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Randomized control trials

Omega-3 polyunsaturated fatty acids in treating non-alcoholic steatohepatitis: A randomized, double-blind, placebo-controlled trial

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SUMMARY

Background: & aims: Few clinical trials have addressed the potential benefits of omega-3 polyunsaturated fatty acids (PUFAs) on non-alcoholic steatohepatitis (NASH). We evaluated the effects of supplementation with omega-3 PUFAs from flaxseed and fish oils in patients with biopsy-proven NASH.
Methods: Patients received three capsules daily, each containing 0.315 g of omega-3 PUFAs (64% alpha-linolenic [ALA], 16% eicosapentaenoic [EPA], and 21% docosahexaenoic [DHA] acids; n-3 group, $n = 27$) or mineral oil (placebo group, $n = 23$). Liver biopsies were evaluated histopathologically by the NASH activity score (NAS). Plasma levels of omega-3 PUFAs were assessed as a marker of intake at baseline and after 6 months of treatment. Secondary endpoints included changes in plasma biochemical markers of lipid metabolism, inflammation, and liver function at baseline and after 3 and 6 months of treatment.
Results: At baseline, NAS was comparable between the groups ($p = 0.98$). After intervention with omega-3 PUFAs, plasma ALA and EPA levels increased ($p \leq 0.05$). However in the placebo group, we also observed increased EPA and DHA ($p \leq 0.05$), suggesting an off-protocol intake of PUFAs. NAS improvement/stabilization was correlated with increased ALA in the n-3 group ($p = 0.02$) and with increased EPA ($p = 0.04$) and DHA ($p = 0.05$) in the placebo group. Triglycerides were reduced after 3 months in the n-3 group compared to baseline ($p = 0.01$).
Conclusions: In NASH patients, the supplementation of omega-3 PUFA from flaxseed and fish oils significantly impacts on plasma lipid profile of patients with NASH. Plasma increase of these PUFAs was associated with better liver histology. (ID 01992809)

1. Introduction

Non-alcoholic fatty liver disease (NAFLD[1]) encompasses a large spectrum of conditions, ranging from simple steatosis to steatohepatitis (NASH), fibrosis, cirrhosis, and hepatocellular carcinoma [1]. Mechanisms driving the transition from simple steatosis to NASH and the later degrees of NAFLD are multifactorial and seem to involve oxidative stress, lipotoxicity, insulin resistance, and central inflammatory signalling pathways [2,3]. Maintaining a proper body weight and a healthy lifestyle seem to provide benefits for controlling NASH development [4].

Because dietary and lifestyle modifications often fail or cannot be implemented effectively, new pharmacologic agents, designed

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[1] AA, arachidonic acid; ALA, alpha-linolenic acid; ALT, alanine aminotransferase; AST, aspartate aminotransferase; CI, confidence interval; CRP, C-reactive protein; DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; FA, fatty acid; GGT, gamma-glutamyl transferase; HDL, high-density lipoprotein; IL-6, interleukin-6; LDL, low-density lipoprotein; MS, metabolic syndrome; n-3, omega-3; n-6, omega-6; NAFLD, nonalcoholic fatty liver disease; NAS, NASH activity score; NASH, nonalcoholic steatohepatitis; PUFA, polyunsaturated fatty acids.

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and studied with the main goal of improving hepatic histopathology, are needed. In this regard, polyunsaturated fatty acids (PUFAs) play a role in lipid metabolism and inflammation and are potential candidates. Patients with NASH present a higher liver ratio of omega-6 (n-6) to omega-3 (n-3) PUFAs compared to healthy controls, suggesting a possible role for low n-3 PUFA or high n-6 PUFA content in the physiopathology of this disease [5].

Omega-3 PUFAs have biological properties of special interest to the treatment of NASH. They include the essential alpha-linolenic acid (ALA), present in some vegetable sources, and the eicosapentaenoic (EPA) and docosahexaenoic (DHA) acids, abundantly found in cold-water marine fish. These three n-3 PUFAs may influence hepatic lipid metabolism, adipose tissue function, and immune response through anti-inflammatory effects [6,7].

Due to its lower unsaturation, ALA is less vulnerable to oxidation than EPA and DHA. In addition, ALA can be endogenously converted into EPA and, to a lesser extent, into DHA [8]. Several studies have demonstrated various cardioprotective, glycaemic and lipid-lowering benefits for ALA, but n-3 PUFAs supplementation in NASH was mainly evaluated using EPA and DHA as source [9–12].

Experimentally, n-3 PUFAs were associated with a positive impact on NAFLD treatment by reducing hepatic steatosis, improving insulin sensitivity, and reducing inflammatory markers [13]. Despite these findings, only few randomized trials have addressed the effect of n-3 PUFA supplementation on liver histology in patients with NAFLD or NASH, and those studies suffer limitations including, poor study design, small samples, lack of omega-3 intake markers and use of techniques that measure only indirectly liver histology, mainly ultrassonography [14–19]. We performed a double-blind, randomized, and controlled study assessing the effect of oral supplementation of n-3 PUFAs derived from flaxseed and fish oils on the treatment of NASH, with emphasis on liver histology.

2. Materials and methods

2.1. Ethical considerations

This study was performed according to the ethical standards of the World Medical Association's Declaration of Helsinki. The protocol was approved by institutional ethics board review and registered on www.ClinicalTrials.gov (ID 01992809). Written informed consent was obtained from each patient prior to trial participation.

