) [76,77].

6. METABOLIC AND NUTRITIONAL ASPECTS OF N-3 PUFAs

EPA and DHA exert a relevant participation in lipid metabolism, especially in the liver [68,78]. In addition, EPA has an important role in the control of vascular homeostasis [79], whereas DHA participates in brain and retinal development and growth and in the protection against neurological deterioration [80]. N-3 LCPUFAs are ligands that have a high affinity for PPARs [11], the binding to PPAR-α being a key molecular aspect in the regulation of the transcription of genes linked with different metabolic and cellular processes, such as FA β-oxidation and adipogenesis, representing a key mediator of lipid homeostasis, particularly in the liver [81-83]. On the other hand, n-3 LCPUFAs downregulate SREBP-1c activity controlling FA and TG synthesis, an effect that is mediated by AMP-activated protein kinase (AMPK) activation that triggers the inhibition of the post-translational processing of nascent SREBP-1c (Fig. **3**) [83]. Therefore, an inadequate hepatic availability of n-3 LCPUFAs determines a low PPAR-α expression and activity with concomitant elevation in those of SREBP-1c [82], promoting a pro-lipogenic state in the liver [50]. Consequently, preserving the hepatic levels of n-3 LCPUFAs via maintenance of the synthesis capacity [74,77] or by direct supplementation [11,82,83] is a central aspect to maintain an anti-lipogenic hepatic molecular state. Genetic variants in the metabolism (specifically polymorphisms) of n-6 and n-3 fatty acids have a possible role in the increment of the chronic disease risk [84], while n-3 PUFAs may be relevant in diseases such as obesity, type 2 diabetes, certain types of cancer and NAFLD due to their role in mitochondrial activity [85].

7. INTERVENTIONS WITH N-3 PUFAs IN CELLULAR AND ANIMAL MODELS AND IN HUMANS

7.1. Cellular Models

Studies about the effects of the treatment with n-3 PUFAs in NAFLD conducted in cellular models are scarce. Some studies have been based on the preventive effect of ALA on lipotoxicity caused by diet. In particular, certain SFAs such as palmitic acid induce ER stress and apoptosis in the liver in different models causing inflammation and/or degeneration of the liver [86]. Palmitate-induced ER stress is also a mechanism associated with the development of steatosis [87] through the induction of SREBP-1c, PPAR-γ and CCAAT/enhancer-binding proteins (C/EBPs), which are triggered by palmitate incorporation into TGs and phospholipids that disrupts ER morphology and function [88]. Researchers have hypothesized that the cytoprotection provided by ALA is a common feature in primary rat hepatocytes and may be effective against apoptosis mediated by ER stress induced by palmitic acid [89]. Furthermore, Wei *et al.* showed that the SFAs stearate- and palmitate-induced alteration in ER homeostasis is rescued by oleate and linoleate, unsaturated FAs that do not trigger ER stress [88]. In addition to SFAs, liver OS prevailing in NAFLD triggers protein carbonylation [14,73,77,82,90] with the consequent increase in the content of poorly folded or unfolded proteins in the lumen of the ER, which contributes to ER stress development that increases the susceptibility of the liver to injury [87,88,90] and loss of β-cell function that alters the regulation of glucose ho-

meostasis in diabetes (Fig. **3**) [91]. Similarly, primary rat hepatocytes subjected to palmitic acid produced a significant increase in cell death and in the expression of genes associated with the ER stress, namely, *CHOP*, *GRP78* and *GRP94*, an effect that is mimicked by the ER stress inducer tunicamycin and reversed by the concomitant treatment wih ALA [89]. The discussed findings suggest that ALA provides a successful strategy to abolish the lipotoxicity induced by palmitic acid and nutrient overload that accompany obesity and NAFLD. Besides ALA, recent studies using DHA (50-100 μM) and EPA (100-200 μM) added to 3T3-L1 adipocytes incubated for 24 h increased the cellular expression of adiponectin and its secreted levels [92]. These effects involve higher PPAR-γ expression with lower Ser273 phosphorylation over control values [92], a transcription factor known to be upregulated in the liver of obese patiens [6], thus supporting the contention that DHA may be more beneficial than EPA in diminishing risks of non-comunicable diseases such as NAFLD [92]. Moreover, HepG2 cells subjected to EPA (10 μg/ml) counteracted the activation of Δ-9 desaturase and the consequent enhacement in 5,8,11-eicosatrienoic acid (20:3 n-9) levels and TAG accumulation, which are induced by the core protein in chronic hepatitis C, effects that are mimicked by AA as possible tools for the development of new therapeutic devices [93].

