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TOPIC HIGHLIGHT

Giovanni Tarantino, Professor, *Series Editor*

Omega-3 fatty acids for the treatment of non-alcoholic fatty liver disease

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Abstract

Non-alcoholic fatty liver disease (NAFLD) has been recognized as a major health burden. It is the most important cause of chronic liver disease and a major independent cardiovascular risk factor. Lacking a definite treatment for NAFLD, a specific diet and an increase in physical activity represent the most commonly used therapeutic approaches. In this review, major literature data about the use of omega-3 polyunsaturated fatty acids (n-3 PUFAs) as a potential treatment of NAFLD have been described. n-3 PUFAs, besides having a beneficial impact on most of the cardio-metabolic risk factors (hypertension, hyperlipidemia, endothelial dysfunction and atherosclerosis) by regulating gene transcription factors [i.e., peroxisome proliferator-activated receptor (PPAR) α, PPARγ, sterol regulatory element-binding protein-1, carbohydrate responsive element-binding protein], impacts both lipid metabolism and on insulin sensitivity. In addition to an enhancement of hepatic beta oxidation and a decrease of the endogenous lipid production, n-3 PUFAs are able to determine a significant reduction of the expression of pro-inflammatory molecules (tumor necrosis factor-α and interleukin-6) and of oxygen reactive species. Further strengthening the results of the *in vitro* studies, both animal models and human intervention trials, showed a beneficial effect of n-3 PUFAs on the severity of NAFLD as expressed by laboratory parameters and imaging measurements. Despite available results provided encouraging data about the efficacy of n-3 PUFAs as a treatment of NAFLD in humans, well-designed randomized controlled trials of adequate size and duration, with histological endpoints, are needed to assess the long-term safety and efficacy of PUFA, as well as other therapies, for the treatment of NAFLD and non-alcoholic steatohepatitis patients. It is worthwhile to consider that n-3 PUFAs cannot be synthesized by the human body and must be derived from exogenous sources (fish oil, flaxseeds, olive oil) which are typical foods of the Mediterranean diet, known for its beneficial effects in preventing obesity, diabetes and, in turn, cardiovascular events. According to these data, it is important to consider that most of the beneficial effects of n-3 PUFAs can also be obtained by an equilibrate nutrition program.

Key words: Hepatic steatosis; Non-alcoholic fatty liver disease; Omega-3 polyunsaturated fatty acids; Animal models

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INTRODUCTION

Non-alcoholic fatty liver disease (NAFLD) is defined as pathological fat deposition in the liver cells of patients with minimal or no alcohol intake and without any other known cause. It encompasses a wide spectrum of liver damage stages ranging from isolated hepatic steatosis or simple fatty liver (FL), to non-alcoholic steatohepatitis (NASH) or even cryptogenic cirrhosis and hepatocellular carcinoma. In more detail, about 10%-29% of individuals with NASH develop cirrhosis within 10 years[1], and 4%-27% of NASH-induced cirrhosis can ultimately progress to hepatocellular carcinoma[2]. NAFLD affects 10%-35% of the adult population[3] and, because of the increasing incidence of obesity and of type 2 diabetes mellitus, it has been recognized as a major health burden and as the most important cause of chronic liver disease[4]. Overall, NAFLD is considered as the hepatic expression of metabolic syndrome[5,6] and it is associated with an increased risk of cardiovascular disease[7], along with venous[8] and arterial thrombotic events[9]. On the other hand, the impact of NAFLD on overall cardiovascular mortality is still widely challenged[7]. Nowadays, there is no definite treatment for NAFLD and NASH, as their physiopathology and natural history are not completely understood. Indeed, treatment is based on general approaches such as diet and physical activity[10]. The aim of this review is to describe major literature data about clinical and pre-clinical studies evaluating the effects of omega-3 polyunsaturated fatty acid (n-3 PUFAs) supplementation on NAFLD.

MOLECULAR MECHANISMS

The pathophysiology of NAFLD is multifactorial and not completely understood. According to the "two-hit" hypothesis[11], insulin resistance and visceral obesity promote the synthesis of fatty acids from glucose and inhibit β-oxidation of fatty acids. The excess of fatty acids leads to triglyceride synthesis and to their intrahepatic accumulation. Overall, these changes lead to FL (first hit), which is a relatively benign clinical condition[12].

