Treating liver fat and serum triglyceride levels in NAFLD, effects of PNPLA3 and TM6SF2 genotypes: results from the WELCOME* trial

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*WELCOME, **W**essex **E**valuation of fatty **L**iver and **C**ardiovascular markers in NAFLD with **OM**acor th**E**rapy.

Key words: Omega-3 fatty acid, DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; PNPLA3, patatin-like phospholipase domain-containing protein-3; TG, triglyceride; NAFLD, non alcoholic fatty liver disease; liver fat; WELCOME, Wessex Evaluation of fatty Liver and Cardiovascular markers in NAFLD with OMacor thErapy.

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Abbreviations: CI, confidence interval; DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; HA, hyaluronic acid; MetS, metabolic syndrome; MRI, magnetic resonance imaging; MRS, magnetic resonance spectroscopy; NAFLD, non-alcoholic fatty liver disease; NASH, nonalcoholic steatohepatitis; PIINP, procollagen-III N terminal propeptide; PNPLA3, patatin-like phospholipase domain-containing protein-3; SD, standard deviation; TG, triglyceride; TM6SF2, transmembrane 6 superfamily member 2 protein (E167K).

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ABSTRACT

Background and Aims: Genetic variation in both patatin-like phospholipase domain-containing protein-3 (PNPLA3) (I148M) and the transmembrane 6 superfamily member 2 protein (TM6SF2) (E167K) influences severity of liver disease, and serum triglyceride concentrations in non-alcoholic fatty liver disease (NAFLD), but whether either genotype influences the responses to treatments is uncertain.

Methods: 103 patients with NAFLD were randomised to omega-3 fatty acids (DHA+EPA) or placebo for 15-18 months in a double blind placebo-controlled trial. Erythrocyte enrichment with DHA and EPA was measured by gas-chromatography. PNPLA3 and TM6SF2 genotypes were measured by PCR technologies. Multivariable linear regression and analysis of covariance were undertaken to test the effect of genotypes on omega-3 fatty acid enrichment, end of study liver fat percentage and serum triglyceride concentrations. All models were adjusted for baseline measurements of each respective outcome.

Results: 55 men and 40 women (Genotypes PNPLA3 I148M, CC=41, CG=43, GG=11; TM6SF2 E167K CC=78, CT+TT=17 participants) (mean ±SD age, 51±11 years) completed the trial. Adjusting for baseline measurement, measured covariates and confounders, PNPLA3 (I148M) GG genotype was independently associated with % DHA enrichment (B coefficient -1.02 (95%CI -1.97, -0.07), p=0.036) but not % EPA enrichment (B coefficient -0.31 (95%CI -1.38, 0.75), p=0.56). This genotype was also independently associated with end of study % liver fat (B coefficient 9.5 (95%CI 2.53, 16.39), p=0.008), but not end of study triglyceride concentration (B coefficient -0.11 (95%CI -0.64, 0.42), p=0.68).

Conclusions: PNPLA3 (I148M) GG genotype influences the changes in liver fat and DHA tissue enrichment during the trial but not the change in serum triglyceride concentration.

INTRODUCTION

Non-alcoholic fatty liver disease (NAFLD) is strongly associated with type 2 diabetes, metabolic syndrome (MetS) (1) and weight loss (2). Omega-3 polyunsaturated fatty acid (PUFA) treatment (3) and weight loss (4) can be effective in decreasing liver fat and also triglyceride concentrations. Recently in patients with NAFLD we have suggested that the omega-3 fatty acid docosahexaenoic acid (DHA, 22:6n-3) may be more effective that the omega-3 fatty acid eicosapentanoic acid (EPA; 20:5n-3) in decreasing liver fat (5). In this randomised placebo-controlled trial that tested the effects of high dose purified omega-3 fatty acids, we showed an association between high levels of erythrocyte DHA, but not EPA, enrichment and decreased liver fat. Similarly, and in support of our findings, others have shown a benefit of omega-3 fatty acids to decrease liver fat, both in adults (6) and in children (7) with NAFLD. Although these findings are all consistent with the results of a recent systematic review and meta-analysis showing a benefit of omega-3 fatty acid treatment on liver fat in NAFLD (8), a recent trial that used high doses of EPA only, failed to show an improvement in NAFLD histological score (9). The reason for this apparent discrepancy in findings between the effects of DHA and EPA in NAFLD is uncertain, but there is considerable evidence that EPA and DHA have different biological effects in man (10-13) (14).

In NAFLD, patients with the homozygous GG (I148M) gene variant of the patatin-like phospholipase domain-containing protein-3 (PNPLA3) have higher levels of liver fat accumulation (15, 16) and lower serum fasting TG concentration, compared to those without the homozygous gene variant (17). The precise mechanism by which variation in PNPLA3 genotype induces hepatic lipid accumulation and decreased fasting triglyceride concentration is not clear. With regard to regulation of liver lipid, PNPLA3 exhibits both

acyltransferase activity and TG hydrolase activity (18). The PNPLA3 I148M GG genotype has been shown to reduce secretion of large TG-rich VLDL particles in obese men with hepatic steatosis and to impair apoB100 secretion (19). Moreover, the PNPLA3 I148M GG genotype is known to facilitate the differential incorporation into liver lipid droplets of different types of fatty acid, for example vaccenic and palmitoleic acids, thereby altering the fatty acid composition of the lipid droplets (20). Recent data suggest that in humans PNPLA3 I148M regulates the efflux and remodelling, but not the influx, of lipid into hepatic lipid droplets (17, 20-22). In mice, Peter et al. (23) have shown that PNPLA3I148M GG genotype is characterised by a decrease in lipolytic activity and lysophosphatidic acid acyl-CoA transferase activity, with a greater than twofold preference for PUFAs. These investigators also showed that there was a reduction of DHA in the hepatocytes of mice overexpressing PNPLA3 I148M GG genotype. Additionally, Ruhanen et al (20) have shown that fatty acid composition was altered in cells expressing PNPLA3 I148M GG genotype with an increase of 18:1 n-7 and 16:1 n-7 (vaccenic and palmitoleic acids, respectively).