7.2. Animal Models

The treatment with n-3 LCPUFAs has consistently have shown to reduce hepatic steatosis in the mouse model [13]. In a model of parenteral nutrition that induces steatosis and abnormal liver enzymes, the administration of n-3 LCPUFAs exhibited anti-steatotic effects and improved fatty liver in obese mice deficient in leptin [94]. These findings were confirmed in numerous other studies for both a mixture of EPA and DHA or given separately [13]. The administration of n-3 LCPUFAs has also shown to increase the expression of resolvins and protectins, which are n-3 LCPUFAs-derived mediators considered as insulin sensitizers, improving insulin sensitivity (Fig. **3**) [95]. There is also evidence that supplementation with these n-3 LCPUFAs (i) reduces reactive oxygen species (ROS) levels [96] and the content of leukotrienes and thromboxanes and modulate the inflammatory response due to the diminution of TNF-α (Fig. **3**); and (ii) increases adiponectin, a powerful insulin-sensitizing agent produced in adipose tissue (Fig. **3**) [92]. Thus, n-3 LCPUFAs supplementation restores PPAR-α and adiponectin levels decreased by TNF-α and improved hepatic steatosis and liver injury [97]. In addition, n-3 LCPUFAs given as fish oil supplementation significantly diminished or suppressed parameters associated with both hepatic lipogenesis and steatosis induced by fructose overconsumption in mice [24,25], although intake of complex lipids with high unsaturated/saturated FA ratios were more effective [24].

Moreover, the combined treatment with DHA (139 mg/day) and EPA (25 mg/day) was employed in mice subjected to a HFD for one month, fed with normal or high calorie diet, with or without n-3 LCPUFA supplementation for another month (post-treatment group) [98]. Under these conditions, mice fed the HFD, with or without supplementation, presented average liver disease (granular or vacuolar degeneration of hepatocytes) and hypertriglyceridemia, with n-3 LCPUFA supplementation decreasing hypertriglyceridemia and serum levels of glucose and cholesterol along with lower hepatic lipid peroxidation. The post-treatment group with hypercaloric diet exhibited medium hepatopathy, whereas those mice treated with normocaloric/normolipidic diet and n-3 LCPUFAs reverted histopatological features to normal and had better metabolic parameters [98]. In another study in C57BL/6 mice subjected to a diet rich in fish oil for 8 weeks was performed along with either a standard diet, dietary fish oil, HFD or a diet high in fish oil (40 g of soy oil and fish oil 238 g, 50% of the content of the diet as lipids) [99]. The results obtained indicate that the HFD group exhibited obesity, metabolic syndrome, and liver damage, along with hypertriglyceridemia, hepatic IR and steatosis, changes that were not observed after supplementation with the diet high in fish oil that remained similar to controls. Besides, animals fed a HFD showed increased hepatic lipogenesis and decreased β-oxidation, whereas opposite effects were observed in those given high in fish oil diet, preventing liver damage (Fig. **3**) [99]. From these studies it was concluded that n-3 LCPUFAs have a hepatoprotective effect in NAFLD when associated with a normocaloric/normolipidic diet [98], improving IR, lipogenesis and β-oxidation, with prevention of liver damage and NASH [99].

Concerning the molecular mechanisms involved in hepatoprotection, natural PPAR-α ligands such as n-3 LCPUFAs concomitantly activate FA oxidation and inhibit *de novo* lipogenesis, therefore, activation of PPAR-α is likely to improve NAFLD (Fig. **3**). Larter *et al.* [100] compared a diet with fish oil to a diet with olive oil in PPAR-activation, a model of steatosis induced by a methionine and choline deficient (MCD)

diet. As expected, supplementation with fish oil led to a significant activation of hepatic PPAR-α in with decreased expression of genes involved in the synthesis of FAs in mice fed the MCD diet, with reduction in hepatic lipid accumulation compared to animals with the same diet but supplemented with olive oil. However, liver injury was further exacerbated in animals fed MCD with fish oil that developed NASH, in comparison to animals with the same diet but supplemented with olive oil. Thus, diets enriched in n-3 LCPUFAs activates liver PPAR-α and suppresses *de novo* lipogenesis, but does not prevent NASH development induced by MCD. Under these conditions, high levels of hepatic lipoperoxides observed may suppress the protection conferred by PPAR-α activation and possibly will also lead to liver injury, inflammation and lipotoxic hepatocellular recruitment [100]. Subsequently, changing this diet to a normal calorie diet supplemented with n-3 LCPUFAs for 8 weeks reversed higher adiposity, adipocyte hypertrophy, hepatic OS, steatosis and SREBP1-c/PPAR-α ratios induced by the HFD [101]. Diminution in the hepatic SREBP-1c/PPAR-α ratio by n-3 LCPUFA suggest a major change in the metabolic state of liver, stimulating FA oxidation and improving liver steatosis (Fig. **3**) [101].