The increased levels of fatty acids and triglycerides are associated with the production of free radicals[13,14], which, by causing lipid peroxidation and activating pro-inflammatory and fibrogenic cytokines[15], lead to NASH establishment (second hit)[16].

In particular, oxidative stress could be considered the result of an imbalance between pro-oxidant and anti-oxidant processes. In fact, the excess of intra-hepatic triglyceride induces high rates of mitochondrial β-oxidation, with the consequent production of reactive oxygen species (ROS), such as superoxide radical ($O_2^{\bullet}$) and hydrogen peroxide (H_2O_2). These reactive molecules, by inactivating the apoptotic caspase system, determine necrotic cell death[17]. Moreover, the increase in pro-oxidant activity is associated with a decrease in the antioxidant potential (superoxide dismutase activity and glutathione content)[13,18].

Following such an increase in pro-oxidant activity[12], the progression from NAFLD to NASH is mediated by the activation of different transcription factors, such as sterol regulatory element binding protein 1c (SREBP-1c), peroxisome proliferator-activated receptor γ (PPARγ) and carbohydrate responsive element-binding protein (ChREBP), which activate the expression of a series of genes essential for lipogenesis[19-23].

Other mechanisms are involved in the pathogenesis of NASH, such as increased secretion by the adipose tissue of proinflammatory and prothrombotic adipocytokines [interleukin 6 (IL-6) and tumor necrosis factor α (TNF-α)] and the reduced production of adiponectin, a potent anti-inflammatory, insulin-sensitizing adipocytokine[24,25]. Inflammation is a component of the wound healing process that leads to the deposition of extracellular matrix and fibrosis in the liver. There is much evidence supporting a central role for pro-inflammatory cytokines, particularly TNF-α and IL-6, in the development of NASH. In fact, increased cytokines levels are found in the liver and blood of patients with NASH[26], and their inhibition improved NAFLD in animal[27] and human models[28].

Considering their beneficial impact on cardiometabolic clusters (hypertension, hyperlipidemia, endothelial dysfunction and atherosclerosis)[29], n-3 PUFAs are emerging as a potential treatment of liver steatosis. They cannot be synthesized by the human body and, thus, must be derived from exogenous sources (fish oil, flax seeds, *etc*.).

n-3 PUFAs, especially eicosapentaenoic acid (C20:5n3, EPA) and docosahexaenoic acid (C22:6n3, DHA), by regulating gene transcription factors (i.e., PPARα, PPARγ, SREBP-1, ChREBP), can control key pathways involved in hepatic lipid metabolism[30,31]. In more detail, n-3 PUFAs are potent activators of PPARα, which up-regulates several genes involved in the stimulation of fatty acid oxidation[32-35] and down-regulates pro-inflammatory genes, such as TNF-α and IL-6[36]. Moreover, n-3 PUFAs activate PPARγ, resulting in increased fat oxidation and improved insulin sensitivity[37].

Besides enhancing hepatic beta oxidation, n-3 PUFAs can also decrease endogenous lipid production by inhibiting the expression and processing of SREBP-1, which, in response to increased glucose and insulin levels, stimulates the transcription of several lipogenic and glycolytic genes[38-42]. Moreover n-3 PUFAs can inhibit hepatic glycolysis and lipogenesis and suppress the ac-

tivity of ChREBP, another regulator of glycolytic, and lipogenic genes, such as *L*-pyruvate kinase and fatty acid synthase[43].

Forthcoming studies show a growing amount of other genes are involved in NAFLD pathophysiology and, in turn, in the effect of n-3 PUFAs[44,45].

ANIMAL MODELS

A series of animal models have been used to study NAFLD. Most of them found that fat intake and obesity are strictly related to fatty liver development. In more detail, the Western lifestyle, with a high fat content diet and sedentary behavior, was found to lead to liver damage in animals[46,47]. Further models showed that the "cafeteria diet" (a feeding regimen similar to fast food) is strictly associated with NAFLD development and subsequent hepatic necro-inflammatory changes in mice[37]. By evaluating the mechanisms by which diet impacts NAFLD development, an increase in dietary cholesterol, sucrose or fructose was found to induce hepatic lipogenesis in mice through the up-regulation of SREBP-1 expression, which stimulates the transcription of a series of lipogenic genes[48-51].

After proving the strict relation between diet and NAFLD, the effects of n-3 PUFAs were tested in a series of animal models.

