Four-Week Omega-3 Supplementation in Carriers of the Prosteatotic PNPLA3 p.I148M Genetic Variant: An Open-Label Study

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### Keywords
Elastography · Free fatty acids · Hepatic steatosis · Ketones · Nonalcoholic fatty liver disease

### Abstract
**Background/Aims:** The PNPLA3 loss-of-function variant p. I148M is a strong genetic determinant of nonalcoholic fatty liver disease. The PNPLA3 protein functions as an intracellular lipase in the liver, with a greater activity on unsaturated fatty acids. This study aimed to determine whether short-term supplementation with omega-3 fatty acids impacts hepatic steatosis differently in PNPLA3 p.148I wild-type individuals as compared to homozygous carriers of the PNPLA3 p.148M variant.

**Methods:** Twenty subjects with hepatic steatosis (50% women, age 18–77 years) were included. Ten subjects homozygous for the PNPLA3 148M variant were matched to 10 wild-type individuals. The subjects received 4 g omega-3 fatty acids (1,840 mg eicosapentaenoic acid and 1,520 mg docosahexaenoic acid) a day for 4 weeks. Transient elastography with a controlled attenuation parameter (CAP) was used to quantify liver fat before and after the intervention. Body composition, fibrosis, liver function tests, serum free fatty acids (FFA) and glucose markers were compared.

**Results:** Patients homozygous for the PNPLA3 p.148M variant (risk group) demonstrated no significant changes in CAP compared to baseline (284 ± 55 vs. 287 ± 65 dB/m) as did the control group (256 ± 56 vs. 262 ± 55 dB/m). While serum liver enzyme activities remained unchanged in both groups, the risk group displayed significantly (\(p = 0.02\)) lower baseline FFA concentrations (334.5 [range 281.0–431.0] vs. 564.5 [range 509.0–682.0] μmol/L), which markedly increased by 9.1% after the intervention. In contrast, FFA concentrations decreased significantly (\(p = 0.01\)) by 28.3% in the wild-type group.

**Conclusions:** Short-term omega-3 fatty acid supplementation did not significantly alter hepatic steatosis. The nutrigenomic and metabolic effects of omega-3 fatty acids should be investigated further in carriers of the PNPLA3 148M risk variant.

### Introduction
PNPLA3, a member of the patatin-like phospholipase family, represents a clear independent risk for NAFLD [1] and is primarily expressed in the liver. PNPLA3 3 rs738409, encoding p.148M, significantly influences the hepatic fat content and liver injury. Carriers of PNPLA3 148M...
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(wher a C>G change leads to the substitution of isoleucine with methionine at codon 148) are more likely to present with increased liver stiffness and have a higher risk of developing advanced liver fibrosis and cirrhosis [2]. Indeed, homozygous carriers have shown a 3.3-fold increased risk of NAFLD as compared to controls without this variant [3] and ~50% of NAFLD patients are believed to carry at least one variant of the PNPLA3 gene [4]. A meta-analysis with 2,937 subjects reported a 73% higher liver fat content in carriers of the PNPLA3 148M variant than in individuals with the 148 I/I genotype [5].

PNPLA3 has triglyceride (TG) hydrolase activity with a higher affinity for unsaturated fatty acids (UFA) [6, 7]. In vitro studies have indicated that the 148M variant is a loss-of-function mutation with regard to TG hydrolysis [6]. Conversely, Kumari et al. [8] reported a gain of function with lipogenic activity. A Mediterranean diet, which is naturally rich in UFA, is increasingly advocated for patients with NAFLD [9] since guidelines continue to recommend weight loss through dietary changes and increased physical activity [10, 11]. Studies support a positive role for omega-3 polyunsaturated fatty acids (PUFA) in decreasing liver fat, as confirmed in a meta-analysis [12].