Similarly, rats and mice that exhibit a depletion of n-3 LCPUFAs for two generations show several features of metabolic syndrome, including hepatic steatosis [102,103]. However, the biochemical mechanisms underlying liver disorders that occur under depletion of n-3 LCPUFAs remain unknown [31]. In this respect, Pachikian *et al.* [103] investigated the effect in the hepatic lipid composition and metabolism of an n-3 PUFAs depleted diet (DEF) in mice *in vitro* and *in vivo*. For this purpose, a drastic drop in n-3 PUFAs in liver phospholipids was induced by feeding C57BL/6J mice with DEF for 3 months as compared with a control diet differing only in the n-3 FAs content. Under these conditions, DEF elicited (i) depletion of n-3 FAs in phospholipid fractions and an increase in the liver endocannabinoids content; (ii) decreased hepatic FA oxidation with lipid synthesis and storage promotion; (iii) enhancement in FA and cholesterol synthesis at the expense of FA oxidation in the liver; (iv) upregulation of SREBP-1c expression; (v) increased hepatic glucose production under insulin stimulation and IR; and (vi) an increase in the activity of lipogenic liver X receptor (LXR) [103]. These data suggest that LXR activation is related to the overexpression of SREBP-1c, with depletion of n-3 PUFAs in liver phospholipids promoting SREBP-1c activation, lipogenesis and hepatic steatosis [103], a condition that favours SREBP-1c processing [88].

In order to determine whether ALA could prevent the development of fatty liver and IR induced by CLA, mice were subjected to a control diet, control diet plus CLA, and control diet plus CLA plus chia oil for 8 weeks [43]. It was found that a relatively small amount of ALA (0.3 g/100g diet) versus CLA (0.5 g/100g diet) completely prevented IR and attenuated fatty liver induced by CLA. Also, ALA increased n-3 and n-6 PUFAs with marked diminution in the ratio n-6/n-3 PUFAs in liver lipids, effects that were independent of changes in leptin and adiponectin levels. These studies suggest that some adverse effects of CLA may be due to deficiency of n-3 PUFAs, which can be corrected by increasing in ALA intake [43].

A different approach for the study of NAFLD is the fat-1 transgenic mouse expressing the Caenorhabditis elegans fat-1 gen, which encodes an n-3 FA desaturase enzyme that converts n-6 to n-3 FAs and that is absent in most animals, including mammals [104]. Consequently, the fat-1 mouse exhibits an abundance of n-3 PUFAs that reduces the levels of n-6 PUFAs in body tissues under a normal fat diet [104]. The study of the effect of endogenously generated n-3 PUFAs in fatty liver disease induced by diet, using wild type (WT) and the fat-1 mice maintained on a HFD for 5 months, revealed that weight gain and diet-induced fatty liver was most prominent in WT mice than in fat-1 animals [105]. Histological and metabolic analysis indicated that: (i) WT mice fed with a HFD developed moderate to severe macrovesicular steatosis, which was minimal in the liver of fat-1; (ii) ballooned hepatocytes and fibrosis induced by HFD was alleviated in fat-1 mice; (iii) serum AST and ALT levels were within normal ranges in HFD fed fat-1 mice, whereas both enzymes were significantly elevated in WT mice fed with a HFD; and (iv) fat-1 mice showed a significant decrease in serum lipids, including TAGs, total cholesterol (TC), high-density lipoprotein (HDL) cholesterol and LDL cholesterol compared to WT mice, with the increase in VLDL cholesterol and chylomicrons detected in WT mice fed with a HFD being completely annulled in fat-1 mice fed with a HFD [105]. These findings are accompanied by n-3 LCPUFA-induced upregulation of genes involved in cholesterol uptake (*Ldlr*), bile acid synthesis (*Cyp7a1*) or excretion (*Abcg5, Abcg8*) and metabolism (transcription factors *Pgc1α, Lxrα, Hnf4α*) ameliorating fatty liver and hypercholesterolemia, thus favouring lipid and energy homeostasis [105].

Interestingly, Depner *et al.* [106] reported that DHA was more efficient than EPA in reversing NAFLD using the mouse model of LDL receptor$^{-/-}$ (LDLR$^{-/-}$) induced by western diet. Under these conditions, the dietary intake of EPA and DHA attenuates NASH progression by diminishing multiple processes, including the content of sphingomyelin and phosphoglycerate lipid in membranes, nuclear content of key transcription factors (NF-κB, SREBP-1c), the expression of genes involved in lipid metabolism, inflammation, OS and fibrosis, with improvement in glucose metabolism and detoxification of methylglyoxal [107]. Furthermore, dietary EPA and DHA increase the formation of different oxidized lipids that are hepatoprotective, namely, epoxy- acid and/or di-hydroxy-FA derivatives of EPA and DHA. (Fig. **3**) [106]. In addition, recent findings show that epoxides derived from n-3 PUFAs may be regulatory molecules of inflammation and could help prevent hepatic steatosis [107,108]. In order to evaluate the effects of ALA in liver steatosis modulation, rats were fed with HFD (55% energy) containing either canola oil with high oleic acid content, canola oil, a blend of canola oil/linseed (C/L 3:1), sunflower oil, soybean oil or lard [109]. After 12 weeks of treatment, the C/L group exhibited 20% less hepatic lipid content and higher levels of total n-3 and EPA in liver phospholipids, higher DHA and lower concentrations of AA. These findings ratify that C/L diet with the highest content of ALA attenuated hepatic steatosis and FAs profile disorders of liver phospholipids [109], supporting the development of non-traditional vegetable oils rich in ALA (up 45%) as a new strategy to increase consumption of ALA.