The first interesting data were that n-3 PUFAs depletion was found to promote steatosis and insulin resistance in rodents. Thus, rats fed with a low n-3 PUFAs diet rapidly developed NAFLD[52,53]. In a recent study[54], a drastic drop in n-3 PUFAs was induced by feeding C57Bl/6J mice for 3 mo with a n-3 PUFAs depleted diet. The animals showed insulin resistance and hepatic steatosis, which was associated with a decrease in fatty acid oxidation. Compared to the animals following the control diet, which only differed in the n-3 PUFAs content, analysis of the liver tissue revealed higher expression of all enzymes involved in lipogenesis, as well as increased expression and activation of SREBP-1. On the contrary, supplementing the diet with n-3 PUFAs prevented or reversed hepatic steatosis in animals. Recently, it has been reported that rats fed with a high fat diet combined with n-3 PUFAs supplementation were protected against severe NAFLD development. In fact, significantly increased lipid peroxidation was seen in the group fed with the same diet without n-3 PUFAs supplementation[55].

In a further experimental model to confirm the protective effect from NAFLD development in mice[56], n-3 PUFA administration was also found to reverse already established hepatic steatosis in leptin deficient obese mice[57].

Marsman *et al*[58] induced hepatic steatosis by a 3 wk methionine/choline deficient diet in rats, and then administered n-3 PUFAs, standard lipid solution, or NaCl for 2 wk. Compared with control animals receiving a standard diet, n-3 PUFAs treated animals showed histological evidence of mild macrovesicular steatosis. On the contrary, severe macrovesicular steatosis was found in both standard lipid solution and saline diet groups. In the same study, liver ischemia/reperfusion injury was evaluated by clamping vessels for 40 min. At 6 and 24 h from reperfusion, n-3 PUFA treated rats showed lower alanine aminotransferase (ALT) serum levels, lower hepatic TNF-α levels and a higher anti-oxidative capacity compared with both standard lipid solution and saline diet groups. Overall, these findings suggest that n-3 PUFA treatment both reduces hepatic steatosis and attenuates hepatic ischemia/reperfusion injury in rats.

Other experimental studies analyzed further mechanisms by which n-3 PUFAs could impact on NALFD. In particular, a diet enriched in n-3 PUFAs was shown to improve insulin sensitivity, and reduce intrahepatic triglyceride content and steatohepatitis, in both mice[56,59] and rats[60,61] with fatty liver. Sekiya *et al*[59] exposed ob/ob mice to a dietary supplementation of n-3 PUFAs, obtaining a down-regulation of the *SREBP-1* gene and a reduction of hepatic lipogenesis, with an improvement of insulin-dependent metabolism (reduction of glucose, insulin and free fatty acid serum levels).

Similarly, Levy *et al*[61] found that the "Quantitative Insulin Sensitivity Check Index" was higher in fish oil fed Fischer Rats than in the control animals. A possible explanation for these results comes from a murine model, in which n-3 PUFAs supplementation in obese mice induces an up-regulation of the genes involved in insulin sensitivity (PPARγ), glucose transport (GLUT-2/GLUT-4), and insulin receptor signaling (IRS-1/IRS-2)[37].

Apart from the effects on metabolic homeostasis, in experimental NAFLD murine models, by influencing the production of eicosanoids, prostaglandins, and of leukotrienes, n-3 PUFAs also showed anti-inflammatory properties[62].

Overall, these results suggest that n-3 PUFAs improve insulin sensitivity and reduce markers of inflammation, all major events in NAFLD development[37,62].

Moreover, n-3 PUFAs supplementation improves hepatic steatosis in obese animals by modifying the genetic expression of key enzymes[63]. It has been shown that n-3 PUFAs are the natural ligands of PPARα, which modulates lipid metabolism in hepatocytes[64]. In fact, by inducing the expression of proteins with peroxisome proliferator response elements in their promoting region[64], PPARα regulates fatty acid binding and their export in very low density lipoprotein[65,66]. In PPARα (-/-) knockout animals, hepatic steatosis, which was minimal under normal conditions, drastically increased after a fasting period. The absence of PPARα likely impaired mitochondrial β-oxidation in the liver during fasting, leading to hepatic steatosis development[65]. Furthermore, there is evidence from other studies that n-3 PUFAs reduce hepatic ROS levels[67]. n-3 PUFAs seem to improve the tolerance to oxidative stress, IRS-2 activity in the liver, brain and uterus of rats[68]. Consequently, they may have a potential protective role against ROS-induced oxidative cellular damage in rat organs, especially in the liver. Re-

