Effect of Omega-3 Polyunsaturated Fatty Acids on Lipid Metabolism in Patients With Metabolic Syndrome and NAFLD

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Nonalcoholic fatty liver disease (NAFLD) is the most common chronic liver disease. n-3 polyunsaturated fatty acids (n-3-PUFAs) have been reported to ameliorate the progression of NAFLD in experimental studies; however, clinical trials have yielded contradictory results. The aim of our study was to assess the effects of n-3-PUFA administration on lipid metabolism and the progression of NAFLD in patients with metabolic syndrome. Sixty patients with metabolic syndrome and NAFLD were randomized in a double-blind placebo-controlled trial (3.6 g/day n-3-PUFA vs. placebo). During the 1-year follow-up, the patients underwent periodic clinical and laboratory examinations, liver stiffness measurements, magnetic resonance spectroscopy of the liver, and plasma lipidomic analyses. After 12 months of n-3-PUFA administration, a significant decrease in serum GGT activity was recorded compared with the placebo group (2.03 ± 2.8 vs. 1.43 ± 1.6; P < 0.05). Although no significant changes in anthropometric parameters were recorded, a significant correlation between the reduction of liver fat after 12 months of treatment—and weight reduction—was observed; furthermore, this effect was clearly potentiated by n-3-PUFA treatment (P < 0.005). In addition, n-3-PUFA treatment resulted in substantial changes in the plasma lipidome, with n-3-PUFA-enriched triacylglycerols and phospholipids being the most expressed lipid signatures. Conclusion: Twelve months of n-3-PUFA treatment of patients with NAFLD was associated with a significant decrease in GGT activity, the liver fat reduction in those who reduced their weight, and beneficial changes in the plasma lipid profile. (Hepatology Communications 2022;6:1336-1349).

Nonalcoholic fatty liver disease (NAFLD) includes a spectrum of potentially progressive liver diseases that consist of simple steatosis, nonalcoholic steatohepatitis (NASH), variable degrees of fibrosis, and ultimately cirrhosis. NAFLD is strongly associated with type 2 diabetes (T2DM) and abdominal obesity, and is recognized as a hepatic manifestation of metabolic syndrome.1 NAFLD has emerged as a leading cause of chronic liver diseases worldwide, as its incidence in the adult population (17%-46%) parallels the epidemics of obesity and T2DM2 and is becoming one of the most frequent causes of cirrhosis and liver transplantation worldwide.3,4 Moreover, its prevalence is expected to increase in the future as a consequence of the increasing adoption of unhealthy dietary habits and sedentary lifestyles.5 The presence
of NAFLD is associated with increased risks of both cardiovascular disease and cancer, regardless of the type of liver lesion.\(^{(6,7)}\)

In addition to a hypercaloric, Western-type diet representative of the most important pathogenic factor, several genetic modifiers of NAFLD development have also been identified. Among others, a strong genetic association has been reported for \(TM6SF2\) (transmembrane 6 superfamily member 2), \(MBOAT7\) (membrane-bound O-acyltransferase domain-containing 7), and especially the \(PNPLA3\) (patatin-like phospholipase domain containing 3) gene, hydrolyzing triacylglycerols (TG) in hepatocytes, and retinyl esters in hepatic stellate cells.\(^{(8)}\) \(PNPLA3\) polymorphism (rs738409[G], I148M) was identified as a modifier of NAFLD development and severity.\(^{(9,10)}\) Furthermore, based on lipidomic studies, \(PNPLA3\) genetic variants in patients with NAFLD appear to be associated with specific plasma lipid signatures,\(^{(11)}\) and even with different responses to n-3 polyunsaturated fatty acids (n-3-PUFA) treatments,\(^{(12)}\) indicating their role in lipid homeostasis.

In spite of the high frequency of NAFLD in the population, and its consequences for overall mortality, the treatment of NAFLD is subject to many controversies. The first-line treatment for NAFLD is making lifestyle changes, including modification of dietary habits and increased physical activity aimed at reducing body weight. However, the percentage of patients willing/able to adopt these long-term lifestyle modifications is low, underlining the importance of a pharmacologic therapy for NAFLD. Despite intensive efforts and an increasing number of clinical trials, no pharmacological treatment for NAFLD has yet been approved. The only available pharmacotherapy is based on vitamin E and pioglitazone, whose effects were described on a relatively small cohort\(^{(13)}\) and have not yet generally been accepted.\(^{(14)}\)