Nobili et al. [13], however, observed an interaction between dietary fatty acids dependent on the PNPLA3 148M homozygosity of the subjects. Specifically, the omega-3 fatty acid docosahexaenoic acid (DHA) was associated with a greater degree of hepatic steatosis in homozygous carriers of the PNPLA3 148M risk allele compared to carriers of the PNPLA3 wild-type genotype. A similar finding was demonstrated in adults after long-term supplementation with omega-3 fatty acids [14].

This study investigates the impact of short-term omega-3 fatty acid supplementation on hepatic steatosis as quantified by transient elastography with a controlled attenuation parameter (CAP) in subjects who are homozygous for the PNPLA3 148M minor (risk) allele matched with subjects homozygous for the major allele. We hypothesized that a deterioration in CAP would be observed in the risk subjects.

Materials and Methods

Patients

This open-label intervention study recruited 20 subjects between May 2015 and May 2016 from the Department of Internal Medicine II at the Saarland University Medical Center. The recruitment method included both self-referrals and referrals from medical health professionals. Adults aged >18 years were included if they had an NAFLD diagnosis, were able to provide informed consent, and had valid transient elastography measurements. Subjects were excluded for the following reasons: known infection with hepatitis B or C virus or HIV; diabetes type 2 (HbA1c ≥7.5%); alcohol consumption >21 and >14 drinks/week in men and women, respectively [11], or diagnosis of alcoholic fatty liver disease; presence of liver cirrhosis based on liver stiffness measurements (LSM; transient elastography ≥13.0 kPa); prescribed medications known to influence hepatic fat (e.g., statins); and being a vegetarian or a vegan.

This study was conducted in accordance with the Declaration of Helsinki and approved by the local research ethics committee (reference No. 148/14), and it was prospectively registered at the DRKS (German Clinical Trials Registry, registration No. DRKS00007781). All of the participants provided written informed consent before participation.

Study Design

Ten subjects who were homozygous carriers of the PNPLA3 148M allele were matched with 10 homozygous carriers of the 148 L-allele. Individuals were also matched for sex, age (±5 years), CAP (±40 dB/m), and, if possible, BMI category (normal, overweight, or obese). Compliance was assessed through a pill count.

A 2-week run-in phase preceded the intervention to assess normal dietary and exercise habits. The participants were instructed to follow their regular dietary habits and to record all food and beverages consumed in a 5-day food diary. The participants were also asked to wear a pedometer to document the daily number of steps. During the intervention, the subjects received a daily dose of 4 g Omacor split into 2 doses (2 g in the morning and 2 g in the afternoon). One gram of Omacor contains 460 mg eicosapentae- noic acid (EPA) and 380 mg DHA as ethyl esters. In conjunction with the supplementation regimen, the participants were encouraged to choose predominantly food rich in polyunsaturated instead of saturated fats.

During the first week of supplementation, the subjects were once again instructed to keep a 5-day food diary and to document the daily number of steps with a pedometer. Patients were contacted by telephone once a week during the intervention to ensure that they were following the study guidelines and accurately documenting their food and exercise data in their diaries. Subjects were considered compliant when >80% of the assigned omega-3 supplement dose was taken, which is a threshold selected in previous studies [15, 16].

Biochemical Analyses

Fasting venous blood was drawn to measure the following parameters: alanine aminotransferase, aspartate aminotransferase, alkaline phosphatase, γ-glutamyl transpeptidase, bilirubin, and albumin. HbA1c (≥6.5%) and fasting plasma glucose concentrations (≥100 mg/dL) were used to evaluate the presence of diabetes type 2. Insulin resistance and pancreatic β-cell function were assessed based on an oral glucose tolerance test and via a routine blood analysis including fasting plasma glucose and fasting serum insulin levels, from which the homeostatic model assessment measuring the insulin resistance (HOMA-IR) index was calculated (fasting glucose [mg/dL] × fasting insulin [µU/mL]/405). Total serum (nonesterified) free fatty acids (FFA) were measured enzymatically (DiaSys Diagnostic Systems GmbH, Holzheim, Germany). Total ketones, as well as acetoacetate and β-hydroxybutyrate, were quantified individually using an in vitro assay with cyclic enzymatic reactions (Wako Diagnostics GmbH, Neuss, Germany).
**Noninvasive Quantification of Liver Steatosis and Fibrosis**