2.2. Patients and experimental design

Adult outpatients (18–75 years old) attending the Division of Clinical Gastroenterology and Hepatology of the Hospital das Clínicas of the University of São Paulo School of Medicine (Sao Paulo, Brazil) from April 2009 to 2011 were screened for eligibility. Criteria for inclusion were men and women with a proven histological diagnosis of NASH. Criteria for exclusion were: a history of any other acute or chronic liver or biliary disease; substance abuse, especially intake of >100 g alcohol/week; use of hepatotoxic drugs (e.g. corticosteroids, high-dose oestrogens, methotrexate, tetracycline, calcium channel blockers, amiodarone, or tamoxifen); neurologic or psychiatric dysfunctions; any allergy or food intolerance, including intolerance to any ingredient of the supplemental capsules; and refusal to give informed consent.

A randomization sequence with two branches of 40 patients each (1:1 allocation) was generated by computer (GraphPad statistical software; QuickCalcs, La Jolla, CA–USA) before initiation of the study by an independent dietician (MCGD), to assign participants to either the n-3 or the placebo group. With the exception of this independent dietician, investigators and clinical staff remained blinded to each study participant's assignment until the end of the statistical analysis phase of the trial. Included patients were enrolled in the study by two trained investigators (CPMSO and MAN) following this randomization sequence.

Treatment was performed in a double-blind manner. Identical capsules containing omega-3 fatty acids or mineral oil were packed in identical bottles and labelled with 10 different codes (5 for each treatment). The n-3 group received appropriate labelled bottles with capsules containing 0.315 g of n-3 PUFAs (64% ALA, 16% EPA, and 21% DHA; Table 1) and instructions to ingest 3 capsules daily, comprising a total daily intake of 0.945 g of n-3 PUFAs. The placebo group received proper labelled bottles with identical capsules, each containing 2 mL of mineral oil, with identical intake instructions. Two capsules of each labelled bottle (5 omega-3 and 5 mineral oil) were kept sealed by MCGD in order to certify the blinding procedures after the study end.

The intervention was implemented for 6 continuous months and, at the end, the patients were readmitted for a new liver biopsy. Each patient's plasma lipid profile was analysed to assess treatment compliance. The primary endpoint was a change in liver histopathology compared to baseline (before treatment), according to the NASH activity score (NAS), after 6 months of treatment. Secondary endpoints included changes over baseline scores in biochemical and anthropometric data after 3 and 6 months of treatment.

2.3. Analysis of plasma fatty acids

Plasma lipid gas chromatography was performed using an Agilent 7890A GC chromatograph System and J&W DB-23 columns of 60 m × 250 μm × 0.15 μm (Agilent Technologies, Santa Clara, CA, EUA) with plasma samples obtained at baseline and after 6 months of treatment. Methyl esters of fatty acids (FAs) were obtained as previously described [20]. Chromatographic conditions were set as previously described [21], with some specific adaptations to optimize study samples according to parameters of selectivity, linearity, precision, accuracy, and limits of detection and quantification (injection split mode = 50:1, injector temperature = 250 °C, detector temperature = 280 °C, and chromatographic column described above). The column was operated at an initial temperature of 80 °C for 1 min, followed by an increase to 230 °C for 5 min, with a total run time of 45 min. To obtain the calibration curve, FAs were assessed by external standardization using reference standards (Sigma–Aldrich; St. Louis, MO, USA).

2.4. Histopathologic analysis

All liver biopsy slides were stained with hematoxylin and eosin. Two liver pathologists, experts in NAFLD scoring (VAFA & BC),

Table 1
Composition of each capsule offered to the omega-3 group.

Component	Amount[a]	Total intake/day
Calories (kcal)	8	24
Carbohydrates (g)	0	0
Proteins (g)	0	0
Total fat (g)	1	3
Saturated fat (g)	0	0
Monounsaturated fat (g)	0	0
Polyunsaturated fat (g)	0.315	0.945
Omega-3 EPA (g)	0.065	0.195
Omega-3 DHA (g)	0.050	0.15
Omega-3 ALA (g)	0.200	0.6
Cholesterol (g)	0	0

EPA: eicosapentaenoic acid; DHA: docosahexaenoic acid; ALA: alpha-linolenic acid.
[a] Data provided by the manufacturer (Amway; Buena Park, CA, USA).

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assessed the slides by optical microscopy in a blinded manner and occasional disagreements between them were resolved through the individual case discussion to reach a final common conclusion. Slides were classified according to the NAS devised by the Pathology Committee of the NASH Clinical Research Network, which includes assessments of macro- and microvacuolar fatty changes, zonal distribution, necrotic foci, portal and perivenular fibroses, and inflammatory and fibrotic infiltrates with zonal distribution [22]. The single-variable grades of steatosis (0–3), lobular inflammation (0–3), fibrosis (0–4), and ballooning (0–2) obtained from the NAS evaluation were also analysed separately. For all histologic parameters, improvement or worsening was evaluated. As NASH is a progressive disease, stabilization of any parameter was considered as an improvement.

2.5. Analysis of biochemical data

Biochemical data were assessed at baseline and after 3 and 6 months of treatment. Serum total cholesterol, high- and low-density lipoprotein cholesterol, triacylglycerol, alanine aminotransferase (ALT), aspartate aminotransferase (AST), gamma-glutamyl transferase (GGT), glycated haemoglobin, alkaline phosphatase, C-reactive protein, serum amyloid A, total and direct bilirubin, fasting glucose, and insulin levels were assessed by automated methods (Cobas equipment, Roche, Rotkreuz, Switzerland) at the Central Laboratory of the Hospital das Clínicas of the University of São Paulo School of Medicine. The homeostasis model assessment-insulin resistance (HOMA-IR) was used for the background population [23].

Plasma interleukin (IL)-6 levels were assessed at the LIM-51 Laboratory of the School of Medicine of the University of Sao Paulo. Human IL-6 enzyme-linked immunosorbent assay (ELISA) kits (R&D, Minneapolis, MN, USA) were used, according to a technique previously described by our team [24]. IL-6 assays were performed in duplicate, and average values were used.