In the last few years, a growing interest has been directed toward the potentially favorable effects of n-3-PUFAs on NAFLD. As reported in recent systematic review papers (covering four meta-analytical studies), n-3-PUFA supplementation generally results in improvements in the biochemical aspects of NAFLD, as well as amelioration of liver steatosis\(^{(15-18)}\), however, additional better designed/more robust randomized studies are certainly needed to confirm these effects. In addition, there is a lack of metabolomic data describing the effects of n-3-PUFA administration in patients with NAFLD, although detailed metabolomic analysis provides a powerful tool for improvements in diagnosis as well as our understanding of the mechanisms of this complex disease.\(^{(19)}\) Indeed, based on this metabolomic approach, patients with NAFLD patients can be differentiated into several subgroups with different prognoses and specific, targeted therapies.\(^{(20)}\)

Thus, the aim of our study was to assess the effects of a 12-month n-3-PUFA treatment on the biochemical parameters and plasma as well as on liver...
metabolism in Czech patients with NAFLD and metabolic syndrome. The primary endpoint was to test whether administration of n-3-PUFAs over a period of 12 months can decrease the liver fat content. Secondary endpoints were to test whether administration of n-3-PUFAs can improve the laboratory markers of NAFLD, affect the plasma lipidomic profile, and to assess the progression of liver fibrosis, frequency of major genetic modifiers (PNPLA3, TM6SF2, and MBOAT7), as well as their impact on the progression or regression of NAFLD.

Materials and Methods

STUDY DESIGN AND INCLUSION/EXCLUSION CRITERIA

The study was based on a single-center, double-blind, randomized trial of patients with NAFLD and metabolic syndrome, who were treated for 12 months either with n-3-PUFAs or a placebo (registered at www.ClinicalTrials.gov under No. NCT02647294). The patients were recruited from the outpatient clinics of the Fourth Department of Internal Medicine (Hepatology/Gastroenterology, Diabetic, and Metabolic Disease Centers), General University Hospital, First Faculty of Medicine of Charles University, and they consisted of 60 consecutive patients diagnosed with NAFLD and metabolic syndrome who were referred to the Hepatology unit in the period February 2016 to February 2017 (see flow diagram in the Supporting Information). A diagnosis of NAFLD was based on clinical and laboratory parameters according to American Association for the Study of Liver Diseases practice guidelines, including (1) evidence of hepatic steatosis, either by imaging or histology, and (2) lack of secondary causes of hepatic fat accumulation. Other etiologies of liver disease such as viral hepatitis, drug-induced liver disease, autoimmune liver disease, biliary diseases, and inherited metabolic diseases were excluded by specific laboratory and radiologic examinations, as well as by the patients’ medical histories. Significant alcohol abuse (140 g/week in women and 210 g/week in men) was ruled out via the patients’ personal history, a short questionnaire, stable gamma-glutamyltransferase (GGT) activities documented in the patient’s documentation, and determination of either serum carbohydrate-deficient transferrin and/or urine ethyl-glucuronide levels, if needed. The presence of any malignancy was another exclusionary criterion. Metabolic syndrome was diagnosed on the basis of the diagnostic criteria stated in the International Societies Consensus of 2009. The healthy controls (n = 168) for the PNPLA3 gene polymorphism were recruited from consecutive healthy blood donors of the General University Hospital, First Faculty of Medicine of Charles University. Inclusion criteria for the healthy subject group were the absence of any apparent liver disease (as also evidenced by physiological values of standard liver enzyme activity), diabetes mellitus, or metabolic syndrome.

The study was carried out in full accordance with the Helsinki Declaration of 1975, as revised in 1983, and was approved by the Institutional Ethics Committee. Informed consent was obtained from all subjects before entering the study.

N-3-PUFA ADMINISTRATION

The patients with metabolic syndrome and NAFLD were randomly allocated for 12 months treatment with either n-3-PUFAs (3.6 g/day; n = 30) or the placebo (n = 30). The study’s medication was purchased from SVUS Pharma (Hradec Kralove, Czech Republic). One tablet with the active substance contained 450 mg of n-3-PUFAs (the main components: eicosapentaenoic acid [EPA] 215 mg [48%] and docosahexaenoic acid [DHA] 155 mg [34%]), and the dosage was 2 tablets four times a day. Capsules containing 450 mg of soya oil and having the same appearance as the capsules containing the active substance were used as the placebo. All of the patients, physicians, nurses, and the technicians performing the imaging techniques (ultrasonography, proton magnetic resonance spectroscopy [1H MRS]) were blind to the treatment assignment throughout the study. Adverse events were recorded on all follow-up visits.