This study employed vibration-controlled transient elastography (VCTE) ( FibroScan®; Echosens, Paris, France) to measure hepatic steatosis using a controlled attenuation parameter (CAP) and liver fibrosis with LSM. The transient elastography method measures the shear wave velocity. Specifically, it generates a shear wave via the transducer's mechanical impulse, and the ultrasound signals sent by the instrument's probe monitor the dispersion of this shear wave. Higher CAP values reflect an increased hepatic fat content (expressed in dB/m) and range from 100 to 400 dB/m. LSM ranges from 2.5 to 75 kPa. All measurements were carried out in fasted patients (10–12 h). At least 10 valid measurements had to be obtained for a valid CAP. In order to include LSM results in the analysis, a success rate ≥60% based on at least 10 valid measurements and an IQR/median LSM ≤30% were required [17]. According to a recent study [18], the IQR/LSM ratio was not taken into account in patients with a median LSM <7.1 kPa.

**Body Composition**

Height was determined with the stadiometer (Seca 217; Seca GmbH, Hamburg, Germany). Body composition was assessed via bioelectrical impedance analysis using the medical body composition analyzer mBCA515 (Seca). This 8-electrode segmental multi-frequency bioelectrical impedance analyzer uses empirical linear regression models and includes impedances at multiple frequencies of 5 and 50 kHz to evaluate the following parameters: fat-free mass, total body water, intracellular water, and extracellular water [19].

**Dietary Intake and Physical Activity**

Subjects completed a 5-day food diary at run in (of which two days were weekend days) to determine their normal intake and then again at baseline (T0) and during the last week of the intervention (T1). The dietary intake was analyzed using the software EBISpro® (version 2009; Germany), which is based on the German Nutrition Society (DGE) food composition database. Physical activity during this study was assessed using pedometers to record the daily number of steps, and activity diaries.

**Genotyping of the PNPLA3 Risk Variant**

Genomic DNA was isolated from EDTA-anticoagulated blood samples using the membrane-based QIAamp DNA extraction protocol (Qiagen, Hilden, Germany). Genotyping of the PNPLA3 rs738409 polymorphism was performed using a PCR-based assay with 5′-nuclease and fluorescence detection (TaqMan®; Life Technologies, Darmstadt, Germany; rs738409: C_7241_10).

**Outcome Parameters and Statistical Analyses**

The primary outcomes were within- and between-group changes in CAP after 4 weeks of omega-3 fatty acid supplementation. Secondary outcomes included within- and between-group changes in body composition and biochemical parameters.

Statistical analyses were performed with SPSS 24.0 (IBM, Ehningen, Germany) and GraphPad Prism 7.0 (GraphPad Software, La Jolla, CA, USA). Similar intervention studies assessing the effects of dietary lipid modification on liver steatosis calculated that 10 patients per group were needed to detect a 30% difference in liver fat content with 80% power and 5% significance [20]. Our sample size was based on these numbers despite the different methods used to assess liver steatosis.

Data are presented as means ± SD or medians (IQR), depending on the data distribution. Within-group differences were assessed using a paired t test and the Wilcoxon signed rank test. Student’s t test and the Mann-Whitney U test evaluated between-group differences. Univariate regression analysis using CAP as the independent variable assessed for associations with baseline variables. A two-sided p ≤ 0.05 was considered statistically significant.