2.6. Analyses of dietary intake and anthropometric data

Food intake was assessed at baseline with a 7-day dietary history survey. The Virtual Nutri Plus® software version 2.0 for Windows (FSP-USP, São Paulo, SP, Brazil) was used for nutrient estimation. Anthropometric data were evaluated at baseline and after 3 and 6 months of treatment. Body weight and height were determined by a platform-type weight scale (Filizola®, São Paulo, SP, Brazil) and a stadiometer (Biospace model BSM370; Gangnam-Gu, Seoul, Korea), respectively. Waist circumference was measured from the iliac crest to the lower costal margin with an inelastic plastic tape of 0.7 cm in width [25]. Body mass index was calculated as weight (in kg) divided by height (in m) squared.

2.7. Sample size and statistical analysis

Sample size was calculated on the basis of a 2006 study by Capanni et al., which assessed the effect of n-3 PUFAs in NASH [14]. That study found histologic or biochemical improvement of any degree in 64% of patients from the treatment arm, and no benefits in individuals from the control arm [17]. We estimated that improvements would be observed in 15% of patients in the placebo group and 64% of patients in the n-3 group. Given a significance level of $\alpha = 0.05$ and test power of 80%, we computed a minimum of 14 patients in each group. Assuming an approximate 50% risk of patient drop-out, patient enrolment was closed after 60 patients were randomly distributed into the two groups.

All data were evaluated with the Statistical Analysis System (SAS) software package (version 9.1, Cary, NC, USA.). Data were analysed by *t*-tests, Mann–Whitney tests, Fisher's tests (to compare groups), and Wilcoxon tests (to analyse each group separately), as appropriate. Quantitative variables were analysed with the paired *t*-test and confidence interval (CI) for the mean method. When necessary, the nonparametric Kruskal–Wallis and Mann–Whitney tests were performed. For qualitative variables, the chi-squared and Fisher's exact tests were used. For post-hoc analysis, a correlation between changes in the plasma PUFA concentration and histopathologic NAS evaluation was performed with Fisher's test. All analyses were based on a 5% level of significance.

3. Results

Sixty patients meeting the inclusion criteria were enrolled in the study, but 10 patients did not complete the study protocol (Fig. 1). Drop-outs were due to: voluntary drop-out not associated with any discomfort or side effect of the protocol treatment (n-3 group, $n = 1$; placebo group, $n = 3$), voluntary drop-out after reference of epigastric pain (placebo group, $n = 1$), patient refusal to undergo the second biopsy (n-3 group, $n = 1$; placebo group, $n = 1$), occurrence of neoplasia (n-3 group, $n = 1$), and unfeasibility of liver biopsy for histological analysis (n-3 group, $n = 2$).

The placebo and n-3 groups were comparable at baseline in terms of their dietary intake habits, demographic data, laboratory tests, metabolic parameters, and histologic conditions (Table 2), except that the baseline AST level was higher in the placebo group. Most patients presented metabolic syndrome (MS) with at least three diagnostic components, according to the MS criteria of the United States National Cholesterol Education Program Adult Treatment Panel III (Table 2) [26].

3.1. Plasma fatty acids

Patients from the n-3 group had higher final plasma concentrations of ALA and EPA compared to baseline, and lower concentrations of the n-6 arachidonic acid (AA) compared to baseline and the placebo group ($p < 0.05$, Fig. 2). The placebo group evidenced higher final levels of EPA and DHA compared to baseline ($p < 0.05$, Fig. 2). The n-3 ($p = 0.02$) and placebo ($p < 0.01$) groups presented higher final levels of total n-3 PUFAs compared to baseline (Fig. 2E). The blinded and randomization accomplishment was confirmed at the study end.

3.2. Histopathologic data

Comparison between the final and basal liver histopathologic scores failed to show significant changes between the n-3 and placebo groups regarding NAS, hepatocellular ballooning, liver steatosis, or fibrosis, although lobular inflammation was improved in the placebo group ($p = 0.05$, Table 3).

We also correlated the individual variation of plasma PUFA levels with the percentage of patients who had improvement histopathologic scores for the n-3 and placebo groups. In the n-3 group, a correlation was observed between the increase of individual ALA and EPA plasma levels and the percentage of patients who presented improvement of liver lobular inflammation (ALA, $p = 0.02$; EPA, $p = 0.002$), steatosis (ALA, $p = 0.04$; EPA, $p = 0.05$), and ballooning (ALA, $p = 0.01$; EPA, $p = 0.02$). Individual increase in plasma ALA and DHA levels and decrease in plasma levels of AA were also correlated with the percentage of patients with improvements of NAS ($p = 0.02$, Fig. 3A), lobular inflammation ($p = 0.028$), and ballooning ($p = 0.05$), respectively, in the n-3 group. For patients from n-3 group we also observed a slight non-statistically significant correlation between the individual increase of EPA levels and the percentage of patients with NAS improvement