CLINICAL, ANTHROPOMETRIC, AND BIOCHEMICAL MEASUREMENTS

Clinical, anthropometric, and laboratory parameters were recorded at the baseline, at the end of the study, and at 3, 6, and 9 months during the study. Biochemical analyses were performed on an automatic analyzer (Modular Analyzer; Roche
Diagnostics GmbH, Mannheim, Germany) using standard laboratory assays. The Fatty Liver Index was calculated as described in the literature, along with the AST-to-platelet ratio index (APRI), Fibrosis-4 index (FIB-4) score (based on age, AST/ALT activities, and platelet count), and NAFLD fibrosis score.

ULTRASONOGRAPHY, MAGNETIC RESONANCE IMAGING, AND SPECTROSCOPY

Ultrasonography with liver stiffness measurement (acoustic radiation force impulse; Siemens, Erlangen, Germany) was performed at the baseline, at month 6, and at the end of the study. The $^1$H MRS for visceral and liver fat determination was performed at the baseline and at the end of the study. All $^1$H MRS examinations were performed on a 3T MR scanner (Magnetom Trio; Siemens) as described previously.

PLASMA LIPIDOMICS USING ULTRAHIGH PERFORMANCE LIQUID CHROMATOGRAPHY–HIGH-RESOLUTION MASS SPECTROMETRY

Samples of plasma for lipidomic analysis were prepared by plasma protein precipitation using 2-propanol with two internal standards (phosphatidylcholine 14:0/14:0, $c = 6.67 \mu$g/mL; and fatty acid [FA] 19:0, $c = 13.3 \mu$g/mL) and butylated hydroxytoluene. After centrifugation of the precipitated samples, 150 µL of the supernatant was collected and stored in a 1.5-mL vial for analysis. A quality control sample was prepared by transferring 25 µL of supernatant from every sample.

For the lipidomic analysis, ultrahigh-performance liquid chromatography (UHPLC; Infinity 1290; Agilent, Santa Clara, CA) coupled to a high-resolution mass spectrometer (HRMS) with a hyphenated quadrupole time-of-flight mass analyzer (6560 Ion Mobility Q-TOF LC/MS; Agilent) with the Agilent Jet Stream electrospray (ESI) source were used.

An Acquity BEH C18 column (1.7 µm, 2.1 x 150 mm; Waters, Milford, MA) was used for chromatographic separation. The chromatographic system used with ESI detection was (A) 10 mM ammonium formate and 0.1% formic acid in acetonitrile:water (60:40, vol/vol); and (B) 10 mM ammonium formate and 0.1% formic acid in 2-propanol:acetonitrile (90:10, vol/vol). For chromatographic separation of plasma detected in ESI$^-$ mode, the following mobile phases were used: (A) 10 mM ammonium acetate and 0.1% acetic acid in acetonitrile:water (60:40, vol/vol); and (B) 10 mM ammonium acetate and 0.1% acetic acid in 2-propanol:acetonitrile (90:10, vol/vol). The run time of the method was 18 minutes, and the elution gradient was as follows: start at 40% of mobile phase B; after 2 minutes with initial conditions a linear change to 50% of B in 2 minutes, followed by a linear change to 60% of B in 1 minute and slow linear change to 100% B in 7 minutes. The column was then washed with 100% B for 3 minutes and reconditioned for 3 minutes. The flow rate was constant at 0.300 mL ∙ min$^{-1}$. The column temperature was maintained at 60°C, and the injection volume was increased to 1 µL. The autosampler was kept at 5°C.

GENOTYPING

Genomic DNA was isolated from peripheral blood white cells by the standard salting-out procedure. The specific variants of the PNPLA3 (rs738409 and rs738408), TM6SF2 (rs58542926), and MBOAT7 (rs641738) genes were typed by polymerase chain reaction–restriction fragment length polymorphism (see Supporting Information for details).

DATA PROCESSING AND STATISTICAL ANALYSIS

For randomization, participants were randomized by simple randomization 1:1 to n-3-PUFAs or placebo (Research Randomizer, version 4.0). The randomization, enrollment, and assignment of participants to study group was performed by physician and study nurse not involved in the evaluation of study results. For study sample-size calculation, we estimated that at least 20% more patients in the n-3-PUFA group compared with placebo group will achieve reduction in liver fat.

