RESEARCH PAPER

# *ELOVL2* gene polymorphisms are associated with increases in plasma eicosapentaenoic and docosahexaenoic acid proportions after fish oil supplement

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**Abstract** Fish oil supplementation provides an inconsistent degree of protection from cardiovascular disease (CVD), which may be attributed to genetic variation. Single nucleotide polymorphisms (SNPs) in the elongation-of-very-long-chain-fatty-acids-2 (*ELOVL2*) gene have been strongly associated with plasma proportions of n-3 long-chain polyunsaturated fatty acids (LC-PUFA). We investigated the effect of genotype interaction with fish oil dosage on plasma n-3 LC-PUFA proportions in a parallel double-blind controlled trial, involving 367 subjects randomised to treatment with 0.45, 0.9 and 1.8 g/day eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) (1.51:1) or olive oil placebo for 6 months. We genotyped 310 subjects for *ELOVL2* gene SNPs rs3734398, rs2236212 and rs953413. At baseline, carriers of all minor alleles had lower proportions of plasma DHA than non-carriers ($P = 0.021$–$0.030$). Interaction between genotype and treatment was a significant determinant of plasma EPA ($P < 0.0001$) and DHA ($P = 0.004$–$0.032$). After the 1.8 g/day dose, carriers of *ELOVL2* SNP minor alleles had approximately 30 % higher proportions of EPA ($P = 0.002$–$0.004$) and 9 % higher DHA ($P = 0.013$–$0.017$) than non-carriers. Minor allele carriers could therefore particularly benefit from a high intake of EPA and DHA in maintaining high levels of plasma n-3 PUFA conducive to protection from CVD.

**Keywords** Docosahexaenoic acid ·
Eicosapentaenoic acid · Elongase · Fish oil · Single nucleotide polymorphism

This study was conducted on behalf of the MARINA study team.

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## Introduction

Evidence from epidemiological, observational and clinical trial studies has led to the recommendation of n-3 long-chain polyunsaturated fatty acid (LC-PUFA) consumption to prevent cardiovascular disease (CVD). In the United Kingdom, the Standing Advisory Committee on Nutrition (2004) suggested that one portion of oily fish per week providing 3.2 g n-3 LC-PUFA (0.45 g/day) might be effective in primary prevention. Controlled trials in patients with pre-existing CVD suggest that an additional 0.85–1.8 g/day n-3 LC-PUFA is needed to provide protection (Marchioli et al. 2009; Yokoyama et al. 2007), equivalent to consuming oily fish 2–4 times per week. Powerful cardioprotective effects of eicosapentaenoic acid (20:5n-3, EPA) and docosahexaenoic acid (22:6n-3, DHA) in fish oil have been reported in a large number of human trials (reviewed by Mozaffarian and Wu 2011; Delgado-Lista et al. 2012). Most consistent benefits are seen for coronary heart disease mortality and sudden cardiac death, but potential effects on other cardiovascular outcomes are less well established, with conflicting evidence for effects on non-fatal myocardial infarction, ischaemic stroke and heart failure. Several studies and meta-analyses have failed to demonstrate any significant benefits (Kromhout et al. 2010; ORIGIN trial investigators 2012; Jump et al. 2012; Kotwal et al. 2012; Rizos et al. 2012). Inconsistencies may

relate to power of studies, characteristics of the subjects, the primary outcome, n-3 PUFA dosage and duration of the treatment. Consistent effects on intermediate biomarkers of cardiovascular risk are usually observed only at intake levels above 2 g/day and even at these higher doses, significant inter-individual variability in response has been observed (Minihane 2010). Supplementation with fish oil concentrates in excess of 3 g/day, which is much greater than most dietary levels, in subjects with raised serum triacylglycerol (TAG) show reductions ranging from 19 to 47 % and an increase in low-density lipoprotein (LDL) cholesterol from 10 to 46 % (Bays 2006). This suggests that a range of gene variants may underpin variability in n-3 LC-PUFA metabolism or pathophysiological pathways contributing to CVD risk that are influenced by changes in EPA and DHA availability.