Moreover, recent studies have also identified that genetic variation in the transmembrane 6 superfamily member 2 protein (TM6SF2) at rs58542926 influences NAFLD severity, and contributes to lower levels of fasting triglycerides (24, 25). Although genetic variation in both PNPLA3 and TM6SF2 is known to influence severity of liver disease, and fasting serum triglyceride concentrations in NAFLD, it is presently uncertain whether either genotype influences the responses to treatments for NAFLD. The aim of our study was to test whether either PNPLA3 (I148M) or TM6SF2 (E167K) genotypes affected: a) the level of DHA and EPA enrichment; b) end of study liver fat percentage and c) end of study fasting triglyceride concentration, in patients with NAFLD treated for 15-18 months with DHA+EPA. The present

study was a sub-study of the randomised placebo-controlled WELCOME trial (5, 26) (**W**essex **E**valuation of fatty **L**iver and **C**ardiovascular markers in NAFLD with **OM**acor th**E**rapy; www.clinicalTrials.gov registration number NCT00760513) in which liver fat percentage and fasting serum triglyceride concentrations decreased due to DHA+EPA treatment and weight loss observed during the trial.

PATIENTS AND METHODS

Study design

The WELCOME trial was a double blind placebo-controlled trial where we tested the effect of 4 g per day of Omacor (1 g of Omacor contains 460 mg of EPA and 380 mg of DHA as ethyl esters) or placebo for 15-18 months on liver-related primary outcomes in patients with NAFLD (26). Omacor, also known as Lovaza, was provided free of charge by Pronova BioPharma/Abbott (Pronova BioPharma ASA, Lysaker, Norway; Abbott Laboratories, Southampton, UK).

Subjects and measurements

We randomised 103 participants to either DHA+EPA (n=51) or placebo (n=52) and 95 participants completed the study (DHA+EPA=47; placebo=48). Consort diagram and the baseline characteristics of both placebo and active groups have been reported previously (5). All participants had NAFLD and features of metabolic syndrome. Metabolic syndrome was defined using the International Diabetes Federation criteria (27), the criteria for defining NAFLD were reported previously (26). Blood pressure was measured using a Marquette Dash 3000 monitor (GE Healthcare, Little Chalfont, Bucks, UK) in the non-dominant arm. Waist circumference was measured over bare skin, midway between the costal margin and the iliac crest. Hip circumference was measured at the widest part between the greater trochanter and lower buttock level. Radiological assessments of body fat (dual-energy X-ray absorptiometry, DEXA) were undertaken at both baseline and end of study. DEXA scanning was undertaken with a Delphi W instrument (Hologic, Bedford, MA, USA) to assess percentage body fat, fat distribution and lean mass. Glucose, insulin, total cholesterol, HDL-cholesterol, TG, alanine aminotransferase (ALT) and aspartate aminotransferase (AST) and cytokeratin-18 (CK-18 M65 as a marker of liver apoptosis (28))

were measured in fasting serum. For measurement of serum TG concentration, the intra-assay coefficient of variation (CV) was 1% and the inter-assay CV was 1.7%, for TG concentrations < 88.5 mg/dL. Magnetic resonance imaging (MRI) of abdominal visceral and subcutaneous fat and MR spectroscopy of hepatic lipid content were undertaken at the beginning and at the end of the study. Any change in diet during the study was assessed by food frequency questionnaire and we also generated a 'prudent diet score' as a healthy diet index, using principal component analyses (29). Drug medication was recorded at each study visit. At 6 months, 12 months and end of study, participants returned all used study capsules. Returned capsules were counted to assess compliance to allocated DHA+EPA or placebo.

DHA and EPA enrichment in erythrocytes

We measured DHA and EPA in erythrocytes at the beginning and at the end of the treatment by gas chromatography (30, 31). Enrichment was defined as the difference between end of study and baseline measurements. Total lipids were extracted from thawed packed erythrocytes (300 µl) with 5 ml chloroform:methanol (2:1 v/v) containing butylated hydroxytoluene (50 mg/L) (32). Fatty acid methyl esters (FAMEs) were synthesised by heating the total lipid extract with 1 ml methanol containing 2% (vol/vol) H_2SO_4 at 50°C for 2 h (30), FAMEs were extracted with hexane and analysed using a Hewlett Packard 6890 gas chromatograph fitted with a BPX-70 fused silica capillary column (30 m x 0.22 mm x 0.25 µm) with flame ionisation detection (30). FAMEs were identified by comparison of retention times with those of authentic standards. Intra-assay CVs for EPA and DHA were 3.0% and 2.0% respectively. Inter-assay CVs for EPA and DHA were 5.0% and 2.2% respectively.