The results, other than from lipidomic analyses, are presented as mean values with SD. Either two-sample paired $t$-test or the Mann-Whitney rank test for non-Gaussian distributed variables was used to estimate intergroup differences. The correlations between different parameters were evaluated by calculation.
of Pearson or Spearman correlation coefficients and linear regression analyses. All tests were two-sided, with $P < 0.05$ considered as statistically significant. The statistical analyses were performed using BDMP Statistical Software version PC90 (Cork Technology Park, Ireland) and Statistica CZ version 12 (StatSoft, Tulsa, OK).

The plasma lipidomic data were processed with the LipidMatch suite, which uses MZmine 2 for feature extraction and an R script for lipid identification based on MS/MS in silico libraries. Statistical analysis of the lipidomics data was performed in both web-based and R-based MetaboAnalyst. Before building statistical models, sum normalization, logarithmic transformation, and Pareto scaling was applied to ensure normal distribution and higher significance of low abundance compounds. First, Principal Component Analysis (PCA) was used to overview the data. As the next step, tools of univariate and multivariate statistics were applied to find and describe variables important for changes in lipidome. A Student’s $t$ test was used to filter out all insignificant features (false discovery rate [FDR] $P$ value $< 0.01$), to reduce the feature-to-sample ratio before multivariate analysis. A selected subset of lipids was loaded into SIMCA software (Sartorius) to build an orthogonal partial least-squares discriminant analysis (OPLS-DA) model. A variable importance plot (VIP) score higher than 1 was required to include the feature in the final markers list. The value of the area under the curve from the receiver operating characteristic curve was calculated for each variable on the final list to assess its classification strength.

## Results

### Baseline Characteristics

The basic characteristics of the 60 randomized patients are given in Table 1. All patients enrolled in the study had metabolic syndrome and NAFLD, with 5 patients already suffering from liver cirrhosis (diagnosis was made using liver elastography; in addition, a liver biopsy was performed in 4 of 5 patients in the past). The patients with cirrhosis had a functional stage Child-Pugh A without a history of cirrhosis decompensation. At the start of the study, the patient groups (n-3-PUFA vs. placebo) did not differ in any of the observed basic clinical parameters (Table 1).

### Effect of n-3-PUFA Administration on Anthropometric and Biochemical Parameters

After the 1-year follow-up, no significant changes in anthropometric data (weight, waist circumference, or body mass index) were observed in the patients enrolled in the study (Table 1). The year-long n-3-PUFA treatment resulted in a significant decrease in GGT activity in the n-3-PUFA group ($2.27 \pm 2.5$ vs. $1.43 \pm 1.6$ µkat/L; $P < 0.05$), with no change in the placebo group ($2.11 \pm 3.1$ vs. $2.03 \pm 2.8$ µkat/L; $P < 0.05$) (Table 1).

All other biochemical markers observed remained unchanged in both groups. During the follow-up, liver elastography parameters did not change in either group (Table 1).

Similarly, no effect on selected noninvasive parameters of NASH and liver fibrosis (APRI score, FIB-4 score, Fatty Liver Index, and NAFLD fibrosis score) were observed after a 1-year treatment with n-3-PUFAs (Table 1).

### Effect of n-3-PUFA Administration on Liver and Abdominal Fat Content

The $^1$H MRS data were obtained in 59 patients at the beginning of the study (1 patient was unable to undergo the examination because of claustrophobia) and in 51 patients at the end of the study (lost from follow-up and/or refusal of regular controls). After 12 months of n-3-PUFA administration, no significant changes were observed in any of the $^1$H MRS-analyzed parameters, although a nonsignificant trend in the reduction of liver fat content after n-3-PUFA supplementation was observed (Table 1). Reduction of liver fat for more than 10% was observed in 15 of 27 (56%) patients in the n-3-PUFA group and in 8 of 24 (33%) patients in the placebo group.

Despite the fact that the patients’ weights from the start to the end of the study did not change in the whole group ($96.9 \pm 15.3$ kg vs. $96.8 \pm 15.3$ kg; $P > 0.05$), a nonsignificant trend in weight reduction in the n-3-PUFA treatment group was observed at the end of the study ($96.2 \pm 16.7$ kg vs. $94.7 \pm 15.2$ kg; Table 1). When comparing the reduction of liver fat (assessed by $^1$H MRS) to the weight reduction,
A strong correlation in the patient group as a whole was found (Spearman correlation, $P < 0.001$, $r = 0.5228$). Surprisingly, the reduction of liver fat strongly correlated with the weight reduction exclusively in the n-3-PUFA treatment group ($P = 0.002$, $r = 0.5943$). In the placebo group, a correlation between the weight loss and decrease in liver fat content was also observed, but missed the significance ($P = 0.054$, $r = 0.416$; Fig. 1).