Studies by our group addressed the evaluation of ALA, EPA and DHA levels in phospholipids extracted from erythrocytes, liver, kidney, short bowel, heart, quadriceps and brain from rats fed an isocaloric diet for 21 days (20% protein, 10% fat and 70% carbohydrate), where the total fat in each group was exclusively provided by sunflower oil (SFO-1% ALA), canola oil (CO-10% ALA), Rosa Canina oil (RCO-33% ALA), Sacha Inchi oil (SIO-49% ALA) or chia oil (CHO-64% ALA) [110]. In this setting, animals fed SIO and CHO showed the greatest increase in ALA content with a diminution of AA in all tissues, except brain. EPA content increased in erythrocytes, liver, kidney, small intestine, heart and quadriceps but not in the brain, whereas levels of DHA were enhanced in the liver, small intestine and brain. These findings demonstrate that ALA, when provided in sufficient quantities in mice, can be effectively converted into n-3 LCPUFAs, mostly to DHA in liver and brain, suggesting that oils rich in ALA, such as SIO and CHO are good sources of ALA and improve n-3 LCPUFAs levels in several tissues [110]. Alternatively, the role of Rosa mosqueta oil (RMO) in preventing HFD–induced oxidative stress and inflammation was studied in male C57BL/6J mice divided into 4 groups and fed for 12 weeks, namely, control diet (10% fat, 20% protein and 70% carbohydrates), control diet + RMO (1.94 mg ALA/g body weight), HFD (60% fat, 20% protein and 20% carbohydrates) and HFD + RMO [111]. The results showed that RMO supplementation prevents the obese phenotype observed in HFD-fed mice, with downregulation of inflammatory cytokine expression and secretion and stimulation of hepatic antioxidant and FA oxidation markers [111]. These findings confirm the effectiveness of RMO in preventing HFD-induced insulin resistance and metabolic alterations in a fashion similar to dietary EPA and DHA, validating the use of this oil as a nutritional source of both ALA for the synthesis of n-3 LCPUFAs and phytochemicals with a high antioxidant potential [112,113].

The use of diets differing in the content of ALA and n-3 LCPUFAs both in WT and Δ-6 desaturase null (D6KO) mice revealed that (i) D6KO mice consuming CD diet (canola il + ARASCO, with 8% ALA) or FD (flax seed oil + ARASCO, with 55% ALA) exhibiting n-3 LCPUFA depletion and ALA enrichment, had lower liver steatosis and inflammation over controls given LD (lard with 0% n-3 LCPUFA); and (ii) animals subjected to MD (menhaden oil, with 30% n-3 LCPUFA) presented the lowest levels of steatosis and inflammation, regardless of the period of supplementation and genotype [114]. These findings indicate that, although MD rich in n-3 LCPUFAs is the most protective, protection by ALA is afforded by mechanisms acting independently on its conversion to EPA and DHA [114].

NAFLD may progress into NASH, fibrosis, cirrhosis and hepatocellular carcinoma [2], and recent studies addressing the effects of n-3 PUFAs in disease progression into fibrosis are available in animal models. Using a high-fat/high fructose diet (HFHSD) for 14 days in mice followed by D-galactosamine administration (DGalN) or vehicle, exacerbation of the serum levels of AST, ALT and soluble TNF-α receptor, and hepatic ROS production and TAG accumulation, with enhanced expression of the fibrogenic genes for tissue inhibitor of matrix metalloproteinase 1 (TIMP-1), connective tissue growth factor (CTGF) and osteopontin (OPN) was obsered, parameters that were significantly diminished by highly purified EPA [115]. In a model of

NASH in melanocortin 4 receptor-deficient (MC4R-KO), EPA administration prevented liver necrosis, fibrosis and hepatocellular carcinoma [116], with hepatic crown-like structures accelerating inflammation and fibrosis being prevented by EPA [117]. Also, n-3 LCPUFAs were shown to attenuate carbon tetrachloride (CCl_4)–induced liver fibrosis in mice, which is mediated by downregulation of the expression of profibrogenic genes in activated hepatic stellate cells by promoting the degradation of transcriptional factors Yes-associated protein (YAP) and coactivator with PDZ-binding motif (TAZ) (Fig. **3**) [117]. Recently, anti-fibrogenic effects were reported for EPA + DHA in rats with thioacetamide-induced liver fibrosis [118] or for DHA in primary keloid fibroblast cultures from patients with dermal fibroproliferative disorders [119]. In adition, dietary supplementation with n-3 LCPUFAs for 4 weeks prevented liver steatosis and reproductive disturbances induced by HFD in male rats [120].