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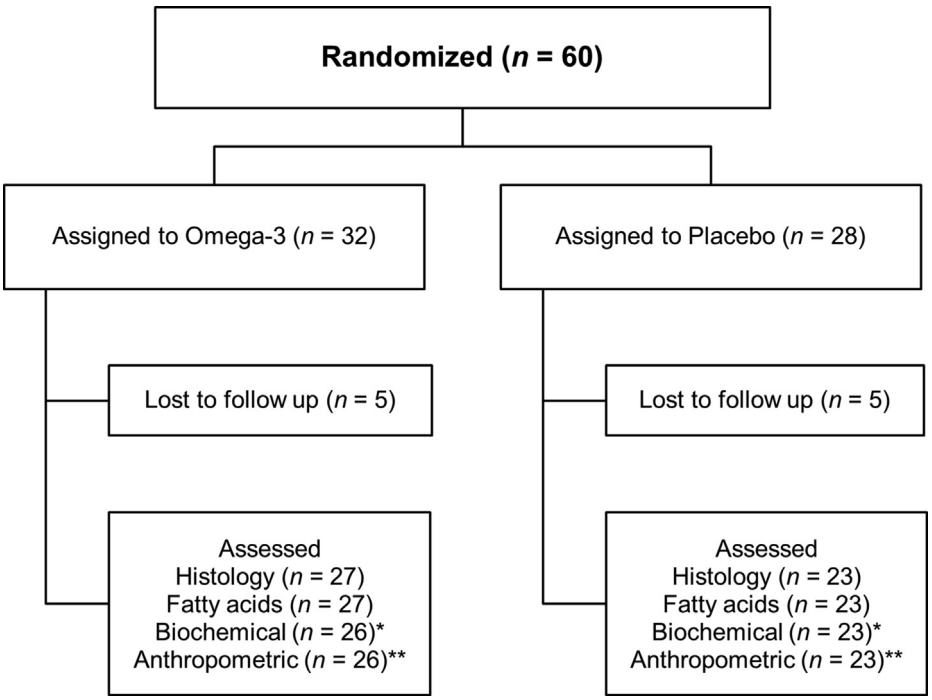

Fig. 1. Consort diagram of nonalcoholic steatohepatitis patients randomized to receive oral omega-3 fatty acids supplementation or placebo. * Except for waist circumference (omega-3: $n = 17$; placebo: $n = 20$) ** Except for: serum amyloid A (omega-3: $n = 12$; placebo: $n = 9$), low-density lipoprotein and triacylglycerol (omega-3: $n = 27$), glycated haemoglobin (omega-3: $n = 25$; placebo: $n = 22$), interleukin-6 (omega-3: $n = 19$; placebo: $n = 17$), and C-reactive protein (omega-3: $n = 23$; placebo: $n = 20$).

($p = 0.07$, Fig. 3B). Furthermore, a correlation was found between the individual increase of EPA and DHA and the percentage of patients with NAS improvement and plasma levels in the placebo group (EPA: $p = 0.04$; DHA: $p = 0.05$, Fig. 3B and Fig. 3C, respectively).

A post-hoc analysis was performed to correlate the individual variation of plasma PUFA levels with the percentage of patients who demonstrated improvement or worsening of histopathologic data, regardless of treatment received. An association was observed between the individual increase of plasma DHA levels and the percentage of patients with improvement of lobular inflammation ($p = 0.01$).

3.3. Biochemical and anthropometric data

A significant reduction in serum triglyceride levels compared to baseline ($p = 0.01$, Table 4) was observed after 3 months of treatment in the n-3 group, but not in the placebo group (Fig. 4). No additional significant changes in serum aminotransferases, fasting lipid profile, fasting glucose, anthropometric parameters, or plasma levels of IL-6 were observed within or between the two groups ($p > 0.05$, Table 4).

4. Discussion

Six-months supplementation with n-3 PUFAs from flaxseed and fish oil increased plasma ALA and EPA levels in patients with NASH. However, elevated plasma EPA and DHA levels were also observed in placebo-treated patients. This finding is highly suggestive of the off-protocol intake of fish oil supplements by placebo-treated patients, as both groups had the same self-reported dietary intake of PUFAs, and jeopardizes our direct determination of possible liver histologic benefits in the n-3 PUFA group.

Available NASH clinical studies looking after n-3 PUFAs effect have not typically control its intake. One exception is the Welcome

Table 2
Clinical and laboratory characteristics of nonalcoholic steatohepatitis patients from placebo and n-3 groups at baseline.

Variables	Placebo group[a]	n-3 group[a]	*P*
Demographic data			
Age (years)	53.9 ± 6.8	52.5 ± 7.2	0.47
Women (%)	78.3	85.2	0.72
Serum biochemical levels			
Alanine aminotransferase (U/L)	46.8 ± 28.2	45.4 ± 35.3	0.27
Aspartate aminotransferase (U/L)	37.9 ± 15.0	31.1 ± 16.7	0.01
γ-glutamyl transferase (U/L)	65.9 ± 49.5	73.6 ± 87.5	0.57
Alkaline phosphatase (U/L)	79.2 ± 26.6	88.1 ± 31.4	0.29
Serum amyloid A (mg/L)	9.4 ± 6.1	8.6 ± 6.3	0.54
Total bilirubin (mg/dL)	0.6 ± 0.2	0.7 ± 0.4	0.27
Direct bilirubin (mg/dL)	0.2 ± 0.1	0.2 ± 0.1	0.98
Glycated haemoglobin (%)	6.7 ± 1.0	6.4 ± 0.9	0.32
C-reactive protein (mg/L)	5.7 ± 5.3	5.6 ± 6.3	0.89
Interleukin-6 (ng/L)	4.0 ± 2.6	4.3 ± 2.0	0.72
Serum lipid levels			
Triglycerides (mg/dL)	148.6 ± 44.7	165.6 ± 76.2	0.80
Total cholesterol (mg/dL)	199.0 ± 46.3	199.4 ± 39.2	0.97
High-density lipoprotein (mg/dL)	52.1 ± 14.2	50.6 ± 16.6	0.49
Low-density lipoprotein (mg/dL)	116.5 ± 39.5	117.7 ± 33.0	0.91
Metabolic factors			
Fasting plasma glucose (mg/dL)	112.0 ± 29.6	103.6 ± 24.9	0.38
Insulin (μU/dL)	13.3 ± 4.6	14.3 ± 7.6	0.59
Body weight (kg)	77.6 ± 10.1	76.9 ± 12.0	0.38
Body mass index (kg/m^2)	30.3 ± 4.4	31.1 ± 4.6	0.93
Waist circumference (cm)	103.1 ± 10.3	104.6 ± 11.1	0.72
Metabolic syndrome (%)	95.6	88.5	1.00
Liver histology			
Total NAFLD activity score	5.6 ± 1.4	5.0 ± 1.4	0.15
Steatosis	2.2 ± 0.8	2.0 ± 0.8	0.34
Lobular inflammation	1.7 ± 0.8	1.4 ± 0.7	0.28
Hepatocellular ballooning	1.7 ± 0.5	1.6 ± 0.5	0.32
Fibrosis stage	1.3 ± 1.0	1.1 ± 0.8	0.61
Dietary intake			
Polyunsaturated fatty acids	9,3 ± 0.8	10.4 ± 2.6	0.53