**DETAILED LIPIDOMIC ANALYSIS USING UHPLC-HRMS/MS**

Based on the UHPLC-HRMS/MS analysis, 312 confirmed lipid signatures were identified in the plasma of our patients with NAFLD. PCA revealed clustering of samples into two groups based on the type and time of treatment (Fig. 2). All samples from time 0 clustered together with the placebo-treated patients, whereas the lipidome of PUFA-treated patients (other than time 0) changed (Supporting Fig. S1 shows 15 randomly selected patients with a marked shift of plasma lipids after 3 months of n-3-PUFA supplementation).

To determine those lipids that significantly contributed to lipidome changes after n-3-PUFA treatment, the samples were divided into two groups according to the PCA. The group with unaffected lipidome consisted of all samples from time 0 and placebo-treated samples (0, 3, 6, and 12 months). The second group consisted of patients with affected lipidome after 3-12 months of the n-3-PUFA treatment. Based on univariate ($t$-test FDR $P$ value $< 0.01$) and multivariate statistics (OPLS-DA VIP score $> 1$), 42 lipids that differed significantly between the two groups were identified.
filtered out. This approach revealed that 23 lipids increased and 19 decreased in the n-3-PUFA-treated group, compared to the group with unaffected lipidome (Table 2). All but one from the abundant lipids containing n-3-PUFAs (DHA, EPA)—including TG, phospholipids (phosphatidylcholines), and free fatty acids (FFAs)—were increased in the group with an affected lipidome. On the other hand, most of the decreased features in the treated group were lipids containing at least one n-6-PUFA.

Interestingly, a detailed lipidomic analysis had already provided clear evidence of enrichment of lipids

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**FIG. 1.** Correlation between weight change and liver fat content. Correlation between weight change and liver fat content change before and after treatment in the n-3-PUFA group (A) and in the placebo group (B). Liver fat content reduction was potentiated by n-3-PUFA treatment ($P = 0.0017$).
by n-3-PUFAs in the treated group after 3 months. The observed changes persisted for the remaining study period in all subjects (Fig. 3).

**N-3-PUFA SUPPLEMENTATION TOLERABILITY AND PATIENTS’ COMPLIANCE**

In total, 51 patients finished the whole study (27 patients in the n-3-PUFA treatment group and 24 patients in the placebo group). Among the 9 patients who did not complete the study, 3 patients finished the study prematurely due to their refusal of regular clinical controls; 2 patients refused 1H MRS examination; and 4 patients were lost in the follow-up. All patients randomized to the n-3-PUFA or placebo groups started the treatment.

No severe adverse events were observed either in the n-3-PUFA or placebo groups. Mild and transient adverse events were recorded in 6 patients in the n-3-PUFA treatment group, and in 5 patients in the placebo group; these included intermittent diarrhea, flatulence, and a feeling of fullness of the abdomen. No interruption of treatment due to these events was reported in any group, and no study discontinuation was related to the treatment (Table 3).

The lipidomic analysis revealed that in 6 patients in the n-3-PUFA group (14%), the plasma lipids were unaffected by n-3-PUFA treatment and could be viewed as noncompliant. In the placebo group, we expected no changes in the PUFA composition between the baseline and end of the study in all participants. Nevertheless, 1 patient in this group (4%) reached the threshold values for the n-3-PUFA group,
Table 2. Significantly Changed Lipidome Markers Between Placebo and N-3-PUFA-Treated Group