DNA analyses

Blood was collected from participants at baseline for determination of polymorphisms in the gene encoding PNPLA3. DNA was extracted from 200 µl whole blood using QIAamp blood DNA blood mini kit (Qiagen, 51106), as per manufacturer's instructions. All samples were eluted in 200 µl DNAse free water. Quality and quantity of DNA was confirmed by spectrophotometry using a Nanodrop 2000 (Thermo Scientific), where all samples had a 260/280 ratio ≥1.8. PCR primers (Table. 1) were designed using PyroMark Assay Design 2.0 software (Biotage) to investigate the SNP rs738409 in the human *PNPLA3-I148M* gene variant. PCR was carried out on 25 ng DNA using 25 µl KAPA2G Robust Hot Start Taq (Anachem, KK5702) and 0.2µM of forward (5' AGCAGAGAAAGCCGACTTACCAC 3') and reverse (5' GGGTGCTCTCGCCTATAACTTC 3') primers in a 50 µl reaction. PCR products were immobilised on streptavidin–sepharose beads (GE Healthcare UK Ltd., 17-5113-01), washed, denatured and released into annealing buffer containing the sequencing primer (5' ATGTTCCTGCTTCAT 3'). PNPLA3 SNP genotype was analysed using Pyromark ND 1.0 Software (Biotage). TM6SF2 genotype was determined using TaqMan C_89463510_10 Genotyping assay (Life technologies 4351379) and TaqMan Genotyping Master Mix (Life Technologies, 4371353) and analysed using LightCycler 480 SW 1.5.1 software (Roche). The rs58542926 C>T (E167K, TM6SF2) single-nucleotide polymorphisms were assessed in duplicate. Analytical pass rate was 100%.

Statistical analyses

All statistical analyses were performed using SPSS for Windows (version 21.0; SPSS). We examined baseline and end of study characteristics in DHA+EPA and placebo groups. Data are reported as means and 95% CIs or SDs for normally distributed variables, or as median

and interquartile range (IQR), or ranges with maxima and minima for non-normally distributed variables. Comparisons of means between groups were performed by using t-tests for normally distributed variables and Mann Whitney U for non-normally distributed unpaired variables. Comparisons of paired data were tested by paired t tests, Wilcoxon signed rank and McNemar's tests. Multivariable linear regression modelling with backwards elimination was undertaken to test independent associations with these outcome variables: a) end of study TG concentration, b) end of study liver fat % c) DHA or EPA percentage enrichment in erythrocytes (end of study % minus baseline %). In each of these separate regression models, we adjusted for the baseline measurement of the outcome measure in question. Covariates, potential confounders and change in potential confounders between baseline and end of study were included, to test the independence of associations between key exposures and the outcome in question. Explanatory variables that were included in the models were continuous or categorical. For PNPLA3 and TM6SF2 genotype, a binary indicator variable was created and coded as CG+CC=0 and GG=1, or CC=0 and CT+TT=1 respectively. Differences between variables in PNPLA3 genotype groups and in TG quartile groups were tested by ANOVA or Kruskal Wallis depending on whether variables were normally or non-normally distributed. A P value of < 0.05 was considered to be statistically significant. ANCOVA analyses were also undertaken test the effects of allelic variation of the genotypes for PNPLA3 and TM6SF2 on the study outcomes and to estimate the adjusted mean differences, according to genotype, between baseline and end of study in the study outcomes. Each ANCOVA model was adjusted for the same potential confounders as shown in the linear regression models.

RESULTS

Subject characteristics

Table 1 shows the baseline and end-of-study results for the main anthropometric and biochemical variables, according to randomization group. Ninety-five participants completed the study (55 men and 40 women) and 32 of these had diabetes. At baseline, fasting TG concentration and BMI were higher in the DHA+EPA group compared with the placebo group (p=0.04 and p=0.02, respectively). At baseline, there were no differences between the groups in MRI visceral fat measurements, waist circumference and weight. At baseline, 7.4% (n=7) of participants were taking fibrates, 6.3% (n=6) were taking ezetimibe, 44.2% (n=42) were taking statins, 4.2% (n=4) were taking orlistat and 9.5% (n=9) were taking Levothyroxine. At the end of the study, there were 9.5% (n=9) of people taking fibrates, 5.3% (n=6) people were taking ezetimibe, 49.5% (n=47) people taking statins, 1.1% (n=1) were taking orlistat (Table 1), 10.5% (n=10) were taking Levothyroxine. With regard to other metabolic and biochemical variables, there were no significant differences between randomisation groups (Table 1). Diet (assessed by prudent diet score) and alcohol intake did not change significantly during the study in either group. Capsule count at 6 months, 12 months and at end of study confirmed that compliance with treatment was at least 78% of all allocated capsules in all participants during the duration of the study. No serious adverse events occurred that were attributed to DHA+EPA or placebo.

Table 1 also shows fatty acid percentages in erythrocytes at baseline and at the end of study in the placebo and DHA+EPA groups. Despite very good compliance with the DHA+EPA intervention in the treatment arm, there was very variable DHA and EPA enrichment between individuals. Some individuals had only limited enrichment of either fatty acid while others had excellent enrichment, which did not relate to numbers of capsules consumed.

For example, for DHA and EPA, the range of change in concentrations in erythrocyte membranes was from -1.8% to +5.6% (DHA) and from -0.9% to +6.2% (EPA). In the placebo group, there was no change in enrichment in EPA and there was a very small increase in DHA enrichment.

In our cohort, at baseline 42 participants had the PNPLA3 (I148M) CC genotype, 43 had the CG genotype and 13 had the GG genotype. **Table 2** describes the baseline characteristics of the cohort stratified by PNPLA3 genotype. There were between group differences for DHA % (*p*=0.03). With regard to TM6SF2 (E167K) genotype, 80 participants had the CC genotype, 15 had the CT genotype and 3 had the TT genotype (**Table 3**).