cently, using the methionine/choline deficient model of steatohepatitis, the effect of EPA, one of the most important long chain PUFAs, on hepatic fibrosis and ROS production was investigated in rat livers. For the study in question, steatosis was induced in 20 Wistar rats by a 20 wk methionine/choline deficient diet, followed by oral administration of EPA in 10/20 rats from week 12; a time at which hepatic fibrosis was already established. Control animals instead received a methionine/choline sufficient diet. At histology evaluation, EPA treatment was found to suppress hepatic fibrosis in liver sections, with repressed macronodular formation and decreased hepatic triglycerides content. EPA also suppressed the increase of hepatic fibrogenic factors, such as α-smooth muscle actin, TGF-β1, procollagen, and connective tissue growth factor. The attenuation of hepatic fibrosis by EPA was significantly related to hepatic ROS levels. EPA also suppressed increases in hepatic ROS levels and reduced serum oxidative markers, such as 8-isoprostane and ferritin[69].

All the aforementioned animal models show that omega-3 depletion can promote steatosis and insulin resistance. On the other hand, n-3 PUFAs supplementation, by inducing SREBP-1 up-regulation and lipogenic genes expression reduction, improving glycemic control, insulin levels and insulin sensitivity, reducing the oxidative stress, and exerting an anti-inflammatory effect, is able to prevent, or even at reverse, hepatic steatosis.

INTERVENTIONAL STUDIES

Although several clinical trials have been conducted, due to a wide variability in treatment duration, and the different n-3 PUFAs doses and preparations used, the efficacy of n-3 PUFAs in the treatment of NAFLD in humans has not yet wholly defined. The first clinical trial (Table 1) providing encouraging evidence about the efficacy of n-3 PUFAs in the treatment of NAFLD was performed by Capanni *et al*[70]. They evaluated the efficacy of prolonged n-3 PUFAs supplementation in 56 patients with an ultrasonographic (US) diagnosis of NAFLD. 1 g/d of n-3 PUFAs was administered to 42 subjects for 12 mo. The 14 subjects refusing the same treatment served as controls. The primary outcome was the US appearance of the liver, including a quantitative measurement of fat storage on the basis of the Doppler perfusion index (DPI)[71]. At the end of the treatment, subjects showed a significant ($P = 0.0001$) improvement of NAFLD compared with controls. A concomitant increase of DPI, proof of a hemodynamic improvement, was also reported in the treatment group, but not in the control group. In addition, n-3 PUFA supplementation was associated with a significant reduction of liver enzymes ($P = 0.003$), fasting glucose ($P = 0.02$) and triglyceride ($P = 0.02$) levels. However, it should be noted that this prospective study has some limitations, such as the absence of blindness and randomization. In a subsequent study[72], the effectiveness of n-3 PUFAs supplementation was demonstarted on-top of a validated diet (Table 1). In this trial, 40 patients with NAFLD randomly received an American Heart Association (AHA) recommended diet[73] plus n-3 PUFAs 2 g/d, or only the AHA diet, for 6 mo. Primary outcomes included: changes in fatty liver severity assessed by abdominal US, and liver ALT and triglyceride levels. Interestingly, inflammatory and metabolic markers such as TNF-α serum levels and insulin resistance assessed by homeostatic model assessment (HOMA) were also evaluated in this study. At the end of the treatment, patients who received diet plus n-3 PUFA supplementation had a significant reduction in ALT ($P < 0.01$), triglycerides ($P < 0.01$), TNF-α ($P < 0.05$), and HOMA ($P < 0.05$) levels. In addition, 33.4% of them showed a complete fatty liver regression. On the contrary, none of the patients receiving the diet alone showed a complete regression of the fatty liver. Indeed, this trial showed some design weaknesses, such as the lack of placebo and the lack of blindness of both participants and investigators. At variance with the latter reported studies, enrolling relatively few patients, Zhu *et al*[74] performed a randomized clinical trial with a large sample size (Table 1). In 144 patients with NAFLD and mixed hyperlipidemia, the efficacy of n-3 PUFA from seal oils was evaluated. Patients were randomly assigned to two groups of treatment: Group A received an AHA recommended diet[73] and 2 g of seal oils (rich in EPA, DHA, and DPA) × 3/daily, while Group B received the recommended diet and 2 g placebo × 3/daily. The treatment duration was 24 wk. Primary endpoints were changes in ALT and serum lipid levels, symptom scores (liver discomfort or pain, weakness, abdominal distention, and nausea) and modifications in fatty liver assessed by US. At the end of the treatment period, total symptom scores, ALT and triglycerides levels decreased more significantly ($P < 0.01$) in Group A than in Group B. At the abdominal US, a normal liver echo pattern and a significant liver steatosis improvement compared with the baseline was found in 19.7% and 53.03% of patients in Group A, respectively. On the other hand, in Group B only 7.35% of subjects achieved complete regression ($P = 0.04$) and 35.29% had some degree of liver steatosis improvement ($P = 0.04$), with no change being observed in the remaining 64.71% of patients in the group. It is noteworthy that some patients only on the diet ameliorated. Although only having a small sample size of the population, the results of a study performed by Sofi *et al*[75] are of particular interest. It aimed to assess the efficacy of the administration of olive oil (rich in PUFAs) in patients with NAFLD. As many as 6 subjects received 5 mL/d of olive oil for 1 year, while 5 were selected as controls (Table 1). Outcome measurements were serum liver biochemistry, serum lipids, adiponectin levels, and the appearance of the liver with US and Doppler investigation. In this study, at variance with all the others, n-3 PUFAs were administered in olive oil instead of in capsules. Thus, this could be considered a "nutritional" rather than a "therapeutic" study. Since olive oil is one of the staples of the Mediterranean diet, it is interesting to note that, at the