**Results**

**Patient Characteristics**

In total, 23 patients were screened for this intervention study. Two patients did not meet the inclusion criteria and 1 patient was removed from this study due to an adverse event (gout flare-up during the intervention). No other adverse events were reported during this study, though several patients complained of eructation. Thus, 20 of the 23 screened patients were included (50% women) in the analyses. From these, 10 patients were homozygous for the PNPLA3 148M risk (minor) allele and 10 were homozygous for the major allele, with an average age of 41.6 ± 16.4 years and 42.5 ± 17.5 years, respectively. One patient was underweight (BMI <18.5), 6 patients had a normal BMI (≥18.5 but < 25), 8 patients were overweight (BMI ≥25 but <30), and 5 patients were obese (BMI ≥30). Three patients were taking thyroid medication for hypothyroidism. Other medications included: proton pump inhibitors (n = 2), angiotensin receptor blockers (n = 2), β-blockers (n = 1), calcium antagonists (n = 1), and L-dopa (n = 1).

**Comparison of Baseline Parameters**

No significant differences in baseline characteristics between the 2 groups were detected (Table 1), apart from serum FFA concentrations. Specifically, the homozygous variant carriers (risk group) displayed significantly (p = 0.02) lower FFA concentrations than the matched controls, with an average difference of 40.7% (334.5 [280.8–554.5] vs. 564.5 [508.5–720.0] μmol/L).

**Effects of a Short-Term Omega-3 Intervention on Liver Fat**

All of the patients complied with this study and adherence to the intervention was 100%. No significant changes in CAP compared to baseline were observed for participants homozygous for the 148M risk allele (risk group: 284 ± 55 vs. 287 ± 65 dB/m; p > 0.05) or for the wild-type participants (control group: 256 ± 56 vs. 262 ± 55 dB/m; p > 0.05). LSM and serum surrogate markers for liver injury remained stable in both groups.
Effects of an Omega-3 Intervention on Body Composition and Metabolic Parameters

After the 4-week intervention, body weight, BMI, fat mass, and waist circumference did not differ significantly either within or between the 2 groups (Table 1). The risk group displayed a modest increase in fat-free mass (in kg) from baseline ($p = 0.001$); however, when comparing the percentage of fat-free mass at baseline (68.2%) to that at follow-up (68.7%), this was not significant ($p = 0.051$).

Blood pressure remained unchanged after the intervention. Despite not being significant, glucose homeostasis-related markers shifted in opposite directions in the 2 groups after the intervention. Specifically, markers such as nonfasted glucose and insulin concentrations tended to increase after the intervention in the risk group, whereas they decreased in the control group (Table 1). In addition, follow-up analysis revealed changes in FFA concentrations that occurred after the intervention, which differed based on genotype. As shown in Figure 1, FFA concentrations increased by 9.1% ($p = 0.7$) in the risk group, in contrast to patients without PNPLA3 mutation, in whom they decreased significantly ($p = 0.01$) by 28.3%. In contrast, ketone markers revealed nonsignificant reductions for total ketone concentrations, as well as individually for acetoacetate and for