The effects of PNPLA3 (I148M) and TM6SF2 (E167K) genotypes on DHA and EPA enrichment

We then tested whether PNPLA3 (I148M) GG genotype was independently associated with either erythrocyte DHA or EPA % enrichment between baseline and end of study. For DHA enrichment, the multivariable linear regression model included % change in erythrocyte DHA enrichment (end of study – baseline % enrichment) as the outcome, and age, sex, baseline liver fat %, PNPLA3 (I148M) GG genotype, TM6SF2 genotype CT+TT, BMI, diabetes, % DHA enrichment at baseline, total fat mass, change in CK-18 M65 (between end of study and baseline), triglyceride concentration at baseline, use of orlistat, L thyroxine, fibrates, beta blockers, and thiazide diuretics at baseline and capsule count. In this model, PNPLA3 (I148M) GG was independently associated with % DHA enrichment (unstandardized B coefficient enrichment -1.02 (95%CI -1.97, -0.07), p=0.036). In contrast, when this regression model was adjusted to test associations between PNPLA3 (I148M) GG genotype and % EPA

enrichment, there was no significant association (unstandardized B coefficient -0.31 (95%CI -1.38, 0.75), p=0.56). TM6SF2 genotype was not independently associated with either EPA or DHA enrichment.

Effect of PNPLA3 and TM6SF2 genotypes on end of study liver fat percentage and end of study triglyceride concentration

Between baseline and end of study the change in liver fat % was -7.0 (14) (median and IQR) in the PNPLA3 (CC+CG) genotype groups compared with +1.2 (9.0) (median and IQR) in the GG group (p=0.027). The change in liver fat was -4% (20) (median and IQR) in the TMS6SF2 CC genotype and in the TMS6SF2 CT+TT genotype it was 0.75 (12.3) (median and IQR). Between baseline and end of study the change in serum triglyceride concentration was (median and IQR) -0.1 (0.8) in the PNPLA3 (CC+CG) genotype groups compared with +0.3 (0.9) in the GG group (p=0.22). In presence of the TMS6SF2 CC genotype the changes in triglycerides were -0.1 mmol/L (0.8) (median and IQR) and -0.1 mmol/L (0.6) (median and IQR) in the TMS6SF2 CT+TT genotype group respectively.

The effect of PNPLA3 (I148M) GG genotype on end of study liver fat percentage and end of study triglyceride concentration was then investigated adjusting for covariates and potential confounders. From the results of univariate analyses (data not shown), for liver fat %, factors included in the regression modelling included age, sex, baseline liver fat %, PNPLA3 genotype, TM6SF2 genotype CT+TT, BMI, diabetes, % DHA enrichment (end of study % – baseline % DHA), baseline serum triglyceride, total fat mass, change in M65 (between end of study and baseline), use of orlistat at baseline, use of L thyroxine at baseline and capsule count. **Table 4** shows the only factors that were independently associated with end of study

liver fat %. DHA enrichment and decrease in weight (kg) during the 15-18 months of the trial, were both independently associated with end of study % liver fat and baseline % liver fat was also independently associated with end of study % liver fat. Additionally, PNPLA3 (I148M) GG genotype was independently associated with end of study % liver fat (B coefficient B coefficient 9.5 (95%CI 2.53, 16.39), p=0.008). Overall this model (**Table 4**) accounted for 54% of the variance in end of study % liver fat (R^2=0.54, p<0.0001).

The effect of PNPLA3 (I148M) GG genotype on end of study triglyceride concentration was then investigated. From the results of univariate analyses (data not shown), factors included in the model, were age, sex, baseline liver fat %, MRS difference, PNPLA3 genotype, TM6SF2 genotype CT+TT, BMI, diabetes, % EPA enrichment (end of study – baseline % enrichment), total fat mass, change in M65 (between end of study and baseline), triglyceride concentration at baseline, and use of orlistat, L thyroxine, fibrates, beta blockers, and thiazide diuretics at baseline, and capsule count. Factors that were independently associated with end of study triglyceride concentrations are shown in **Table 5**. In contrast to the data for end of study % liver fat, PNPLA3 genotype GG (I148M) was not associated with end of study triglyceride concentration (B coefficient -0.11 (95%CI -0.64, 0.42), p=0.68). Additionally, % EPA enrichment **(**B coefficient -0.19 (95%CI -0.31, -0.07), p=0.002) and not % DHA enrichment was independently associated with end of study triglyceride concentration. Factors included in the regression model shown in **Table 5** explained 56% of the variance in end of study triglyceride concentration (R^2=0.56, p<0.0001). We did not find any relationship between TMS6SF2 genotype and MRS liver fat or fasting triglyceride concentrations. However, we observed a significant difference in fasting TG at baseline by genotype; for the TMS6SF2 CT+TT genotype, triglycerides at baseline were lower (1.5

mmol/L (0.7) (median and IQR)) than for the TMS6SF2 CC genotype (1.8 mmol/L (1.4) (median and IQR)) (p=0.02).

Since the evidence suggests that PNPLA3 (I148M) G/G genotype influences hepatic fat accumulation and liver damage with an additive effect of each G allele (33, 34), we tested the additive effective of allelic variation for PNPLA3 (I148M) genotype on change in liver fat percentage and change in fasting serum triglyceride between baseline and end of study. Each ANCOVA model **(Table 6)** was adjusted for the same potential confounders as shown in Tables 4 and 5. These data show that although there was a change in liver fat % between baseline and end of study in each PNPLA3 genotype the adjusted mean difference in liver fat % was greater for PNPLA3 I148I C/C and for PNPLA3 I148IM C/G (with a decrease in liver fat % between baseline and end of study), compared with the PNPLA3 I148M G/G group where there was a small increase in liver fat %. For change in triglyceride concentration between baseline and end of study, there was no significant effect of PNPLA3 genotype (in keeping with the results of regression modelling presented in Table 5).