Table 1 Summary of trials design and results

Ref.	Study design	Intervention	Population	Outcome measurements	Results	Comments
Capanni *et al*[70]	Open-label	Oral administration of n-3 PUFA, 1-g capsule/d for 12 mo	56 patients with NAFLD (42 subjects receiving therapy; 14 controls)	AST, ALT, GGT, TG, FG, n-6/n-3, liver echo texture by US and liver perfusion by DPI	↓AST (P = 0.003) and ALT (P = 0.002), ↓ GGT (P = 0.03), ↓ TG (P = 0.02) and FG (P = 0.02) in comparison with controls. Circulating arachidonate and n6: n3 ratio was reduced (P = 0.0002, and P = 0.0001 respectively) in treated patients. Improvement of liver echo texture (P = 0.0001), and increase of DPI (P = 0.001)	Limits of this study are the absence of blinding and randomization, and the use for comparison of a self-selected small group consisting of those patients who had been declined entry to the treatment arm
Spadaro *et al*[72]	Randomized; open-label	AHA diet + 2 g/d n-3 PUFA (Group DP) *vs* AHA diet (Group D) for 6 mo	40 patients with NAFLD (Group DP, n = 20; Group D, n = 20)	Liver fat assessed by abdominal US, ALT, AST, TNF-α serum levels, and HOMA	In DP group: ↓ ALT (P < 0.01), TG (P < 0.01), serum TNF-α (P < 0.05) and HOMA (IR) (P < 0.05). Complete fatty liver regression in 33.4% of patients, and an overall reduction in 50%; In the D group: no significant modification of laboratory tests; no patient achieved complete regression of fatty liver, whereas some amount of reduction occurred in 27.7% of patients	Limits of the study are lack of placebo, and the non blinding of participants and investigators
Zhu *et al*[74]	Randomized	AHA diet + 2 g/d n-3 PUFA from seal oil (Group A) *vs* AHA diet + 2g of placebo (Group B) for 6 mo	144 patients with NAFLD and hyperlipidemia (Group A = 72; Group B = 72)	Liver fat assessed by symptom scores, ALT and serum lipid levels after 8, 12, 16, and 24 wk; fatty liver assessed by US at weeks 12 and 24 after treatment	Group A *vs* Group B showed ↓ of total symptoms score, ALT, TG, LDL (P < 0.05); complete fatty liver regression in 19.7% *vs* 7.35% (P = 0.004); In both groups there was a tendency in improvement in AST, GGT, TCHO and HDL levels (P < 0.05)	
Tanaka *et al*[77]	Open label	EPA 2.7 g/d for 12 mo	23 patients with biopsy proven NAFLD	ALT, FFA, plasma soluble TNF receptor 1 and 2 levels, and serum ferritin and thioredoxin levels, body weight, blood glucose, insulin, and adiponectin concentrations; fatty liver infiltration assessed by histology	↓ ALT, AST, TG, TCHO, HOMA-IR, plasma thioredoxin; change in histological grade: steatosis: 2.4 (SD 0.5) *vs* 1.7 (SD 0.5); fibrosis: 1.7 (SD 1.1) *vs* 0.7 (SD 0.5); lobular inflammation: 2.1 (SD 0.7) *vs* 1.1 (SD 0.7); ballooning: 1.6 (SD 0.5) *vs* 0.9 (SD 0.4); NAS: 6.1 (SD 1.3) *vs* 3.7 (SD 1.4); Hepatic steatosis grade on the US changed from 2.1 ± 0.9 at baseline to 1.6 ± 1.1 after treatment (P = 0.004)	Limits of the study are the absence of a control group and small sample size
Sofi *et al*[75]	Randomized	Dietary recommendation + 6.5 mL/d of olive oil enriched with n-3 PUFA (0.83 g n-3 PUFA, of which 0.47 g EPA and 0.24 g DHA) for 12 mo *vs* dietary recommendation alone	11 patients with NAFLD assessed by US (intervention group, n = 6; control group, n = 5)	Liver fat content assessed by B-mode US and DPI; liver enzymes, TG and adiponectin levels	Intervention group *vs* controls showed a ↓ of AST (P = 0.02), ALT (P = 0.03), GGT (P = 0.03), TG (P = 0.04) levels; ↑ of HDL (P = 0.03), adiponectin (P = 0.04). There was a significant (P = 0.02) improvement of DPI in the intervention group, while no change was observed in the control group. Improvement of liver steatosis on US in the intervention group (% of patients at T0 and T12): absent (from 0% to 16.7%); mild (from 16.7% to 50%); moderate (from 33% to 0%); severe (from 50% to 33%)	