| Table 1. Baseline and follow-up data for each PNPLA3 genotype group |
|---------------------------------------------------------------|
| **Baseline** MM ($n = 10$)                                    | **Follow-up** MM ($n = 10$)                      | **Baseline** II ($n = 10$)                        | **Follow-up** II ($n = 10$)                      |
|---------------------------------------------------------------|
| **Transient elastography**                                    |                                              |                                              |                                              |
| CAP, dB/m                                                     | 287±65                                       | 284±55                                       | 262±55                                       | 256±56                                       |
| LSM, kPa                                                       | 6.3 (5.1–6.4)                                | 5.2 (4.0–7.3)                                | 5.4 (4.3–6.3)                                | 5.1 (4.0–6.1)                                |
| **Body composition**                                          |                                              |                                              |                                              |
| Weight, kg                                                    | 80.0±16.8                                    | 80.5±16.7                                    | 81.4±15.6                                    | 81.9±15.4                                    |
| BMI                                                           | 26.7±4.1                                     | 26.9±4.1                                     | 27.7±5.6                                     | 27.9±5.8                                     |
| FM, kg                                                        | 25.0±8.6                                     | 24.8±8.8                                     | 27.8±12.2                                    | 27.8±12.3                                    |
| FFM, kg                                                       | 54.9±15.5                                    | 55.6±15.6                                   §§ | 53.5±10.8                                    | 57.1±10.4                                    |
| WC, cm                                                        | 85.3±10.7                                    | 85.2±11                                     | 86.3±12.1                                    | 86.8±11.4                                    |
| **Serum biochemistry**                                        |                                              |                                              |                                              |
| ALT, U/L                                                      | 44.5 (24.0–64.0)                             | 53.0 (26.0–69.0)                             | 31.0 (21.0–53.0)                             | 27.5 (19.0–38.0)                             |
| AST, U/L                                                      | 27.5 (21.0–32.0)                             | 27.5 (22.0–44.0)                             | 24.5 (23.0–27.0)                             | 23.5 (20.0–30.0)                             |
| γ-GT, U/L                                                     | 34.0 (27.0–42.0)                             | 31.5 (28.0–46.0)                             | 36.5 (24.0–101.0)                            | 43.5 (27.0–64.0)                             |
| AP, U/L                                                       | 54.5 (47.0–94.0)                             | 51.0 (47.0–92.0)                             | 67.0 (48.0–77.0)                             | 63.0 (51.0–77.0)                             |
| Bilirubin, mg/dL                                              | 0.6 (0.4–0.6)                                | 0.5 (0.3–0.5)                                | 0.6 (0.3–0.7)                                | 0.5 (0.4–1.0)                                |
| Albumin, g/L                                                  | 44.5±2.9                                     | 44.6±3.6                                     | 45.5±2.4                                     | 44.1±2.2                                     |
| FFA, μmol/L                                                   | 334.5 (289.0–431.0)*                         | 380.5 (253.0–478.0)                         | 564.5 (509.0–682.0)                          | 436.5 (382.0–553.0)*                         |
| Ketones (total), μmol/L                                      | 52.0 (39.0–65.0)                             | 67.5 (50.0–78.0)                             | 61 (44.0–96.0)                               | 40.5 (34.0–91.0)                             |
| Acetocetate, μmol/L                                           | 15.0 (15.0–19.0)                             | 23.0 (22.0–29.0)                             | 19.5 (14.0–34.0)                             | 16.0 (12.0–36.0)                             |
| β-hydroxybutyrate, μmol/L                                    | 37.0 (24.0–46.0)                             | 43.0 (28.0–49.0)                             | 40.0 (30.0–62.0)                             | 27.0 (20.0–58.0)                             |
| Glucose (fasted), mg/dL                                       | 87.4±7.4                                     | 89.5±11.0                                    | 89.9±8.3                                     | 90.9±8.3                                     |
| Glucose (1 h), mg/dL                                          | 136.2±38.5                                   | 149.3±47.4                                   | 145.1±47.6                                   | 139.8±50.2                                   |
| Glucose (2 h), mg/dL                                          | 115.8±16.9                                   | 117.3±33.6                                   | 121.1±35.3                                   | 117.4±35.5                                   |
| Insulin (fasted), μIU/mL                                      | 8.0±6.3                                      | 12.6±9.4                                     | 8.2±7.1                                      | 9.9±8.7                                      |
| Insulin (2 h), μIU/mL                                         | 83.6±65.7                                    | 68.2±63.3                                    | 53.2±50.5                                    | 41.6±15.0                                    |
| C-peptide (fasted), ng/mL                                     | 2.1±1.2                                      | 3.7±1.9                                      | 1.5±1.0                                      | 1.7±0.8                                      |
| HOMA-IR                                                       | 2.6±1.3                                      | 2.7±2.6                                      | 1.5±1.3                                      | 2.2±2.0                                      |
| **BP**                                                        |                                              |                                              |                                              |                                              |
| Systolic, mm Hg                                               | 118.3±12.8                                   | 118.3±14.6                                   | 119.5±16.3                                   | 121.5±16.7                                   |
| Diastolic, mm Hg                                              | 73.2±8.2                                     | 71.0±10.2                                     | 73.7±15.3                                    | 75.5±10.1                                    |
| Heart rate, beats/min                                         | 70.6±15.8                                    | 68.8±12.6                                    | 73.0±6.4                                     | 73.2±7.2                                     |