DHA has a potent anti-inflammatory activity through the G-protein coupled receptor 120 (GPR120) (Fig. **3**) and dietary DHA is able to suppress hepatic markers of OS, inflammation and fibrosis as assessed in monocytic 246.7 cells and primary intraperitoneal macrophages [121]. In this context, studies in obese WT and GPR120 knockout mice fed a HFD with or without n-3 LCPUFA supplementation revealed that n-3 LCPUFAs inhibited inflammation and enhanced systemic insulin sensitivity in WT mice, but were without effect in GPR120 knockout mice [121]. Besides, they reported (i) improvement of histopathological parameters such liver steatosis; (ii) reduction in hepatic progenitor cell activation that correlated with histopathological parameters; (iii) diminution in the number of inflammatory macrophages; (iv) increase in the expression of hepatocyte GPR12O; and (v) attenuation in the nuclear translocation of NF-κB in hepatocytes and macrophages, findings point to the interaction between DHA, GPR120 and NF-κB as a crucial event affording anti-inflammatory response (Fig. **3**) [121].

7.3. Studies in Humans

7.3.1. Interventions in Adult Patients with NAFLD

Hepatic steatosis may be predictive of death by various cardiovascular diseases (CVD) in patients with hepatocellular carcinoma, as well as of the onset of type 2 diabetes, thus strongly indicating the need to treat each state of NAFLD. Currently, there is no an effective pharmacological treatment for NASH and its natural history [122]. Consequently, therapies for patients with NAFLD have typically been focused on the management of associated conditions such as obesity, diabetes mellitus and hyperlipidemia, therefore, specific medications for NAFLD are needed in future research [122].

Several studies have shown that in patients with NAFLD, the treatment with n-3 PUFAs added to the diet decreases the hepatic fat content, the serum levels of ALT and TNF-α, and improves IR. Accordingly, prevention or attenuation of fatty liver development by n-3 PUFAs may constitute a specific drug for liver NASH in the future, through limiting NAFLD progression, representing an attractive alternative to other therapies due to its nutritional and therapeutic benefits (Fig. **3**) [109,122-124]. Studies on the effects of prolonged supplementation with n-3 LCPUFAs in pateints with NAFLD were initiated in 2006 through clinical assessments, blood tests and ultrasound of the liver [125]. In a group of 56 patients, 42 consumed daily one capsule of 1 g of n-3 PUFAs for 12 months and 14 subjects remained untreated (control group). As compared with the control gorup, treated patients showed a significantly decrease in serum AST, ALT, gamma glutamyl transpeptidase (γ-GGT), TAG and fasting glucose levels. The circulating levels of AA and the n-3/n-6 ratio were diminished, with improvement in liver echotexture after treatment with PUFAs and increased Doppler perfusion index [126]. Later, Zhu *et al.* [124] investigated the efficacy and safety of n-3 PUFAs from seal oils in 134 patients with NAFLD associated with hyperlipidemia in a controlled trial for 24 weeks randomly assigned to two groups, one group receiving dietary recommendations and 2 g of n-3 PUFAs three times a day and another group receiving recommendations and 2 g of placebo three times daily (controls), with assessments at 0, 8, 12, 16 and 24 weeks. Total symptom scores, ALT and TG levels decreased more significantly in the supplemented group than in control group ($P< 0.05$), but no significant differences were found between the two groups with respect to those of AST, γ-GGT, TC and HDL cholesterol [124]. Interestingly, a complete regression of fatty liver was observed in 20 % of all patients after treatment, with an overall reduction in 53% of patients given n-3 PUFAs, whereas 7% of the placebo group achieved complete regression of steatosis and 35% showed some improvement of fatty liver. Considering that no adverse events occurred in the patients who completed the treatment, it was suggested that n-3 PUFAs from seal oils are safe and effective for patients with NAFLD associated with hyperlipidemia, enhancing scores of total symptoms, ALT, serum lipid levels and normalization of ultrasound evidence [124]. Similarly, Spadaro