Nobili *et al*[78]	Randomized	DHA (250 and 500 mg/d) *vs* placebo for 6 mo	60 children with biopsy-proven NAFLD randomly assigned to receive DHA 250 mg/d (n = 20), DHA 500 mg/d (n = 20) or placebo (n = 20)	Primary: change in liver fat content as detected by US; secondary: changes in ISI, ALT, TG and BMI	DHA 250 mg *vs* placebo: odds of more severe *vs* less severe steatosis (OR = 0.01, robust 95% CI: 0.002 to 0.11, P < 0.001); ↑ of ISI (P < 0.01), ↓TG (P < 0.05); ALT and SDS of BMI; DHA 500 mg *vs* placebo: (OR = 0.04, 0.002 to 0.46; P = 0.01); ↑ of ISI (P < 0.01), ↓TG (P < 0.05); ALT and SDS of BMI; DHA 250 mg *vs* DHA 200 mg: NS	
Vega *et al*[79]	Open label	9 g/d of fish oil for 8 wk	22 patients with previous elevated liver fat on MRS (17 patients completed the trial)	Liver fat content assessed by B-mode US and DPI; liver enzymes, TG and adiponectin levels	↓ of plasma triglyceride level by 46% (P < 0.03), VLDL + IDL by 21% (P < 0.03), ApoB by 15% (P < 0.03). Liver fat content 7.9% pre-treatment; 8.0% after PUFA (NS)	Causes of liver disease other than NAFLD were not excluded and alcohol intake was not reported. It is unclear whether study participants received any other interventions such as diet or lifestyle advice

NAFLD: Non-alcoholic fatty liver disease; AST: Aspartate transaminase; ALT: Alanine transaminase; GGT: γ-glutamyl transpeptidase; TG: Triglycerides; FG: Fasting glucose; US: Ultrasonographic; DPI: Doppler perfusion index; AHA: American Heart Association; PUFA: Polyunsaturated fatty acid; TNF: Tumor necrosis factor; HOMA: Homeostatic model assessment; IR: Insulin resistance index; TCHO: Total cholesterol; HDL: High-density lipoprotein; MRS: Magnetic resonance spectroscopy; VLDL: Very low density lipoprotein; ISI: Insulin sensitivity index; EPA: Eicosapentaenoic acid; DHA: Docosahexaenoic acid; DPI: Doppler perfusion index; BMI: Body mass index; SDS: Standard deviation score; NS: Not significant.

end of treatment, patients showed a significant (P < 0.05) improvement in liver echo-texture and DPI, a significant improvement of liver enzymes, and triglycerides (P = 0.04) and adiponectin levels (P = 0.04).