NAFLD: nonalcoholic fatty liver disease.
[a] Data are expressed as the mean ± standard deviation, except for gender and metabolic syndrome.

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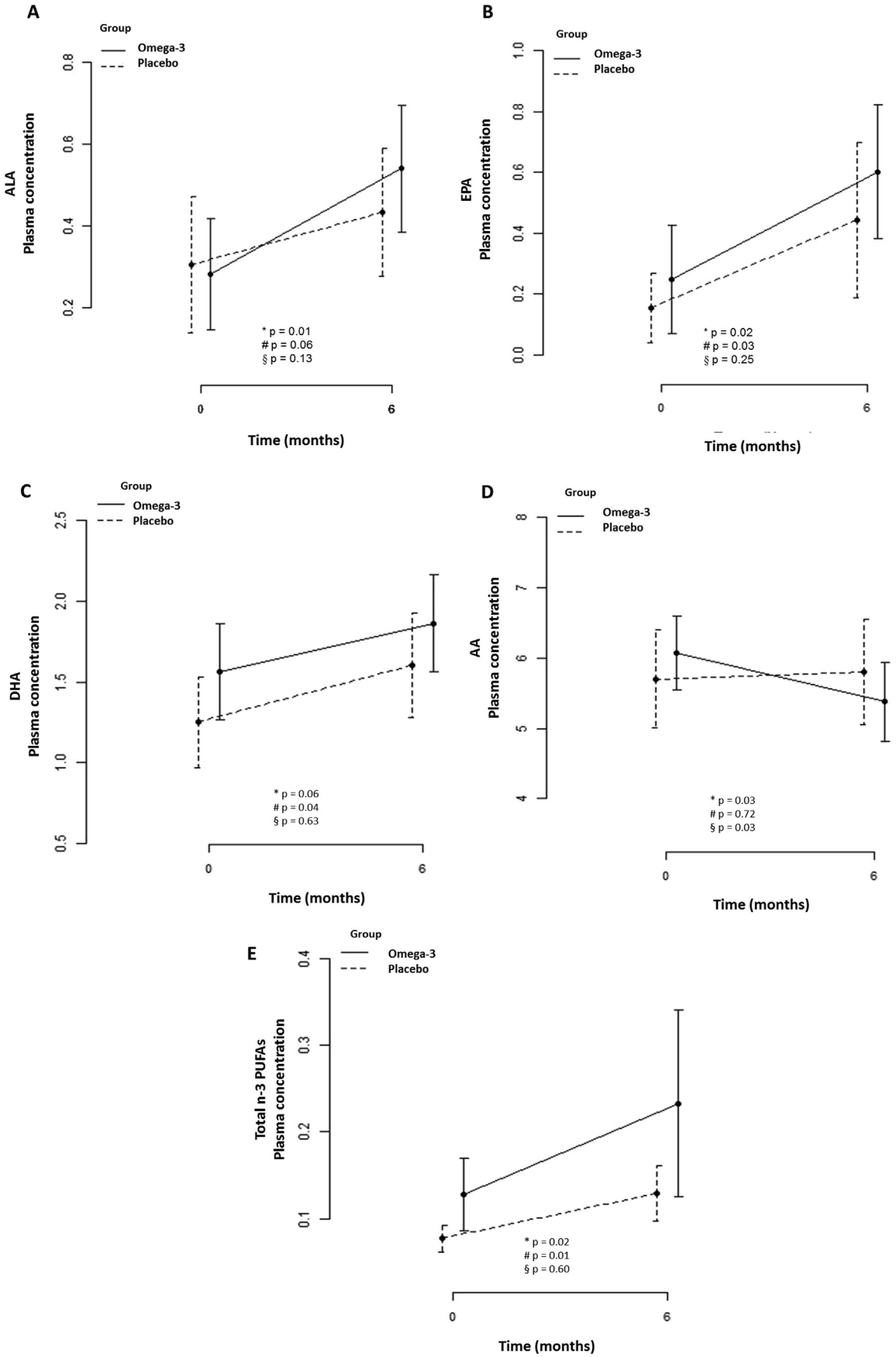

Fig. 2. Plasma concentrations of (A) alpha-linolenic acid (ALA), (B) eicosapentaenoic acid (EPA), (C) docosahexaenoic acid (DHA), (D) arachidonic acid (AA), and (E) total omega-3 polyunsaturated fatty acids (n-3 PUFAs) from patients with nonalcoholic steatohepatitis at baseline and after 6 months of oral intake of omega-3 fatty acids or placebo. Symbols for *p* values: (*) baseline vs. 6 months of n-3 PUFA intake; (#) baseline vs. 6 months of placebo intake; and (§) variation from baseline after 6 months of n-3 PUFA vs. placebo intake.