A significant proportion of fatty acids of dietary or endogenous origin undergo elongation by membrane-bound enzymes in the endoplasmic reticulum (Guillou et al. 2010). The synthesis of LC-PUFA from $C_{20}$ substrates involves two consecutive elongation steps and a delta-6 desaturation step, followed by β-oxidation. Members of the elongation-of-very-long-chain-fatty-acids (ELOVL) gene family encode elongases specific for substrate chain length and degree of unsaturation. Human elongase-5 (ELOVL5) elongates $C_{18}$ and $C_{20}$ PUFA (Leonard et al. 2000) and elongase-2 (ELOVL2) is active with $C_{20}$ and $C_{22}$ PUFA (Leonard et al. 2002). In the n-3 LC-PUFA biosynthetic sequence, the delta-5 desaturase FADS1 catalyses the formation of EPA. ELOVL2 catalyses the two sequential elongation reactions converting EPA to docosapentaenoic acid (DPA, 22:5 n-3) thence to 24:5 n-3. The delta-6 desaturase FADS2 then converts 24:5 n-3–24:6 n-3, the precursor of DHA. The pathway is shown in online resource 1.

A number of genome-wide association studies (GWAS) have identified single nucleotide polymorphisms (SNPs) in the chromosome 6 region that includes ELOVL2 to be strongly associated with plasma fatty acid proportions (Aulchenko et al. 2009; Sabatti et al. 2009; Tanaka et al. 2009; Illig et al. 2010). A subsequent meta-analysis confirmed a number of minor allele associations with higher EPA and DPA and lower DHA proportions (Lemaitre et al. 2011). Higher enzyme substrate and lower product proportions imply SNP association with reduced enzyme activity, but to date there is no evidence of functionality.

The aim of the Modulation of Atherosclerosis Risk by Increasing doses of N-3 fatty Acids (MARINA) randomised controlled dietary intervention trial was to investigate whether the recommended consumption of n-3 LC-PUFA equivalent to one portion of oily fish per week was sufficient to modify CVD risk factors in healthy subjects or whether higher intakes were required. In this study of 310 recruits, we first investigated association of ELOVL2 SNPs rs3734398, rs2236212 and rs953413 with plasma proportions of EPA, DPA and DHA at baseline. After randomisation to treatment with placebo or EPA and DHA (1.51:1) at 0.45, 0.9 and 1.8 g/day for 6 months, we first established significant interaction between ELOVL2 SNP genotypes and treatment and then investigated the effect of minor allele carriage on plasma n-3 LC-PUFA proportions.

## Methods

### Subjects

The MARINA trial was a single-centre dietary intervention study of randomised double-blind parallel design, to test the effects of three daily doses of EPA and DHA on endothelial function and CVD risk factors (Sanders et al. 2011). The study was conducted at King's College London between April 2008 and October 2010 and approved by the St Thomas' Hospital NHS Research Ethics Committee (NREC 08/H0802/3). The study was registered at controlled-trials.com as ISRCTN66664610. Written informed consent was given by participants, who were healthy non-smoking men and women aged between 45 and 70 years, recruited through media advertisements and screened as described previously (Sanders et al. 2011). The participants (a total of 367) were randomised to treatment. During an initial run-in period of 4 weeks, participants took olive oil (British Pharmacopoeia specification) placebo capsules whilst restricting oily fish intake, after which baseline measurements of outcome variables were made. During dietary intervention, capsules containing placebo or EPA and DHA (1.51:1) at three doses (0.45, 0.9 and 1.8 g/day) were supplied at regular intervals. The present investigation was based on measurements taken at baseline and after 6 months and compliance was verified by assessment of DHA and EPA in erythrocyte phosphoglycerides at this time. Supply of oil blends and quality control analysis was by Croda Chemicals Europe Ltd. (Hull, UK) and encapsulation in gelatine was by Powerhealth (Pocklington, UK).

### Blood sampling and analysis

Blood samples for analysis of fatty acid proportions were drawn after a minimum 8-h overnight fast preceded by a low-fat evening meal (<10 g fat, 3 MJ) and serum was stored at −45 °C until analysed. Plasma total fatty acids were determined by capillary GLC as previously described (Sanders et al. 2011), substituting toluene for benzene and using pentadecanoic acid as an internal standard (Lepage and Roy 1988).