| Lipid Name                   | OPLS-DA VIP Score | ANOVA FDR P Value | ROC AUC Value |
|------------------------------|-------------------|-------------------|---------------|
| **Lipid markers increased in n-3-PUFA-treated group** |                   |                   |               |
| Plasmenyl-PEP(16:0/20:5)     | 2.463             | 4.30E-33          | 0.91          |
| Plasmenyl-PCP(16:0/20:5)     | 2.311             | 9.48E-34          | 0.91          |
| FA(20:5)                     | 2.262             | 5.22E-31          | 0.90          |
| Plasmenyl-PEP(18:1/20:5)     | 2.199             | 8.33E-32          | 0.90          |
| Plasmenyl-PEP(18:0/20:5)     | 2.138             | 5.29E-33          | 0.90          |
| LPC(20:5)                    | 2.046             | 7.49E-25          | 0.88          |
| DMPE(16:0/20:5)              | 1.890             | 1.12E-29          | 0.90          |
| PC(16:0/20:5)                | 1.851             | 4.90E-28          | 0.89          |
| LPE(20:5)                    | 1.799             | 9.58E-23          | 0.88          |
| PC(18:2/20:5)                | 1.789             | 7.86E-23          | 0.86          |
| PC(18:0/20:5)                | 1.679             | 1.69E-29          | 0.88          |
| TG(16:0/18:2/22:6)           | 1.650             | 7.51E-15          | 0.81          |
| TG(16:0/18:1/22:6)           | 1.536             | 1.89E-14          | 0.81          |
| FA(22:6)                     | 1.441             | 8.59E-18          | 0.82          |
| TG(18:1/18:1/22:6)           | 1.436             | 3.75E-14          | 0.80          |
| TG(18:1/20:4/20:4)           | 1.420             | 1.35E-12          | 0.79          |
| PC(18:0/22:6)                | 1.373             | 2.01E-26          | 0.88          |
| Plasmenyl-PCP(16:0/22:6)     | 1.353             | 9.42E-21          | 0.84          |
| TG(16:0/18:2/20:4)           | 1.224             | 3.61E-09          | 0.74          |
| PC(36:6)                     | 1.176             | 1.61E-16          | 0.82          |
| DMPE(16:0/22:6)              | 1.123             | 5.21E-24          | 0.86          |
| LPC(22:6)                    | 1.104             | 1.73E-13          | 0.79          |
| PC(16:0/22:6)                | 1.019             | 8.20E-25          | 0.86          |
| **Lipid markers decreased in n-3-PUFA-treated group** |                   |                   |               |
| Plasmenyl-PEP(16:0/22:4)     | 1.508             | 8.23E-17          | 0.85          |
| LPC(22:4)                    | 1.477             | 1.43E-16          | 0.84          |
| PC(18:0/22:4)                | 1.399             | 1.43E-16          | 0.85          |
| Plasmenyl-PEP(18:0/22:4)     | 1.351             | 3.89E-11          | 0.80          |
| PC(40:5)                     | 1.349             | 4.42E-16          | 0.87          |
| PC(35:4)                     | 1.338             | 7.11E-14          | 0.81          |
| PC(18:0/20:4)                | 1.314             | 2.53E-16          | 0.85          |
| PE(18:0/20:4)                | 1.241             | 5.32E-14          | 0.85          |
| LPE(20:4)                    | 1.234             | 1.59E-12          | 0.82          |
| PE(18:0/20:4)                | 1.207             | 2.21E-12          | 0.82          |
| PE(16:0/20:4)                | 1.120             | 2.83E-09          | 0.78          |
| Plasmenyl-PEP(18:0/20:3)     | 1.095             | 3.52E-10          | 0.80          |
| Plasmenyl-PCO(20:1/18:3)     | 1.071             | 1.13E-11          | 0.81          |
| PE(16:0/22:5)                | 1.051             | 2.42E-08          | 0.77          |
| PC(16:0/20:3)                | 1.035             | 6.67E-07          | 0.74          |
| LPC(20:3)                    | 1.029             | 3.07E-08          | 0.77          |
| PC(34:4)                     | 1.025             | 2.30E-08          | 0.78          |
| TG(18:1/18:1/18:2)           | 1.008             | 5.65E-07          | 0.77          |
| FA(22:4)                     | 1.004             | 2.56E-08          | 0.74          |

Note: The tools of univariate (t-test FDR P value < 0.01) and multivariate statistics (OPLS-DA) were applied to find and describe variables important to changes in lipidome. Based on these methods, 42 lipids differed significantly between both groups and were filtered out. The value of area under the curve from the receiver operating characteristic curve was calculated for each variable on the final list to assess its classification strength.

Abbreviations: ANOVA, analysis of variance; AUC, area under the curve; DMPE, dimethyl-phosphoethanolamines; FA, fatty acid; LPC, lysophosphatidylcholine; LPE, lysophosphatidylethanolamine; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PEP, phosphatidylethanolamine; and ROC, receiver operating characteristic curve.
suggesting n-3-PUFA supplementation during the study from external sources.