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Table 3
Changes in liver histology of nonalcoholic steatohepatitis patients after 6 months of oral intake of omega-3 fatty acids or placebo.

Variable	Placebo group[a]	n-3 group[a]	*P*
NAS			
Average change in score	−0.2 (1.2)	−0.04 (1.7)	0.80
Stable or improved (%)	78.3 (57.5–90.6)	55.6 (37.3–72.46)	0.14
Steatosis			
Average change in score	0.2 (0.7)	−0.1 (0.8)	0.16
Stable or improved (%)	73.9 (53.1–87.6)	77.8 (58.8–89.6)	1.00
Lobular inflammation			
Average change in score	−0.2 (0.7)	0.2 (0.8)	0.97
Stable or improved (%)	91.3 (71.8–98.6)	66.7 (47.7–81.4)	**0.05**
Hepatocellular ballooning			
Average change in score	−0.2 (0.5)	−0.1 (0.7)	0.58
Stable or improved (%)	95.6 (77.0–100.0)	77.8 (58.8–89.6)	0.11
Fibrosis			
Average change in score	0.1 (0.9)	0.1 (0.7)	0.38
Stable or improved (%)	60.9 (40.7–77.8)	77.8 (58.8–89.6)	0.23

[a] Data are expressed as mean (confidence interval).

study evaluating EPA and DHA supplementation [27]. The authors have controlled n-3 PUFAs intake by measuring EPA and DHA concentration in erythrocyte and also observed its increase in some patients from placebo group. Moreover, one of placebo-treated patient admitted to take cod liver oil and other markedly increased fish consumption during the study.

In our study, a combination of flaxseed and fish oils was used for n-3 PUFA supplementation because of the high availability of ALA in flaxseed, which potentially could reduce treatment costs comparative to exclusive EPA and DHA supplementation. Chromatography profile of plasma fatty acids was evaluated to assess treatment compliance and confirmed the effective ingestion of the PUFAs by patients in the treatment arm. DHA levels were not significantly increased, perhaps owing to the low amount of this PUFA supplied by the studied supplement or its low conversion from ALA [11]. In addition, plasma AA levels were decreased in n-3–treated patients.

In Welcome study DHA enrichment was independently associated with a decrease in liver fat percentage [27]. In a similar approach, in our study the improvement of NAS was significantly correlated with ALA enrichment in n-3–treated patients, and with EPA and DHA enrichment in placebo-treated patients. A post-hoc analysis including total patient sample, regardless of treatment received, showed a significant correlation between DHA enrichment and improved lobular inflammation. These findings suggest that increase of n-3 PUFAs, mainly DHA, may have a positive impact on liver histology. However, we were unable to confirm this possibility directly.

NAS failed to indicate direct changes in liver histopathology after 6 months of n-3 PUFA consumption. Recently, two new studies also failed to report benefits in supplementing n-3 PUFAs from fish oil in NASH patients, and one even reported hepatic steatosis and activity score improvement in placebo-treated patients [28,29]. The main limitation of the last one is a lack of biomarkers for n-3 PUFAs intake.

In contrast, a 2012 meta-analysis conducted by Parker et al. concluded that n-3 PUFA may be effective in reducing liver fat in NASH [30]. Most of the studies included in that meta-analysis assessed liver steatosis by an examiner-dependent method (ultrasonography). Only one study assessed liver biopsies before and after treatment with n-3 PUFAs in NASH, reporting improvements in liver steatosis and fibrosis, hepatocyte ballooning, and lobular inflammation after 12 months of treatment with 2.7 g/day of purified EPA [15]. Differences between that study and ours include a higher dose of EPA for a longer duration, different source of n-3 PUFAs, non-randomized design, no control of n-3 PUFAs intake and inclusion of only seven patients [15].

As the rate of NASH evolution may vary, it is possible that 6 months of n-3 PUFA supplementation may be insufficient time to observe histopathologic benefits. However, after supplementing NASH patients with n-3 PUFAs for an identical period, Sapadaro et al. observed complete fatty liver regression in 33.4% of patients and an overall liver steatosis reduction in 50% of patients, as assessed by abdominal ultrasound [16]. These authors provided double the dose of n-3 PUFAs than that used in the present study.

We did not find statistical significance for several biochemical markers of liver function and glucose metabolism that were previously reported by others after n-3 PUFA supplementation in NASH. In a pilot open study in humans, patients with NAFLD receiving EPA and DHA supplementation exhibited reduced plasma AST, ALT, GGT, triglyceride, and fasting glucose levels, increased plasma PUFA levels, lower plasma n-6:n-3 PUFA ratios, and improvements in liver ultrasound echotexture compared to controls [14]. Another randomized clinical trial in NASH patients found a