DISCUSSION

The novel results of our study are that in patients with NAFLD, the PNPLA3 (I148M) GG genotype, and not either the TM6SF2 (E167K) CT+TT or CC genotypes, was associated with markedly higher end of study liver fat percentage and lower DHA tissue enrichment (after 4 g DHA+EPA intervention for 15-18 months). In contrast, neither PNPLA3 (I148M), nor TM6SF2 (E167K) genotypes were associated with end of study serum triglyceride concentrations. As can be seen in **Table 4**, after adjusting for baseline % liver fat, baseline body fat mass, and other covariates and confounders, the key independent factors associated with end of study liver fat %, were a decrease in weight during the trial, baseline body fat, an increase in tissue % DHA enrichment, PNPLA3 (I148M) GG genotype, and baseline liver fat %. Differences between baseline and end of study of participant characteristics, according to PNPLA3 (I148M) genotype are shown in the Supplementary Table.

Previously, the strength of effect of the PNPLA3 (I148M) GG genotype on liver fat has been assessed in a meta-analysis of 16 studies (2,937 subjects) across different populations with NAFLD. In this analysis, PNPLA3 (I148M) GG genotype was associated with 73% higher liver fat content compared with the CC variant (33). In keeping with this evidence, in our subjects at baseline, median liver fat percentage was 28.5% in subjects with PNPLA3 (I148M) GG compared with 22.6% in subjects with CC or CG. However, to date it is uncertain how PNPLA3 (I148M) GG genotype modifies any response to treatment interventions in NAFLD. Very recently it has been suggested that there was a greater reduction in % liver fat with a 12 month lifestyle intervention in subjects with the PNPLA3 (I148M) GG genotype (35) than in subjects with CG or CC genotype. However, initial baseline mean % liver fat in the

intervention group was surprisingly low (i.e. 5.5%) and was only 4.3% in the PNPLA3 (I148M) GG group. As can be seen from our results and from the unstandardized B coefficient in Table 4, even after adjusting for baseline liver fat %, PNPLA3 (I148M) GG genotype was associated with 10% higher end of study liver fat than seen in subjects with either PNPLA3 CC or CG. Importantly, this effect of PNPLA3 (I148M) GG genotype was independent of any benefit conferred by decrease in body weight and increased %DHA enrichment (Table 4).

DHA+EPA treatment produced highly variable inter-individual DHA and EPA tissue enrichment, and importantly this enrichment was independent of compliance and the numbers of capsules returned unused during the trial. PNPLA3 (I148M) GG was associated with decreased DHA enrichment but was not associated with EPA enrichment. It is known that omega-3 fatty acids are rapidly incorporated into plasma membranes where they affect membrane fluidity and membrane permeability (36) and that measurement of erythrocyte DHA and EPA enrichment is considered a good proxy for omega-3 fatty acid enrichment and bioavailability in liver (37). However, based on our results, PNPLA3 I148M is involved in DHA/EPA mobilization in liver and subjects with PNPLA3 (I148M) GG genotype have lower levels of DHA (38). Additionally, omega-3 fatty acids decrease the expression of sterol response element binding protein 1c (SREBP1c), a key regulatory factor in hepatic lipogenesis (39), and recently it has been shown that carriers of the PNPLA3 148M allele have decreased de novo lipogenesis (22). Thus, it is possible that the lack of response to DHA+EPA treatment in decreasing liver fat % in subjects with PNPLA3 I148 MM could be due to the fact that these subjects already have low levels of de novo lipogenesis. That said, it is not known whether PNPLA3 (I148M) GG genotype affects the incorporation of DHA (or EPA) into the liver lipid droplet, although recent evidence in a small study in children suggests that the PNPLA3 (I148M) GG genotype attenuates the benefit of DHA to decrease liver fat

(40). These data in children are in agreement with, and are extended by, our data, showing PNPLA3 (I148M) GG genotype is associated with lower levels of DHA enrichment. We show for the first time that the PNPLA3 (I148M) GG genotype is associated with a ~1 SD decrease (i.e. a 1.2% decrease) in erythrocyte DHA enrichment (unstandardized B coefficient -1.02 (95%CI -1.97, -0.07), p=0.036). A 1 SD decrease in % DHA enrichment means that most individuals with the PNPLA3 GG genotype do not achieve the 2% DHA enrichment threshold that we have shown was necessary to achieve satisfactory reductions in liver fat in this cohort (5). Since our data show that higher levels of % DHA enrichment are associated with lower levels of liver fat (5), it is noteworthy that most patients with PNPLA3 (I148M) GG genotype do not achieve high levels of % DHA enrichment. Similarly, others have shown an effect of PNPLA3 genotype to modify treatment effect and a recent research study by Dongiovanni et al. (41) has shown that statin treatment was associated with a reduction in liver fat content and inflammation in individuals with NAFLD, carrying the PNPLA3 (I148I) CC genotype. The effect was absent in those with the PNPLA3 (I148I) GG genotype.

The mechanism by which PNPLA3 (I148M) GG genotype affects fasting TG concentration is uncertain. Hyysalo et al. showed in non-obese people with NAFLD that the PNPLA3 (I148M) GG genotype is associated with hepatic hydrolysis of TG, reducing hepatic VLDL secretion (17) but it is uncertain whether PNPLA3 (I148M) GG genotype modifies any change in triglyceride concentrations induced by an intervention. 4 g of DHA+EPA (as Omacor or Lovaza) contains similar amounts of DHA (1520 mg) and EPA (1840 mg) as ethyl esters and our data suggest that a greater TG-lowering effect was associated with EPA enrichment rather than DHA enrichment. Our data (Table 4) show the factors that were associated with end of study fasting serum triglyceride concentrations (adjusting for baseline measurement and other confounders and covariates). These data show that many factors (but not PNPLA3

or TM6SF2 genotypes) were independently associated with triglyceride concentrations and, as expected, capsule count was independently associated with end of study triglyceride concentration. This finding is also consistent with licensing data for Omacor for the treatment for high serum triglyceride concentrations, where 4 g per day is the highest licenced dose, and 4 g is more effective in lowering serum triglyceride concentrations than 2 to 3 g/day. It is possible that PNPLA3 (I148M) genotype only influences VLDL secretion (and thereby fasting triglyceride concentrations), when there are specific hepatic lipids available for incorporation into the secreted VLDL particle (42). Thus, it is plausible that PNPLA3 (I148M) genotype may have little or no effect on VLDL levels when there is a modification in the quality of fatty acids and the type of hepatic lipid content (20) (e.g. as may occur after DHA treatment).