FREQUENCY AND ASSOCIATIONS OF CANDIDATE GENE VARIANTS

The PNPLA3 rs738409 genotype frequencies showed no significant differences between the n-3-PUFA group and the placebo group. No significant relationship between the PNPLA3 rs738409 variants or other candidate gene variants and anthropometric or laboratory parameters was found in the patients with NAFLD. In our control group (healthy volunteers; n = 168), the frequencies of wild-type homozygotes of PNPLA3 rs738409 and rs738408 were higher compared to patients with NAFLD (P < 0.05) (Table 4).

No statistical significance of the PNPLA3 rs738409 gene variant was found in relationship to the n-3-PUFA treatment response. No associations between

FIG. 3. The effect of n-3-PUFA administration on composition of plasma lipids. (A) The enrichment of plasma lipids by n-3-PUFA in the treated group was already proven after 3 months and remained stable until the end of the study, as described on the 40 most significant lipids by analysis of variance P value. (B) The observed changes persisted for the remainder of the study period (months 3, 6, 9, and 12) in all subjects (demonstrated on a selection of four lipids).
variants of other candidate genes (TM6SF and MBOAT7) and the treatment response were observed.

**Discussion**

Due to the rising incidence and prevalence of NAFLD, there is a desperate need for efficient preventive and therapeutic measures. However, apart from lifestyle and personal regime changes, there is no efficient therapy for this disease. Because diet and dietary lipids are crucial factors in the pathogenesis of NAFLD, numerous dietary approaches have been proposed and tested in patients with NAFLD, with n-3-PUFAs being promising preventive dietary substances. Moreover, it has been demonstrated that n-3-PUFAs are depleted in the liver tissue of patients with NAFLD. Despite these data, clinical observations are not entirely convincing, with only mild beneficial effects of improvements in liver-enzyme activities and/or hepatic fat content, as evidenced in recent systematic reviews.

In our randomized, placebo-controlled study with n-3-PUFA supplementation in patients with NAFLD and metabolic syndrome, three very important observations were recorded. First, a 1-year supplementation of n-3-PUFAs led to significant improvement of GGT activities compared with the placebo group. Second, supplementation with n-3-PUFAs potentiated the reduction of liver fat content in patients who reduced their weight. And finally, n-3-PUFA treatment led to a substantial enrichment of major lipid classes with EPA and DHA.

Unlike placebo, n-3-PUFA administration led to marked decrease of GGT activity. Elevated activity of GGT is a typical feature of patients with NAFLD and is also a strong predictor of cardiovascular morbidity, mortality, and diabetes, independent of alcohol intake. Therefore, the reduction of GGT activity may represent an important beneficiary factor for patients with NAFLD, despite the fact that neither of the other biochemical parameters nor the noninvasive liver fibrosis markers/liver elastography changed in the treated group. It is likely that the 12-month follow-up was too short to result in any improvement of liver fibrosis. Our results are thus in accordance with other studies demonstrating the beneficial effects of n-3-PUFA treatment on liver enzyme activities in both adult as well as pediatric patients with NAFLD. It is surprising that plasma triglycerides were not lowered, given the n-3-PUFA dose; only a mild decrease was observed.

The fact that no significant changes in the anthropometric measures were observed in the n-3-PUFA-treated patients was not entirely surprising, as the goal of our study was not to intervene in any lifestyle habits of the patients—only to add n-3-PUFAs as a dietary supplement. Most of the published reports have focused on simple n-3-PUFA supplementation...
without any scheduled physical activity and achieved comparable results. Previous clinical trials related to the effect of n-3-PUFAs on liver fat reduction have yielded contradictory results. The most discussed issues concern the dose (ranging from 0.8 to over 6 g/day) and the treatment period duration. Sanyal et al. reported no benefit of supplemental EPA in any blood or hepatic markers of NASH. Some studies point to the fact that DHA is superior to EPA in controlling steatosis, inflammation, and/or fibrosis, whereas others conclude that it is uncertain whether a longer duration, higher dose, or different composition of n-3-PUFA therapy leads to additional benefits. In our study, neither body weight nor the liver fat content changed significantly after the treatment period; the percent change in the body weight during the study correlated positively with the change in liver fat content. Surprisingly, the significant correlation between body-weight reduction and decrease in liver fat content was only observed in the n-3-PUFA treatment group, not in the placebo group. Hence, it appears that n-3-PUFA supplementation may increase the chance for liver-fat reduction during weight loss in patients with NAFLD. In fact, this feature of n-3-PUFA treatment in patients with NAFLD has been suggested in some previous studies, and points to the need for complex treatment of patients with NAFLD.