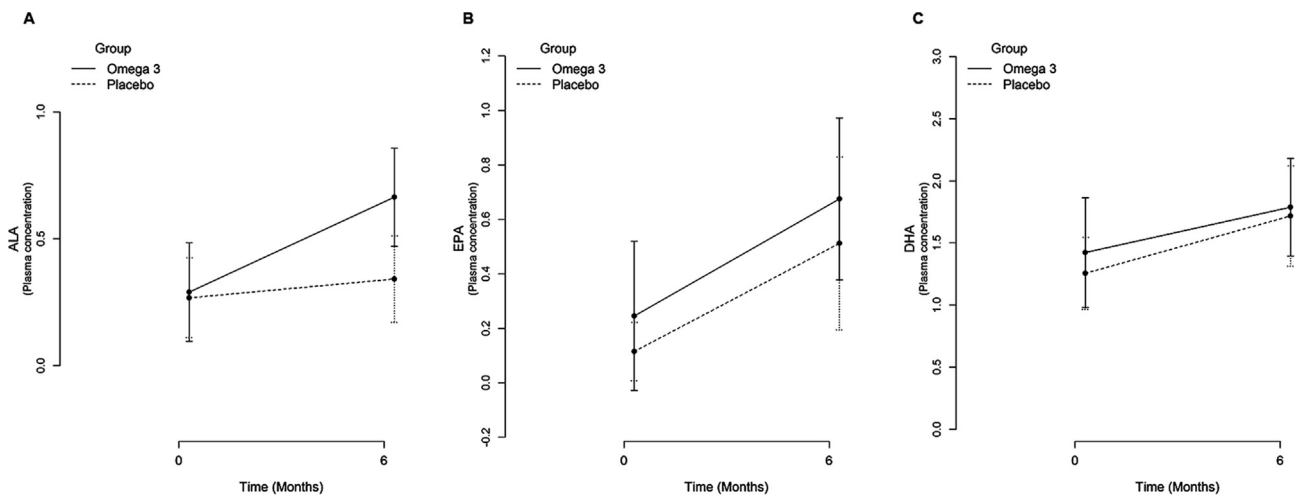

Fig. 3. Correlation between the 6 months' increase of individual omega-3 fatty acids and nonalcoholic steatosis activity score (NAS) improvement in patients with nonalcoholic steatohepatitis A) Alpha-linolenic acid (omega-3, p = 0.02; placebo, p = 0.44); B) Eicosapentaenoic acid (omega-3, p = 0.07; placebo, p = 0.04); and C) Docosahexaenoic acid (omega-3, p = 0.22; Placebo, p = 0.05).

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Table 4
Changes in anthropometric and biochemical outcomes of non-alcoholic steatohepatitis patients after 3 and 6 months of oral intake of omega-3 fatty acids or placebo.

Variable	Patient improvement or stability after 3 months % (95% CI)			Patient improvement or stability after 6 months % (95% CI)		
	Placebo group	n-3 group	*P*	Placebo group	n-3 group	*P*
Anthropometric						
Body mass index	47.8 (29.3–67.0)	57.7 (38.9–74.4)	0.34	47.8 (29.3–67.0)	57.7 (38.9–74.4)	0.34
Weight	47.8 (29.3–67.0)	57.7 (38.9–74.4)	0.34	47.8 (29.3–67.0)	57.7 (38.9–74.4)	0.34
Waist circumference	72.7 (51.49–87.0)	70.6 (46.5–86.8)	0.69	45.0 (25.9–65.8)	64.7 (41.1–82.7)	0.19
Biochemical						
Fasting glucose	63.6 (42.8–80.2)	40.7 (24.6–59.3)	0.97	52.2 (33.0–70.7)	30.8 (16.5–50.2)	0.96
Glycated haemoglobin	65.2 (44.7–81.2)	57.7 (38.9–74.4)	0.80	77.3 (56.0–90.1)	64.0 (44.4–79.7)	0.91
Insulin	39.1 (22.2–59.3)	57.7 (38.9–74.4)	0.16	47.8 (29.3–67.0)	38.5 (22.5–57.5)	0.83
Triglycerides	30.4 (15.5–51.1)	66.7 (47.7–81.4)	**0.01**	30.4 (15.5–51.1)	51.8 (34.0–69.2)	0.11
Total cholesterol	43.5 (25.7–63.2)	40.7 (24.6–59.3)	0.68	39.1 (22.2–59.3)	38.5 (22.5–57.5)	0.63
HDL	60.9 (40.7–77.8)	70.4 (51.3–84.0)	0.34	60.9 (40.7–77.8)	50.0 (32.1–67.9)	0.85
LDL	47.8 (29.3–67.0)	40.7 (24.6–59.3)	0.78	34.8 (18.8–55.2)	33.3 (18.6–52.3)	0.66
C-reactive protein	52.6 (31.8–72.6)	62.5 (42.6–78.8)	0.37	40.0 (21.9–61.4)	52.2 (33.0–70.7)	0.31
IL-6	47.1 (26.2–69.0)	57.9 (36.3–76.8)	0.38	41.2 (21.7–64.0)	52.6 (31.8–72.6)	0.36
Serum amyloid A	41.7 (19.4–68.1)	46.1 (23.3–70.8)	0.57	22.2 (5.6–55.9)	50.0 (25.5–74.5)	0.20
Alkaline phosphatase	69.6 (48.9–84.4)	59.3 (40.7–75.4)	0.85	47.8 (29.3–67.0)	61.5 (42.5–77.5)	0.25
GGT	47.8 (29.3–67.0)	50.0 (32.1–67.9)	0.55	47.8 (29.3–67.0)	38.5 (22.5–57.5)	0.83
AST	78.3 (57.5–90.6)	40.7 (24.6–59.3)	1.00	60.9 (40.7–77.8)	26.9 (13.6–46.4)	1.00
ALT	73.9 (53.1–87.6)	40.7 (24.6–59.3)	1.00	60.9 (40.7–77.8)	34.6 (19.4–53.9)	0.98
Total bilirubin	69.6 (48.9–84.4)	48.1 (30.8–66.0)	0.97	73.6 (53.1–87.6)	42.3 (25.6–61.1)	0.99
Direct bilirubin	69.6 (48.89–84.4)	59.3 (40.7–75.4)	0.85	82.6 (62.1–93.5)	46.1 (28.8–64.5)	1.00

HDL: high-density lipoprotein, LDL: low-density lipoprotein, CRP: C-reactive protein, IL-6: interleukin-6, GGT: gamma-glutamyl transferase, AST: aspartate aminotransferase, ALT: alanine aminotransferase.

decrease in serum ALT, triglycerides, tumour necrosis factor-alpha (TNF-α), and HOMA-IR after n-3 PUFA supplementation (2 g/day) [16]. In their meta-analysis, Parker et al. found benefits in AST levels when using n-3 PUFAs in NASH, but the significance of these benefits disappeared when only randomized trials were considered [30].