Values are presented as means ± SD or medians (IQR), depending on the data distribution. ALT, alanine aminotransferase; AP, alkaline phosphatase; AST, aspartate aminotransferase; BP, blood pressure; FM, fat mass; GT, glutamyl transferase; SMM, skeletal muscle mass; WC, waist circumference. Between-group significance: * $p ≤ 0.05$ and ** $p < 0.01$. Within-group significance: § $p ≤ 0.05$ and §§ $p < 0.01$. 

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β-hydroxybutyrate at follow-up for the latter, whereas a marginal yet nonsignificant increase was observed in the risk group.

**Dietary Intake and Activity Stratified by PNPLA3 Genotype**

Table 2 displays the results of the dietary assessment before and after the intervention, presenting the average dietary intake over a period of 5 days at each time point. Dietary lipids, comprising cholesterol, saturated fatty acids in addition to monounsaturated fatty acids (MUFA), including oleic acid, and PUFA, were assessed in greater detail. The total omega-6 and omega-3 PUFA were individually quantified, as well were linoleic acid and EPA and DHA levels.

At baseline, the risk group consumed more fat than the control group (p = 0.05), yet no other differences were noted. In both groups, however, the dietary fat intake was above the recommended 30% energy intake (36 and 35% for the risk and control groups, respectively). At follow-up the risk group consumed more fat (p = 0.04) and cholesterol (p = 0.03). Both groups exhibited significant increases in overall omega-3 fatty acid intake, as well as EPA and DHA (all p < 0.0001), as a result of the supplementation. A significant (p = 0.004) increase in total omega-6 PUFA was also observed in the risk group.

The pedometers revealed a significantly higher number of steps taken by the risk group at baseline (p = 0.001) and at follow-up (p = 0.027) compared to the control group. No within-group differences were observed at follow-up, indicating that activity levels (based on the step count per day) remained stable during the intervention.

**Discussion**

This study reports no significant changes in hepatic steatosis after short-term omega-3 supplementation in patients with NAFLD and homozygous for the PNPLA3 148M risk allele; however, a different response in terms of serum FFA concentrations was observed. Patients with NAFLD who are homozygous for the PNPLA3 variant have been reported to benefit more from certain lifestyle interventions than heterozygous or wild-type participants [21]. Increasing the omega-3 fatty acid intake is suggested as beneficial for hepatic steatosis, given its ability to reduce the expression of sterol response element binding protein 1c (SREBP1c), which regulates hepatic lipogenesis [22]. Moreover, 4 g EPA and DHA per day for 8 weeks was recently shown to alter the intestinal microbiota and increased production of short-chain fatty acids in healthy volunteers in a randomized open-label crossover trial [23]. However, the use of omega-3 fatty acids in NAFLD remains controversial [24]. A meta-analysis suggested that omega-3 PUFA might decrease liver fat but could not conclude on the optimal study duration (which ranged from 8 weeks to 1 year) because of the marked heterogeneity among trials [12].

The main result of our 4-week supplementation study is in contrast with a long-term randomized controlled trial, reporting an increase in liver fat percentage after providing daily 4 g DHA and EPA supplementation for 15–18 months in 13 patients carrying the PNPLA3 risk genotype and a decrease in liver fat content in carriers of the PNPLA3 major alleles [14]. The identical supplementation regimen was used in both of these studies; however, our shorter supplementation period might be one possible reason for the contrasting results. This indicates that supplementation with omega-3 fatty acids should last longer than 1 month to detect liver-related effects. Furthermore, our matched-design differed from the randomized controlled design used by Scorletti et al. [14], which might be another reason for the observed results.