et al. [125] studied 40 patients with clinical diagnosis of NAFLD that were randomly assigned to two groups receiving the same dietary recommendations, but the treated group also received 2 g of n- 3 PUFAs daily for 6 months. In this setup, supplemented patients showed improvement in serum chemistry with reduced ALT and TAG levels and improved insulin sensitivity and decreased TNF-α, and the assessment of fatty liver by ultrasound improved in 83%, with reversion in 33% of patients to a normal appearance [125]. In a different study conducted in 11 patients with NAFLD, 6 received oral administrations of 6.5 ml of olive oil + n-3 PUFAs for 12 months and 5 were given placebo (controls) [126]. Following olive oil administration, a significant improvement in liver echotexture and perfusion index was observed, whereas no significant changes were found at the end of follow-up period in the control group. Besides, patients who consumed olive oil enriched with n-3 LCPUFAs showed significant improvement in serum liver enzymes and TAGs in a general linear model adjusted for age and gender, with a significant increase in the adiponectin levels [126]. These data corroborate the beneficial effects of n-3 LCPUFAs through long-term consumption of n-3 LCPUFA-enriched EVOO in patients with NAFLD, which involve adiponectin upregulation [126], an anti-steatotic and insulin sensitizer adipokine (Fig. **3**) [94]. Simultaneously, Oya *et al*. [127] evaluated the impact of ALA, EPA and DHA through a cross-sectional study in a Japanese population of NAFLD patients (296 men and 496 women) participants of a screening program of general health, who were apparently healthy and who did not drink alcohol. NAFLD diagnosed by abdominal ultrasonography revealed the presence of fatty liver, and upon completion of the study it was found that dietary EPA and EPA + DHA may be independent and preventive nutrients for NAFLD in Japanese men who generally consume more fish than Westerners, but not in women [127]. A study of 23 patients with NASH confirmed by liver biopsy, who received 2.7 g of EPA daily for 12 months, comprising 7 patients who agreed to repeat the biopsy at the end of the treatment period, completed the study, which did not include a control group. At the end of the study period, 6 of the 7 patients who agreed to repeat the biopsy showed a reduction in liver steatosis, inflammation and fibrosis, serum AST and ALT were significantly improved, cholesterol and FFA levels were significantly decreased, similarly to liver thioredoxin that is related to OS, whereas serum TAGs and LDL levels, fasting glucose, adiponectin levels and IR were unchanged [128]. While these trials provide evidence of the benefits of using n-3 FAs to treat NAFLD, the absence of randomization and of control groups, possible bias, small sample sizes and lack of power, preclude reliable conclusions [13]. A meta-analysis conducted in 2012 revealed that n-3 LCPUFAs may reduce liver fat depots with a consumption of >0.83 g/day [129]. These results are informative for therapeutic procedures for NAFLD, however, researchers have some concerns about the optimal dose, since given the complications of NAFLD, especially CVD, the optimal dose of n-3 LCPUFAs could be much greater than 0.83 g/day [130]. In an intervention trial of long-term secondary prevention after myocardial infarction, a substantial reduction in all-cause mortality and morbidity from CVD is elicited with the supplementation of 1g/day of n-3 PUFAs [131]. Furthermore, an analysis of numerous clinical trials concluded that n-3 LCPUFAs act as beneficial pleiotropic agents to prevent CVD [132].

Petit *et al.* [133] studied the FA profile of erythrocyte membranes in relation to the presence of steatosis in patients with type 2 diabetes based on the hypothesis that increased PUFAs intake could provide protection from hepatic steatosis in a specific population with high risk of NAFLD as patients with type 2 diabetes are. For this purpose, 162 patients with type 2 diabetes were enrolled in the study, the liver fat content was measured using spectroscopy and FA composition of blood cells was determined by gas chromatography. A significant association was found between the concentration of palmitic acid, palmitoleic acid (16:1 n-7) and the palmitoleic acid/palmitic acid ratio with high liver fat content, whereas total PUFAs, dihomo-γ-linolenic Acid (20:3, n-6), DHA and AA were correlated with low-liver fat. These associations suggest that an increase in the content of n-3 LCPUFAs and 20:3 n-6 in erythrocytes are related to low prevalence of steatosis in patients with type 2 diabetes, which would be beneficial in these patients [133]. In addition, Scorletti *et al.* [134] performed a randomized, placebo-controlled trial where 103 participants with histological confirmation of NAFLD were randomized to either 4 g per day of Omacor (1840 mg of EPA and 1520 mg of DHA as ethyl esters) or 4 g per day of placebo (olive oil) for 15-18 months. EPA and DHA enrichment was assessed in the erythrocyte (between baseline and at the end of the study) to test the adherence to the intervention in the DHA + EPA group and monitor contamination with DHA and EPA in the placebo group, concluding that erythrocyte DHA enrichment with DHA+EPA treatment is linearly associated with a decreased liver fat percentage [134]. Finally, Parker *et al*. [130] conducted a systematic literature search for studies pertaining to