In the aforementioned studies, the lack of a liver biopsy for the diagnosis of NAFLD may hamper the relevance of their findings. However, in spite of its inherent operator-dependence, abdominal US analysis is currently thought to provide reliable, careful information about hepatic steatosis. This limits the need for liver biopsy for the diagnosis of NASH and to determine the severity of hepatic fibrosis[76]. Moreover, despite its inherent limitations, US analysis has been validated against histopathological specimens, as well as other imaging methods, for the diagnosis of liver steatosis[76]. In this regard, studies in which the effects of n-3 PUFAs on NAFLD are supported by histology or MRI findings have been also performed. Tanaka *et al*[77] enrolled 23 patients with biopsy proven NASH. They received 2.7 g of EPA daily for 12 mo (Table 1). Outcome measurements were serum liver biochemistry, appearance on US, and liver histology (graded using the NAFLD activity score). All patients completed the trial and showed a significant improvement of laboratory markers of hepatic oxidative stress. The mean US steatosis degree improved significantly and, in 6 out of 7 patients who underwent repeated biopsy, steatosis, inflammation and fibrosis, resulted in significantly reduced levels. Although this was the first human study of n-3 PUFAs fatty acids to have histological data, which are the most valid outcome measurement, the absence of randomization, controls and blindness, along with the small sample size, do not allow us to draw definitive conclusions. In another study[78], in which the diagnosis of NAFLD was confirmed by biopsy, 60 children were randomly assigned to receive DHA 250 mg/d, DHA 500 mg/d or placebo (Table 1). The duration of treatment was 6 mo. The main outcome was the change in liver fat content as detected by US. After 6 mo, DHA supplementation was associated with lower odds of severe steatosis compared to a placebo. In addition, for the groups treated with DHA, where no effects on ALT values were found, there was an improvement of insulin sensitivity and triglycerides levels. Thus, this prospective study showed that, following this therapeutic regimen, both US and metabolic feature improvement occurred.

Therefore, Vega *et al*[79] evaluated the efficacy of n-3 PUFAs on serum and hepatic triglycerides levels, the latter assessed by magnetic resonance spectroscopy (Table 1). Of the 22 patients enrolled, 17 completed the trial. They received a placebo for 4 wk, followed by an 8 wk treatment with 9 g/d of fish oil. Treatment with fish oil significantly reduced the levels of plasma triglycerides by 46% (P < 0.03), very low-density lipoprotein plus intermediate density lipoprotein cholesterol by 21% (P < 0.03), and total apolipoprotein B by 15% (P < 0.03). In contrast to the changes in plasma triglycerides, hepatic triglyceride content was not significantly reduced by fish oil treatment.

In conclusion, NAFLD may be considered the hepatic expression of metabolic syndrome[5] which, in turn, predisposes to cardiovascular events. It is known that n-3 PUFAs have many beneficial effects on most of the metabolic syndrome features. In this regard, there is evidence suggesting that n-3 PUFAs are able to reduce

blood pressure[80,81] and that they have favorable effects on plasma lipids levels[82]. In addition, n-3 PUFAs also showed anti-platelet and anti-inflammatory properties which help to explain their cardio-protective effects[29,82]. Most of the available clinical trials provide encouraging data about the efficacy of n-3 PUFAs as a treatment of NAFLD in humans[9].

In keeping with this, in the era of poly-pills for coronary heart disease prevention, drugs with multifaceted mechanisms of action should be taken into serious consideration[82]. On the other hand, it is worthwhile to consider that a significant amount of n-3 PUFA is contained in fish and in olive oil. All these are typical foods of the Mediterranean diet, which exhibits well known beneficial effects and is able to prevent obesity, diabetes and, in turn, cardiovascular events[83]. For individuals eating low amounts of fish, a 500 mg/d EPA+DHA consumption is recommended in the absence of any type of cardiovascular disease, the suggested dosage being at least 800-1000 mg/d for those with coronary heart disease or congestive heart failure[82].

According to these data, it is important to consider that most of the beneficial effects of n-3 PUFAs can also be obtained by an equilibrate nutrition program.

Well-designed randomized controlled trials of adequate size and duration, with histological endpoints, are needed to assess long-term safety and efficacy of PUFA, as well as other therapies for the treatment of NAFLD and NASH patients. Thus, while waiting for further data, current nutritional recommendations about daily intake should be strictly taken into consideration.

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