Furthermore, Nobili et al. [13] supplemented 60 children with NAFLD and the PNPLA3 148M with 250 or 500 mg DHA or placebo in a randomized double-blind
The PNPLA3 risk group had a greater likelihood (37%; 95% CI 26–48) of more severe steatosis in contrast to carriers of the wild-type genotype (−12%; 95% CI −21 to −3), thus alluding to a gene-dependent effect on fatty acid metabolism. Of note, an increased concentration of the omega-3 PUFA α-linolenic acid, as well as decreased omega-6 PUFA, was observed in the hepatic triacylglycerol content in 52 patients undergoing a liver biopsy [25]. Decreased enrichment of erythrocyte DHA has also been reported in those with the PNPLA3 148M risk variant [13, 14]. These factors might cause the lack of liver fat reduction following omega-3 fatty acid interventions.

Furthermore, the increased amount of liver fat in carriers of the PNPLA3 variant is related to reduced intrahepatic lipid remodeling. Specifically in carriers of the p.I148M mutation, the variant PNPLA3 evades ubiquitylation and proteosomal degradation but accumulates on lipid droplets, which increase in number and median size and display impaired TG mobilization [26]. Therefore, in carriers of PNPLA3 148M, a higher intrahepatic lipid accumulation is likely to occur together with an increased influx of fatty acids as, for example, after omega-3 supplementation. Indeed, Rausch and Mueller [27] referred to the disturbed intrahepatic lipid remodeling as a result of a reduced fatty acid mobilization in PNPLA3 risk allele carriers. Thus, a reduced lipid turnover in the risk group might indeed explain the increased concentrations of free fatty acids detected in serum in these patients.

The PNPLA3 148M risk variant also represents a group of individuals who are at a high risk of NAFLD, independently of metabolic-related perturbations [28]. These observations might explain, at least in part, the significant differences in FFA serum concentrations with lower baseline levels observed in our risk versus control groups. A different response to supplementation with omega-3 fatty acids was also observed between the 2 groups, with a nonsignificant increase of 9% in the risk group compared to a significant reduction of 28% in controls. The significant reduction in serum FFA concentrations observed in the control group reflects the widely reported health benefits of omega-3 fatty acids. In contrast, the potentially deleterious rigid response as observed in the risk group might reflect the aforementioned PNPLA3-dependent mechanisms in these patients. The fact that the difference after the dietary challenge was nonsignificant might be attributed to the sample size and the supplementation period.