## Selection of SNPs

Selection of *ELOVL2* SNPs was based on associations with EPA, DPA and DHA at genome-wide significance threshold ($P < 5 \times 10^{-8}$) reported in a meta-analysis of GWAS of plasma phospholipid n-3 PUFAs in 8,866 participants of European ancestry in five population-based studies from the Cohorts for Heart and Aging Research in Genomic Epidemiology (CHARGE) Consortium (Lemaitre et al. 2011). The three SNPs selected were those having minor allele frequencies >10 % and the highest overall rankings for association with EPA, DPA and DHA as percentage of total fatty acids. The reported *P*-values for genome-wide associations with n-3 PUFA proportions in the CHARGE sample were as follows (*ELOVL2* SNP ranking in parentheses): EPA: rs3734398 $P = 3.99 \times 10^{-12}$ (3rd); rs2236212 $P = 1.97 \times 10^{-12}$ (2nd); rs953413 $P = 1.81 \times 10^{-11}$ (9th); DPA: rs3734398 $P = 9.61 \times 10^{-44}$ (1st); rs2236212 $P = 2.87 \times 10^{-43}$ (2nd); rs953413 $P = 9.07 \times 10^{-42}$ (12th); DHA: rs3734398 $P = 1.65 \times 10^{-15}$ (3rd); rs2236212 $P = 1.26 \times 10^{-15}$ (1st); rs953413 $P = 2.98 \times 10^{-15}$ (4th). SNP rs3734398 (T/C) is located at position 10982973; rs2236212 (G/C) at position 10995015; and rs953413 (G/A) at position 11012859 on chromosome 6 (NCBI Genome Build 37.3, November 2012).

## DNA extraction and SNP genotyping

DNA was extracted from buffy coats as described previously (Al-Hilal et al. 2013). Genotyping was performed on the 310 participants who completed the study and for whom DNA was available by KBiosciences (Hoddesdon, UK), using the KASPar system. Genotype accuracy, as assessed by inclusion of duplicates in the array was 98 % and negative controls (water blanks) were included on each plate. The mean genotyping success rate was 91.46 % (91.35–91.67 %).

## Haplotype analysis

Haplotype analysis was performed using the graphical JAVA interface of the THESIAS software package (Tregouet and Garelle 2007) (available online at http://ecgene.net/genecanvas). This program is based on the maximum likelihood model linked to the SEM algorithm (Tregouet et al. 2004) and used to statistically reconstruct haplotypes in unrelated individuals and perform haplotype-based association analysis of phenotypes. Covariate-adjusted haplotype effects can be investigated.

## Statistical analysis

All genotype distributions were tested for deviation from Hardy–Weinberg equilibrium using a $\chi^2$ test with 1 degree of freedom (*df*) ($P > 0.05$). Inter-locus linkage disequilibrium (LD) based on observed numbers of diplotypes was established using CubeX software (Gaunt et al. 2007) (available online at http://www.oege.org/software/cubex/) represented in a correlation matrix as squared correlation ($r^2$) measures between each pair of SNP loci. Statistical analyses were carried out using the SPSS, version 20.0, for Windows (SPSS Inc, Chicago, IL, USA). Normal distribution of outcome variables was confirmed by Q–Q plots. Differences in quantitative variables between treatment groups at baseline were assessed by one-way analysis of variance (ANOVA). Due to the limited sample size, SNP genotype association analyses were based on a dominant inheritance model. Multivariate ANOVA was used to allow for multiple testing of genotype associations with proportions of the three n-3 LC-PUFA. All *P*-values were adjusted for the covariates body mass index (BMI), age, gender and ethnicity. The presence of any significant difference in SNP genotype frequencies between the four treatment groups was ascertained by $\chi^2$ test with 6 *df* ($P > 0.05$). Univariate ANOVA was used to assess interaction between genotypes and treatment. BMI, age, gender and ethnicity were added to the model as covariates to adjust for possible confounding effects. All data presented in text and tables are expressed as mean ± SD or means (95 % CI). Significance was taken as $P < 0.05$.