the effect of n-3 PUFA supplementation in humans with NAFLD, with primary outcomes being liver fat and the liver function tests ALT and AST. Data were pooled and taken for a meta-analysis using a random effects model in nine studies involving 355 individuals who were administered either n-3 LCPUFAs or control treatment. Beneficial changes in liver fat depots were favored by treatment with n-3 LCPUFAs (effect size: 0.97, 95% CI -0.50 to -1.35, $p < 0.001$), an effect also observed for AST (effect size: -0.97, 95% CI -0.13 to -1.82, $p = 0.02$) but not for ALT (effect size: -0.56, 95% CI -1.16 to 0.03, $p = 0.06$). The sub-analysis of only randomized controlled trials showed a significant benefit for n-3 LCPUFAs versus control in the hepatic fat content (effect size: -0.96, 95% CI -0.43 to -1.48, $p < 0.001$), but not for ALT ($p = 0.74$) or AST ($p = 0.28$), although there was significant heterogeneity between studies. Pooled data suggest that supplementation with n-3 LCPUFAs may decrease the liver fat content; however, the optimal dose is not currently known. Good designs of randomized controlled trials to quantify the magnitude of the effect of n-3 PUFAs supplementation on liver fat are necessary. Data from this search showed that despite the heterogeneity in study design, supplementation of marine n-3 PUFAs in humans is associated with a positive effect on liver fat. Importantly, this effect persists when only randomized controlled trials were examined. In addition to the influence of n-3 LCPUFAs on hepatic steatosis improvement in NAFLD patients, fewer studies are available in relation to its role in hepatic fibrosis in NASH patients. These include the study using n-3 LCPUFAs for 6 months in 78 NASH patients indicating that n-3 LCPUFAs offset several markers including hepatic fibrosis, as shown by histological assessment and the decline in serum levels type IV collagen and procollagen type III pro-peptide [135]. Besides, 7 out of 23 NASH patients given 2.7 g EPA/day for 12 months with post-treatment biopsy showed significant improvement in hepatic steatosis and fibrosis scores [129].

7.3.2. Interventions in Pediatric Patients with NAFLD

A randomized controlled trial of supplementation with DHA (250 mg/day and 500 mg/day) versus placebo in 60 children between 6 and 16 years old with NAFLD for periods of 6, 12, 18 and 24 months showed that decreases in liver fat content obtained after 6 months of treatment, determined by ultrasonography, remained virtually unchanged after 24 months of treatment with DHA, an effect that is independent of the DHA dosage [136]. Moreover, TAG levels were lower in both DHA groups than in the placebo group at any time point of the intervention, ALT was lower in both treated groups at 12 months of treatment, and HOMA index was lower in the group treated with 250 mg/day versus placebo at 6 and 12 months, thus supporting the anti-steatoic effect of DHA in pediatric NAFLD [136]. Other studies have focused on the feasibility of diets enriched with n-3 LCPUFAs as a strategy to increase food intake in this population. The number of foods enriched with n-3 LCPUFAs is increasing particularly in Western countries such as Australia, where milk, cereals and breads are fortified with n-3 LCPUFAs are potential contributors to the intake of n-3 LCPUFAs [137], considering that <7% of Australian children indicate a consumption of foods enriched with n-3 LCPUFAs [138]. Evaluation of the effect of replacing bread, eggs, milk and yogurt by the same foods enriched with n-3 LCPUFAs in the overall intake of n-3 LCPUFAs in diets of Australian children was carried out in food models using data from a nationally representative sample of 4487 children (2240 boys and 2238 girls) aged 2-16 years [137]. This study used the Method of Multiple Points to estimate usual intake of n- 3 LCPUFAs from two 24-hour recalls, corrected for variations between individuals, and revealed that substituting 4 basic foods by alternative foods enriched with n-3 LCPUFAs resulted in an improvement in n-3 LCPUFAs intake, but optimal intake levels were not reached [137], hence, the increase in fish consumption remains the most effective strategy for increasing intake of n-3 LCPUFAs. Finally, a randomized double-blind, placebo-controlled clinical trial in 6 to 9-year-old children diagnosed with NAFLD (n=76) including a group supplemented with n-3 LCPUFAs (fish oil containing EPA+DHA in a range of 450 to 1300 mg/day) and the other group subjected to placebo (sunflower oil) during 24 weeks was performed [139]. All patients received dietary and physical activity recommendations and the intervened group showed a decrease in serum AST and γ-glutamyltranspeptidase (γ-GTP) levels with an improvement on their adiponectin levels [141]. In addition to NAFLD, steatohepatitis is currently a major health problem. Three controlled clinical trials have shown that dietary supplementation with n-3 LCPUFAs has no hepatoprotective effects in patients with NASH [140-142], indicating that the protective effects of n-3 PUFAs would be relevant to hepatic steatosis rather than inflammation, once it is already developed.

CONCLUSIONS AND PROJECTIONS

Currently, NAFLD is a major health problem affecting the liver and is an emerging pathology in several

countries due to the unhealthy lifestyles of the population (Fig. **2**). Several studies in cellular, animal and human prototypes have shown the beneficial effects of n-3 PUFAs, specifically EPA and DHA, against the different components of NAFLD. The relevant advances at the molecular level that explain the mechanisms involved in the favorable effects of the n-3 LCPUFAs discussed in this review (Fig. **3**) identify several molecular mechanisms involved in the onset of NAFLD and its progression, which support n-3 LCPUFA use in NAFLD, although having a limited value in the outcome of NASH [143-146). Besides EPA and DHA, resolution of ongoing inflammation may be elicited by n-3 LCPUFA-derivatives such as resolvins, protectins and maresins [147], in addition to n-3 PUFA-epoxides produced by cytochrome P450 epoxygenase action [106,117,148,149]. While the mechanisms involved in the possible clinical application of these fatty acids in the treatment of NAFLD are robust, nowadays is necessary to carry out additonal clinical trials to establish the doses of n-3 LCPUFA that will be used, the intervention times, patients that can be supplemented or not, as well as possible synergies with other substances, such as natural antioxidants [150], considering that oxidative stress has appeared as a crucial pathological event during NAFLD development and progression [151,152]. Finally, at present, there is a great variety of nutritional supplements that provide n-3 LCPUFAs, for which it is necessary to establish minimum quality and chemical stability requirements of these products.