### Table 2. Dietary intake and physical activity during this study based on PNPLA3 variants

|                     | Baseline M/M (n = 10) | Follow-up M/M (n = 10) | Baseline I/I (n = 10) | Follow-up I/I (n = 10) |
|---------------------|-----------------------|------------------------|-----------------------|------------------------|
| **Energy, kcal/day**| 2,593.9 ± 976.6       | 2,249.8 ± 756.1 §      | 1,923.5 ± 519.6       | 1,740.7 ± 514.0       |
| **Fat, g**          | 104.1 ± 40.2 *        | 87.0 ± 29.3 *          | 74.4 ± 19.8           | 63.4 ± 18.0           |
| **Protein, g**      | 114.5 ± 57.0          | 110.8 ± 47.1 *         | 81.2 ± 26.3           | 59.4 ± 18.0§§         |
| **Carbohydrates, g**| 251.4 ± 105.1         | 251.5 ± 96.3 *         | 219.2 ± 83.5          | 193.8 ± 63.7          |
| **Fiber, g**        | 27.2 ± 9.2            | 23.3 ± 5.8 §           | 23.1 ± 10.4           | 20.1 ± 10.6           |
| **Cholesterol, mg** | 358.2 ± 156.1         | 368.3 ± 148.3          | 267.9 ± 130.7         | 239.5 ± 87.1          |
| **SFA, g**          | 38.7 ± 19.7           | 33.3 ± 14.6            | 28.5 ± 8.4            | 26.9 ± 9.1            |
| **Palmitic acid**   | 18.8 ± 9.3            | 16.5 ± 8.3             | 14.3 ± 4.1            | 13.3 ± 4.4            |
| **Stearic acid**    | 8.2 ± 4.4             | 7.3 ± 4.2              | 6.5 ± 2.1             | 5.8 ± 2.0             |
| **MUFA, g**         | 31.4 ± 13.2           | 26.9 ± 13.3            | 26.5 ± 8.0            | 23.5 ± 9.2            |
| **Oleic acid**      | 29.5 ± 13.2           | 26.3 ± 13.1            | 24.1 ± 7.4            | 21.1 ± 8.6            |
| **PUFA, g**         | 14.0 ± 4.8            | 13.4 ± 3.3             | 14.4 ± 5.9            | 13.9 ± 5.6            |
| **n-6 PUFA**        | 8.8 ± 3.3             | 6.9 ± 2.9 §            | 10.0 ± 5.3            | 7.0 ± 5.0             |
| **Linoleic acid**   | 1.4 ± 0.6             | 1.2 ± 0.6              | 1.7 ± 1.4             | 1.1 ± 0.9             |
| **Arachidonic acid**| 0.1 ± 0.07            | 0.2 ± 0.1              | 0.2 ± 0.2             | 0.2 ± 0.1             |
| **n-3 PUFA**        | 1.4 ± 0.7             | 4.7 ± 0.6 §§           | 2.1 ± 1.5             | 5.7 ± 1.5 §§ §§       |
| **DHA**             | 0.2 ± 0.3             | 2.1 ± 0.3 §§           | 0.3 ± 0.3             | 2.3 ± 0.7 §§ §§       |
| **EPA**             | 0.1 ± 0.2             | 2.4 ± 0.7 §§           | 0.1 ± 0.2             | 2.4 ± 0.7 §§ §§       |
| **Average steps, n/day** | 7,913 ± 1,903 ***     | 7,183 ± 2,135 *        | 4,713 ± 1,665         | 4,955 ± 1,834         |

n-3 PUFA, omega-3 polyunsaturated fatty acids; n-6 PUFA, omega-6 polyunsaturated fatty acids; SFA, saturated fatty acids. Between-group significance: * p ≤ 0.05, ** p < 0.01, and *** p < 0.001. Within-group significance: § p ≤ 0.05, §§ p < 0.01, and §§§ p < 0.001.
Of note, fasting insulin levels markedly increased in the risk group and decreased in controls, respectively. The total and individual ketone concentrations for acetacetate and β-hydroxybutyrate mirrored these changes. Overall, these alterations corresponded to the changes in FFA concentrations; however, since we did not determine the exact composition of FFA in serum, we cannot make firm observations confirming the link that exists between FFA and insulin [29] and ketone production [30].

Despite the matched-pair design, the lack of changes in hepatic steatosis might have been due to the short supplementation period of 4 weeks or to the small sample size, especially as the sample size calculation was based on a study that used a different method to determine hepatic fat contents. Dietary intake, as assessed by food diaries, and physical activity, as assessed based on pedometers and exercise diaries, remained stable, as did body composition, and thus can be excluded as potential confounders.

Regardless of the negative findings herein and because lifestyle interventions continue to be recommended for patients with NAFLD [11], the nutrigenomic and metabolic effects of UFA and omega-3 fatty acid in particular need to be investigated further in carriers of the PNPLA3 p.I148M risk alleles.

**Disclosure Statement**

The authors have no conflict of interests to declare.

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**Author Contributions**

F. Lammert and C.S. Stokes designed this study. C.-S. Kuttner recruited patients and collected the data and, together with C.S. Stokes, analyzed the data. G. Wagenpfeil provided statistical input. C.S. Stokes drafted this paper, which was then critically revised by all of the authors. The final draft submitted was approved by all of the authors.

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