Our study has strengths but also some limitations. The main limitation is the small sample size. However this study is the first study to test the effects of the PNPLA3 (I148M) and TM6SF2 (E167K) genotypes on relevant end points in NAFLD as part of an intervention trial testing the effects of high dose DHA+EPA treatment in adults. This study was a sub-study of the main trial whose primary end point was to test improvements in liver disease per se, rather than changes in fasting TG. We have presented data showing the effects of genotypes on change in % liver fat and change in serum triglyceride in NAFLD, rather than test the effect of genotypes on change in liver fibrosis biomarkers or other biomarkers of NAFLD severity that are known to be affected by PNPLA3 (I148M) and TM6SF2. The reason for this (as reported previously) was that these measures did not change between baseline and end of study during the trial (5). The sample size calculation for the trial has been reported previously (26) and was based on change in liver fat % and not changes in serum TG concentration. The strengths of our study are the randomised double blind placebo-

controlled trial design that also included very detailed phenotyping of the patient cohort that enabled measurement of many potential confounders and a precise, quantitative measurement of % liver fat at baseline and at the end of study.

In conclusion, DHA enrichment and loss of weight during the randomised double blind placebo controlled trial both independently decreased end of study % liver fat, adjusting for baseline % liver fat and all other measured covariates and confounders. PNPLA3 (I148M) GG, but not TM6SF2 genotypes, was strongly and negatively associated with DHA tissue enrichment and was associated with markedly higher (~10%) end of study liver fat levels. In contrast, end of study fasting triglyceride concentrations were strongly associated with % EPA enrichment but not PNPLA3 (I148M) or TM6SF2 (E167K) genotypes.

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Table 1. Baseline and end of study biochemical and anthropometric characteristics

	Placebo group			DHA+EPA group		
Variables	**Baseline**	**End of study**	***p*-value**	**Baseline**	**End of study**	***p*-value**
Age (y)	54.0 (9.6)	55.4 (9.6)	n/a	48.6 (11.1)	50.1 (11.1)	n/a
Sex (M/F)	35/17	32/16	n/a	25/26	23/24	n/a
Weight (kg)	94.5 (15.8)	90.4 (16.3)	0.76	96.5 (17)	94.1 (13)	0.38
BMI (kg/m²)	32.4 (4.5)	30.8 (4.5)	0.74	34.0 (5.8)	33.4 (4.9)	0.30
Prudent Diet Score*	0.09 (0.97)	-0.07(0.9)	0.46	-0.1 (1.0)	0.03 (1.0)	0.55
Alcohol intake (Units/week)	6.3	6.8	0.88	2.3	2.2	0.67
Waist circumference (cm)	108.1 (11.5)	107.7 (10.3)	0.65	114.4 (13.4)	112.3 (10.4)	0.96
Diastolic blood pressure (mmHg)	86.3 (7.4)	82.9 (6.5)	0.75	85.4 (12.3)	81.7 (8.2)	0.006
Systolic blood pressure (mmHg)	137.7 (15.1)	133.9 (11.3)	0.13	138.2 (17.4)	133.3 (13.7)	0.004
Fasting plasma glucose (mmol/L)	6.2 (2.0)	6.7 (3.0)	0.07	6.2 (1.2)	6.1 (2.0)	0.77
Fasting plasma insulin (µUnit/ml)	11.7 (11.2)	10.2 (9.3)	0.68	12.3 (7.1)	13.9 (6.4)	0.18
HbA1c (%)	6.7 (1.2)	6.0 (2.0)	0.18	5.9 (0.9)	5.7 (2.0)	0.64
Serum triglycerides (mmol/L)	1.5 (0.5)	1.8 (0.6)	0.05	1.8 (0.7)	1.5 (1.2)	0.018
Serum cholesterol (mmol/L)	4.5 (0.8)	4.8 (1)	0.28	4.9 (1.1)	4.7 (1.1)	0.17
LDL-cholesterol (mmol/L)	2.7 (0.7)	2.8 (0.8)	0.38	3.0 (0.9)	2.8 (0.9)	0.12
HDL-cholesterol (mmol/L)	1.1 (0.3)	1.1 (0.2)	0.91	1.0 (0.2)	1.1 (0.3)	< 0.0001
ALT (IU/L)	59.5 (45)	48.5 (25)	0.03	55.0 (51)	44.0 (34)	0.89
AST (IU/L)	50.0 (25)	35.0 (17)	0.02	39.0 (24)	30.0 (27)	0.97

DEXA total fat mass (g)	33252 (8734)	30822 (8136)	0.34	38128 (10565)	35694 (8061.6)	0.09
DEXA total lean mass (g)	59017 (11344)	56396 (11237)	0.14	56353 (11564)	56218 (10799)	0.61
DEXA andro/gynoid (ratio)	1.2 (0.2)	1.2 (0.1)	0.13	1.2 (0.1)	1.1 (0.1)	0.72
MRI subcutaneous fat (%)	30.4 (9.7)	28.8 (9)	0.43	35.4 (10.5)	32.0 (9.6)	0.47
MRI visceral fat (%)	16.7 (4.7)	16.5 (5.4)	0.36	15.2 (5.1)	15.9 (4.7)	0.67
MRS liver fat %	21.7 (13.7, 32.3)	19.7 (11.3, 28.0)	0.006	23.0 (12.0, 47.5)	16.3 (9.0, 30.7)	0.01
Erythrocyte DHA (%) enrichment	4.1 (1.6)	5.0 (1)	0.002	3.8 (1.2)	7.1 (1.3)	< 0.0001
Erythrocyte EPA (%) enrichment	0.9 (0.4)	1.0 (0.2)	0.17	0.8 (0.3)	2.4 (1.8)	< 0.0001
Fibrates n	4	4	1.0	3	5	0.5
Statins n	23	23	1.0	19	24	0.06
Ezetimibe n	5	3	0.5	1	2	1.0

Variables that are normally distributed are expressed as mean (standard deviation (SD)). Variables that are non-normally distributed are expressed as median (inter-quartile range (IQR)).