AUTHOR CONTRIBUTIONS

Rodrigo Valenzuela, Macarena Ortiz, María Catalina Hernández-Rodas, Francisca Echeverría and Luis A. Videla performed the analysis of the information and wrote the manuscript. All authors reviewed and approved the final version of the manuscript.

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CONFLICT OF INTERESTS

The authors declare that they have no conflict of interest.

LIST OF ABBREVIATIONS

AA = Arachidonic acid

ACC = Acetyl-CoA carboxylase

AHA = American Heart Association

ALA = α-linolenic acid

ALT = Alanine aminotransferase

AMPK = AMP-activated protein kinase

AP-1 = Activator protein 1

ARA = Arachidonic acid

ASC = Apoptosis-associated speck-like protein

AST = Aspartate aminotransferase

BMI = Body mass index

CDK5 = Activator of cyclin dependent kinase 5

CHO = Chia oil

ChREBP = Carbohydrate regulatory element binding protein

CIC = Citrate carrier

CLA = Conjugated linoleic acid

CO = Canola oil

CTGF = Connective tissue growth factor

CVD = Cardiovascular diseases

C/EBPs = CCAAT/enhancer-binding proteins

DEF = N-3 PUFAs depleted diet

DGalN = D-galactosamine

DHA = Docosahexaenoic acid

D6KO = Δ-6 desaturase null

EPA = Eicosapentaenoic acid

ER = Endoplasmic reticulum

EVOO = Extra virgin olive oil

FAs = Fatty acids

FAS = Fatty acids synthase

FADS1 = Fatty acid desaturase 1

FADS2 = Fatty acid desaturase 2

FFAs = Free fatty acids

FOXO-1 = Forkhead box-containing protein O subfamily 1

GCL = Glutamate cysteine ligase

GPR120 = G-protein coupled receptor 120

GPX = Glutathione peroxidase

GR = Glutathione reductase

GSTs = Glutathione S-transferase

HCV = Hepatitis C virus

HDL = High-density lipoprotein

HFD = High-fat diet

HFHSD = High-fat/high fructose diet

HO-1 = Heme oxygenase-1.

IL-6 = Interleukin 6

IL-8 = Interleukin 8

IL-1β = Interleukin 1β

IR = Insulin resistance

LA = Linoleic acid

LCAD = Acyl CoA dehydrogenase

LCPUFAs = Long-chain polyunsaturated fatty acids.

LDL = Low-density lipoprotein

LXR = Lipogenic liver X receptor

MCD = Methionine and choline deficient

MC4R-KO = Melanocortin 4 receptor-deficient

MD = Menhaden oil

MUFAs = Monounsaturated fatty acids

NAFLD = Non-alcoholic fatty liver disease

NASH = Nonalcoholic steatohepatitis

NF-Kb = Nuclear factor-κB

NLRP3 = Pyrin domain containing 3

Nrf2 = Nuclear factor erythroid-2-related factor-2.

OPN = Osteopontin

OS = Oxidative stress

$O_2^{\bullet}$- = Superoxide radical

PPAR-α = Peroxisome proliferator-activated receptor-α

PPAR-γ = Peroxisome proliferator-activated receptor-γ

PPAR-β/δ = Peroxisome proliferator-activated receptor-β/δ

PUFAs = Polyunsaturated fatty acids

RCO = Rosa canina oil

RMO = Rosa mosqueta oil

ROS = Reactive oxygen species

SFAs = Saturated fatty acids

SFO = Sunflower oil

SIO = Inchi oil

SREBP-1c = Sterol regulatory element binding protein-1c

TAGs = Triacylglycerides

TAZ = PDZ-binding motif

TC = Total cholesterol

TIMP-1 = Matrix metalloproteinase 1

TNF-α = Tumor necrosis factor-α

UPR = Unfolded protein response

VCAM-1 = Vascular cell adhesion molecule 1

VLDL = Very-low-density lipoprotein

WT = Wild type

γ-GGT = Gamma glutamyl transpeptidase

γ-GTP = γ-glutamyltranspeptidase

CONSENT FOR PUBLICATION

Not applicable.

CONFLICT OF INTEREST

The authors declare no conflict of interest, financial or otherwise.

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