Although n-3 PUFA supplementation failed to alter AST and ALT levels in the present study, the placebo group showed improved AST and ALT levels. A specific explanation for these changes is unavailable, because the placebo component (mineral oil) is not absorbed in the bowel to elicit systemic effects and did not have any local side-effects at studied dose. It is possible that adult patients with NASH have heterogeneous levels of these enzymes or that elevated concentrations of EPA and DHA observed in placebo-treated patients may have affected AST and ALT levels. AST levels were higher in the placebo than in the n-3 group at early baseline; therefore, changes in AST may have occurred to the same extent in both groups.

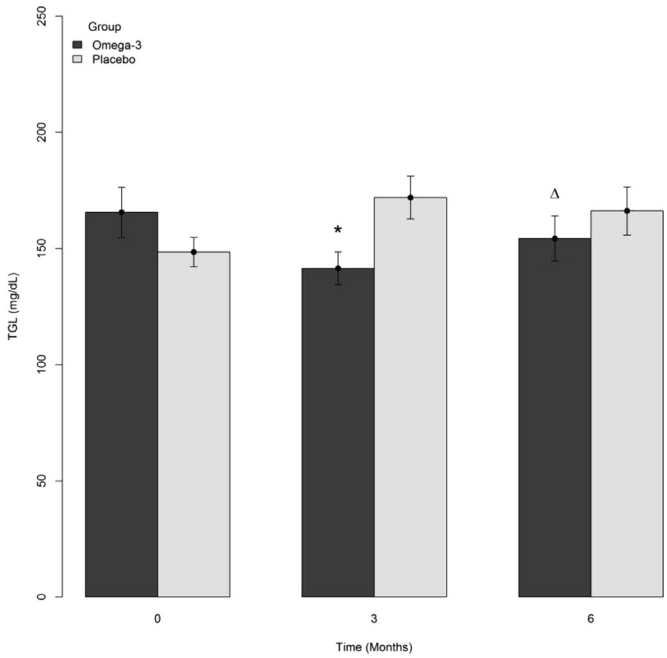

Fig. 4. Changes in serum triglycerides of non-alcoholic steatohepatitis patients at baseline, 3 and 6 after months oral intake of omega-3 fatty acids or placebo. * $p = 0.01$ *vs* baseline, $\Delta p > 0.05$ *vs* baseline.

Other mechanisms in the pathogenesis of NASH include the role of adipose tissue in the secretion of proinflammatory cytokines IL-6 and TNF-α [6]. In the present study, no changes in the plasma levels of IL-6 were found. Likewise, in a study with MS patients, 8 weeks of supplementation with flaxseed or fish oils alone did not change IL-6 levels, independent of the dose or type of n-3 PUFAs supplied [31].

In the present study, a decrease of serum triglycerides was found after 3 months of n-3 PUFA treatment, consistent with the literature [14,16]. NAFLD patients present increased risks of atherosclerosis and cardiovascular disease compared to the general population [32]. Thus, although the effect may be transient, the decreased triglyceride levels with n-3 PUFA treatment may be relevant to improve clinical outcome of NASH patients, most of whom also have MS.

This study included only young adult subjects and involved only one specific mixture, amount, and supplementation time of n-3 PUFAs. Similar results may not be achieved in other age groups or using a different protocol for n-3 PUFAs supplementation. A variant protocol may be the main reason for this study's failure to achieve some of the reported benefits of n-3 PUFA supplementation in NASH patients. Indeed, the lack of study protocol standardization hinders the assessment of the real impact of n-3 PUFAs in the treatment of NASH. For example, Parker et al. evaluated clinical studies that varied greatly in the amount (0.83–13.7 g) and duration (2–12 months) of n-3 PUFA supplementation [30]. We did not perform intent-to-treat analysis, and our placebo group may have ingested an off-protocol source of n-3 PUFAs, preventing the direct determination of possible benefits of n-3 PUFAs on liver histology.

In conclusion, supplementation with n-3 PUFAs from a flaxseed/fish oil mixture significantly impacts the lipid profile of NASH

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patients by increasing plasma n-3 PUFA levels, decreasing levels of the potentially proinflammatory n-6 AA, and decreasing serum triglyceride levels. Plasma increase of these n-3 PUFAs, mainly DHA, was associated with better liver histology.

Statement of authorship

MAN participated in the enrolment, assignment, and clinical evaluation of patients; database management and analysis; and manuscript generation. CPMSO participated in study design and coordination; the enrolment, assignment, and clinical evaluation of patients; data interpretation; and manuscript generation. VAFA and BC performed histological analysis and critically reviewed data for interpretation. JTS participated in study coordination and database management, analysis, and interpretation. LSR participated in the clinical evaluation of patients and anthropometric data collection and performed assays for measuring plasma FAs, data analysis, and critical interpretation. RST participated in the design of randomized and double-blind procedures, in general data interpretation, and in manuscript generation. HB performed the analysis of IL-6 and critically reviewed data for interpretation. FJC participated in study design and coordination and in data interpretation. DLW conceived the study and participated in study design and coordination, database management and interpretation, and manuscript generation. All authors critically reviewed the manuscript for important intellectual content and approved the final version to be published.

Conflicts of interest

The authors have no conflicts of interest to declare.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.clnu.2015.05.001.

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