The primary endpoint of our study has not been met, as we did not demonstrate any significant change in the liver fat content—neither in the n-3-PUFA treatment group nor in the placebo group (only a nonsignificant decrease was observed in the n-3-PUFA group). Nevertheless, beneficial effects of a n-3-PUFA-enriched diet appears to be caused specifically by enrichment of the plasma (and likely also hepatic) lipids, where they exert their possible anti-inflammatory and/or antifibrotic effects reported in experimental studies.

Detailed lipidomic analysis revealed that n-3-PUFA administration significantly affected the plasma lipidomic profile of patients with NAFLD. In n-3-PUFA-treated patients, as many as 23 lipids were increased, and 21 of them contained one of the n-3-PUFAs. These n-3-PUFA-enriched lipids consisted of 3 TGs, 2 FFAs, and 16 phospholipids (Table 2). All of these lipid groups were enriched with DHA and EPA. This confirms the lipidomic data by Lamaziere et al. from a study on rats fed n-3-PUFAs, demonstrating that enrichment of total liver FAs and phospholipids (phosphatidylcholine, phosphatidylethanolamine, and lysophosphatidylcholine) with EPA and DHA decreased lipogenesis. Similar data on the enrichment of plasma FAs were reported in pediatric patients with NAFLD treated with n-3-PUFAs.

In contrast to the n-3-PUFA-treated group, a significant increase of diacylglycerols (DGs) (18:1/18:1) was detected in the placebo group. A high content of DG is a typical disturbance of lipid metabolism in patients with NAFLD (also being associated with insulin resistance); hence, the increase of DGs (18:1/18:1) could be attributed to the progression of NAFLD in untreated patients.

PNPLA3 gene variants, being the major genetic predictor of NAFLD progression, were reported to be associated with an increase in hepatic TGs containing PUFAs. In addition, the PNPLA3 genotype was also shown to affect erythrocyte DHA content in PUFA-treated patients with NAFLD. PNPLA3 gene variations were also linked to GGT heritability, underscoring the role of both variables in predicting the risk of NAFLD. We observed a trend toward higher fat liver content (evaluated by $^1$H MRS) related to the presence of the unfavorable PNPLA3 rs738409 variant, although the difference did not reach statistical significance (data not shown). Consistent with previous reports, the frequency rates of wild-type homozygotes of PNPLA3 rs738409 and rs738408 were higher in our control group, compared to patients with NAFLD. However, we must honestly admit that the sample size (of patients with NAFLD) is limited for the evaluation of gene variants.

Our results suggest that as many as 14% of patients in the n-3-PUFA treatment group had no change in their lipidomic profile, mimicking the placebo group, thus suggesting their nonadherence to the treatment. These suspected noncompliant patients did not have any influence on the study results, as was found in a post hoc analysis (data not shown), not excluding these suspected noncompliant patients from primary lipidomic analyses.

The treatment was well tolerated, as we did not observe any severe adverse events, and all reported adverse events were mild and transient. As many as 85% of the patients finished all study procedures; a 15% dropout rate is acceptable for a 12-month follow-up, 5 clinical controls, and two magnetic resonance procedures during the study. The limitation...
of our study was the treatment length. The duration of our study was 1 year, similar in length to many other studies. Nevertheless, from the viewpoint of the slow progression of NAFLD, it appears that the treatment period should be longer.\(^\text{(44)}\) On the other hand, a longer treatment period would certainly be compromised by a larger dropout of patients. Lack of paired liver biopsies is another limitation. Although liver biopsy is a “gold” standard for diagnosing NASH, it has many drawbacks: patients do not prefer invasive procedures, and for liver fat quantification, MRS is reported the most accurate method. Therefore, we did not find a suitable indication for liver biopsy. Last but not least is the evaluation of a diet rich in n-3-PUFAs. Informed consent included instructions on adherence to dietary habits identical to that before study entry, and the need to follow the same diet pattern during the whole study with avoiding diets rich in n-3-PUFAs.

Despite no marked effects on the anthropometric or most of the standard clinical and laboratory parameters, 12 months of n-3-PUFA treatment in patients with NAFLD was associated with a significant decrease in GGT activity and the liver fat reduction in those who reduced their weight. We also suggest that dietary n-3-PUFAs are associated with beneficial changes in the plasma lipid profile. Therefore, we conclude that a supplementation with n-3-PUFAs could represent a natural way to slow down NAFLD progression in patients with metabolic syndrome. Longer and more robust studies are needed to confirm the possible long-term effects of n-3-PUFA administration for slowing the progression of NAFLD.

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