***Prudent Diet Score (continuous variable derived from food frequency questionnaire, see (26)**

Table 2. Baseline characteristics according to PNPLA3 (I148M) genotype

Phenotypes	Genotypes			
	CC N=42	**CG N=43**	**GG N=13**	***P value (difference between groups)***
Weight (kg)	96.8 (20.2)	97.9 (15.4)	89.3 (10.8)	0.5
BMI (kg/m^2)	34.6 (6.5)	33.2 (4.8)	31.7 (4.5)	0.2
Fasting plasma glucose (mmol/L)	5.3 (1.9)	5.5 (1.2)	5.4 (2.0)	0.3
HOMA-IR	2.5 (2.4)	3.1 (4.6)	2.6 (1.1)	0.3
Serum triglyceride (mmol/L)	1.6 (1.2)	1.7 (0.9)	1.4 (0.3)	0.4
Serum cholesterol (mmol/L)	4.9 (0.9)	4.6 (1.1)	4.6 (0.7)	0.3
HDL-cholesterol (mmol/L)	1.1 (0.4)	1.1 (0.3)	1.1 (0.3)	0.9
LDL-cholesterol (mmol/L)	3.0 (0.9)	2.7 (0.9)	2.9 (0.7)	0.5
ALT (IU/L)	53 (44)	55 (33)	70 (65)	0.4
AST (IU/L)	38 (21)	39 (26)	45 (54)	0.5
MRS liver fat %	26.7 (20.9)	28.8 (19.9)	33.5 (17.5)	0.5
Erythrocyte EPA (%) baseline	0.8 (0.3)	0.9 (0.4)	0.7 (0.3)	0.1
Erythrocyte DHA (%) baseline	3.7 (1.2)	4.3 (1.4)	3.6 (1.4)	0.03

Variables that are normally distributed are expressed as mean (standard deviation (SD)). Variables that are non-normally distributed are expressed as median (inter-quartile range (IQR)). (CC = 148II, C/G=148 IM, GG= 148MM).

Table 3. Baseline characteristics according to TM6SF2 genotype

Phenotypes	Genotypes		
	CC	**CT+TT**	***P value (difference between groups)***
Weight (kg)	94.9 (15.1)	98.5 (20.9)	0.5
BMI (kg/m^2)	32.6 (4.5)	35.4 (8.1)	0.05
Fasting plasma glucose (mmol/L)	6.3 (2.7)	6.1 (1.3)	0.6
HOMA-IR	2.7 (2.9)	4.0 (5.8)	0.7
Serum triglyceride (mmol/L)	1.6 (1.1)	1.5 (0.7)	0.02
Serum cholesterol (mmol/L)	4.7 (1.0)	4.5 (0.9)	0.1
HDL-cholesterol (mmol/L)	1.0 (0.4)	1.0 (0.3)	0.6
LDL-cholesterol (mmol/L)	2.8 (1.0)	2.8 (0.6)	0.3
ALT (IU/L)	55.0 (51)	55.0 (58)	0.8
AST (IU/L)	42.0 (24)	39.0 (40)	0.8
MRS liver fat %	28.5 (20.3)	29.0 (19.1)	0.9
Erythrocyte EPA (%) baseline	1.1 (1.4)	0.5 (0.7)	0.1
Erythrocyte DHA (%) baseline	4.0 (1.4)	4.1 (0.8)	0.6

Variables that are normally distributed are expressed as mean (standard deviation (SD)). Variables that are non-normally distributed are expressed as median (inter-quartile range (IQR)). (CC = 148II, C/G=148 IM, GG= 148MM).

Table 4. Factors independently associated with end of study liver fat percentage with DHA+EPA treatment

Variables	Unstandardised B coefficient	95%CI	*p*-value
Baseline liver fat (%)	-0.39	-0.60, -0.28	<0.0001
Total body fat mass (kgs)	0.58	0.09, 1.07	0.021
Change in body weight (kg)	0.79	0.25, 1.32	0.004
PNPLA3 (I148M) genotype GG	9.5	2.53, 16.39	0.008
% DHA enrichment	-1.50	-2.82, -0.19	0.025

Factors included in the model, age, sex, baseline liver fat %, PNPLA3 genotype, TMS6SF2 genotype, % DHA enrichment (end of study – baseline % enrichment), baseline serum triglyceride, total fat mass, change in M65 (between end of study and baseline), BMI, diabetes, change in weight, use of orlistat at baseline, use of L thyroxine at baseline and capsule count. R2=0.54, p<0.0001.

Table 5. Factors independently associated with end of study fasting triglyceride concentration with DHA+EPA treatment

Variables	Unstandardised B coefficient	95%CI	*p*-value
Age (y)	-0.03	-0.05, -0.02	<0.0001
Being male	0.54	0.13, 0.97	0.011
Baseline triglycerides (mmol/L)	-0.47	-0.60, -0.34	<0.0001
Total body fat mass (kg)	-0.03	-0.05, -0.01	0.003
Capsule count	-0.24	-0.45, -0.03	0.025
Use of beta blockers	0.85	0.07, 1.63	0.034
% EPA enrichment	-0.19	-0.31, -0.07	0.002

Factors included in the model, age, sex, baseline liver fat %, PNPLA3 genotype, TMS6SF2 genotype, % EPA enrichment (end of study – baseline % enrichment), BMI, total fat mass, diabetes, change in M65 (between end of study and baseline), change in weight, triglyceride concentration at baseline, and use of orlistat, L thyroxine, fibrates, beta blockers, and thiazide diuretics at baseline and capsule count. (N.B. PNPLA3 genotype GG (I148M) (B coefficient -0.02 (95%CI -0.52, 0.49, p=0.95). Final model R^2=0.56, p<0.0001).

Table 6. Adjusted mean differences for change in liver fat percentage, change in serum fasting triglyceride concentration and change in DHA percentage enrichment with DHA+EPA treatment, according to PNPLA3 genotype

Phenotypes	Genotypes		
	CC N=42	**CG N=43**	**GG N=13**
Adjusted mean change in liver fat % (95%CI)	-7.05 (-10.77, -3.33)	-7.30 (-10.75, -3.85)	2.75 (-4.22, 9.73)
*Adjusted mean change in triglyceride (mmol/L) (95%CI)	-0.12 (-0.41, 1.62)	-0.09 (-0.36, 0.17)	-0.20 (-0.71, 0.31)
§Adjusted mean change in DHA (%) (95%CI)	1.84 (1.27, 2.41)	2.06 (1.52, 2.60)	0.75 (-0.23, 1.72)

Change in liver fat %: ANCOVA model adjustments: age, sex, baseline liver fat %, PNPLA3 genotype, TM6SF2, BMI, diabetes, % DHA enrichment (end of study – baseline % enrichment), baseline serum triglyceride, total fat mass, change in M65 (between end of study and baseline), change in weight, use of orlistat at baseline, use of L thyroxine at baseline and capsule count.
Pairwise comparisons C/C v GG, p=0.02 and C/G v GG p=0.012.

*Change in triglycerides: ANCOVA model adjustments: age, sex, baseline liver fat %, PNPLA3 genotype, TM6SF2, BMI, diabetes, % EPA enrichment (end of study – baseline % enrichment), total fat mass, change in M65 (between end of study and baseline), change in weight, triglyceride concentration at baseline, and use of orlistat, L thyroxine, fibrates, beta blockers, and thiazide diuretics at baseline and capsule count. Pairwise comparisons C/C v GG, p=0.8 and C/G v GG p=0.71

§Change in DHA % enrichment: ANCOVA model adjustments: , age, sex, baseline liver fat %, PNPLA3 genotype, TM6SF2, BMI, diabetes, % DHA baseline, baseline serum triglyceride, total fat mass, change in M65 (between end of study and baseline), change in weight, use of orlistat at baseline, use of L thyroxine at baseline and capsule count. Pairwise comparisons C/C v GG, p=0.06 and C/G v GG p=0.023

Supplementary Table: Differences between baseline and end of study of participant characteristics according to PNPLA3 genotype

Phenotypes	Genotypes Placebo				Genotypes Active			
	CC	**CG**	**GG**	***P value***	**CC**	**CG**	**GG**	***P value***
Age	54	55	54	0.9	52	45	49	0.1
Sex (Male/Female)	12/7	14/6	7/3	n/a	9/14	13/10	1/2	n/a
Diabetes (y/n)	12/7	15/5	5/5	n/a	15/8	15/8	3/0	n/a
Waist circumference (cm)	2.8 (8.3)	-1.8 (4.4)	0.4 (4.0)	0.07	-0.4 (3.4)	0.8 (5.3)	-5 (2.1)	0.2
Weight (kg)	-2.0 (4.0)	1.6 (4.6)	-0.65 (3.7)	0.03	0.7 (3.6)	0.2 (5.6)	2.8 (1.6)	0.7
BMI (kg/m^2)	-0.6 (1.4)	0.5 (1.6)	-0.3 (1.4)	0.04	0.3 (1.3)	0.1 (1.8)	0.8 (0.6)	0.8
Serum triglyceride (mmol/L)	0.0 (0.6)	0.2 (1.2)	0.3 (0.8)	0.5	-0.1 (1.2)	-0.3 (1.0)	0.0 (n/a)	0.6
ALT (IU/L)	-1.0 (23)	-3 (21.7)	-14 (34.0)	0.4	3.0 (37.2)	-3.0 (20.0)	3.0 (n/a)	0.4
AST (IU/L)	-3.0 (17)	-1.0 (10.7)	-9.0 (31.5)	0.3	-0.5 (20.7)	1.0 (14.0)	-1.5 (n/a)	0.8
MRS liver fat %	-7.6 (10)	-3.7 (7.6)	-0.4 (9.3)	0.1	-6.3 (15.2)	-10.2 (18.8)	8.1 (4.0)	0.3
Erythrocyte EPA (%)	0.1 (0.3)	-0.04 (0.3)	0.1 (0.3)	0.1	1.9 (1.4)	2.1 (1.3)	1.0 (0.3)	0.5
Erythrocyte DHA (%)	3.6 (1.4)	4.7 (1.4)	3.8 (1.4)	0.05	3.8 (0.9)	4.1 (1.5)	3.1 (1.0)	0.3

Variables that are normally distributed are expressed as mean (standard deviation (SD)). Variables that are non-normally distributed are expressed as median (inter-quartile range (IQR)). (CC = 148II, C/G=148 IM, GG= 148MM). *P value (difference between groups)*

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