## Results

### Characteristics of subjects

Table 1 shows the characteristics of subjects after a 4-week run-in on placebo. Data are presented for the 310 participants out of 367 randomised to treatment, who completed the study and for whom DNA was available for genetic analysis. The number of participants allocated and drop-out rates did not differ significantly between treatment groups (Sanders et al. 2011). Women, mostly postmenopausal, outnumbered men by approximately 1.6:1 and about 20 % of the sample was non-white, with similar proportions of Asian and black participants. The average BMI ($25.64 \pm 3.80$ kg/m$^2$) and the mean waist circumferences ($95.31 \pm 9.60$ cm in men and $84.37 \pm 10.67$ cm in women) were slightly greater than cut-offs, indicating risk of metabolic syndrome (BMI > 25 kg/m$^2$ and waist circumference >94 cm in men and >80 cm in women) (Sanders et al. 2011). There were no significant differences in these measures between the four treatment groups at baseline ($P > 0.05$).

### SNP allele and genotype frequencies

Three SNPs in the *ELOVL2* gene, rs3734398, rs2236212 and rs953413 were genotyped. The minor allele and

**Table 1** Characteristics of study participants at baseline by randomised treatment

|  | Placebo $n = 70$ | 0.45 g/day $n = 80$ | 0.9 g/day $n = 80$ | 1.8 g/day $n = 80$ | $P*$ |
|---|---|---|---|---|---|
| Gender |  |  |  |  |  |
| Male (%) $n$ | 31, 44 | 30, 38 | 30, 38 | 29, 36 |  |
| Female (%) $n$ | 39, 56 | 50, 62 | 50, 62 | 51, 64 |  |
| Age, years | 55.37 ± 6.85 | 55.13 ± 6.84 | 55.15 ± 6.60 | 55.02 ± 6.71 | 0.995 |
| BMI, kg/m$^2$ | 26.13 ± 3.72 | 25.14 ± 3.88 | 26.16 ± 4.01 | 25.15 ± 3.50 | 0.172 |
| Waist circumference, cm | 89.50 ± 11.74 | 87.33 ± 11.80 | 89.42 ± 11.87 | 88.03 ± 11.00 | 0.602 |
| Plasma fatty acid, % total |  |  |  |  |  |
| EPA | 1.17 ± 0.59 | 1.10 ± 0.61 | 1.11 ± 0.56 | 1.10 ± 0.57 | 0.901 |
| DPA | 0.65 ± 0.10 | 0.64 ± 0.12 | 0.64 ± 0.13 | 0.65 ± 0.13 | 0.897 |
| DHA | 2.37 ± 0.68 | 2.44 ± 0.77 | 2.36 ± 0.67 | 2.47 ± 0.68 | 0.961 |

Data are presented for subjects who completed the study and for whom DNA was available ($n = 310$). Measurements taken at baseline after 4-week run-in on normal diet with olive oil placebo supplement are shown for each randomised treatment group: placebo and EPA and DHA (1.51:1) at the daily doses shown. Values are $n$ (%) for male/female or mean ± SD for all other variables

*BMI* body mass index; *EPA* eicosapentaenoic acid; *DPA* docosapentaenoic acid; *DHA* docosahexaenoic acid

\* Significance of differences between treatment groups assessed by one-way ANOVA. *P*-value for difference in age, adjusted for BMI, gender and ethnicity; for differences in BMI and waist circumference, adjusted for age, gender and ethnicity; for differences in plasma fatty acid proportions, adjusted for BMI, age, gender and ethnicity

**Table 2** Proportions of EPA, DPA and DHA in plasma stratified by *ELOVL2* SNP genotype at baseline

|  | rs3734398 | | | rs2236212 | | | rs953413 | | |
|---|---|---|---|---|---|---|---|---|---|
|  | TT $n = 90$ | TC + CC $n = 192$ | $P*$ | GG $n = 91$ | GC + CC $n = 191$ | $P*$ | GG $n = 80$ | GA + AA $n = 208$ | $P*$ |
| EPA | 1.15 (1.03,1.26) | 1.10 (1.02,1.19) | 0.528 | 1.15 (1.04,1.26) | 1.11 (1.02,1.19) | 0.533 | 1.16 (1.03,1.28) | 1.10 (1.02,1.18) | 0.559 |
| DPA | 0.65 (0.62,0.67) | 0.65 (0.63,0.66) | 0.950 | 0.65 (0.62,0.67) | 0.65 (0.63,0.66) | 0.877 | 0.64 (0.62,0.67) | 0.65 (0.63,0.66) | 0.620 |
| DHA | 2.53 (2.39,2.68) | 2.37 (2.27,2.46) | 0.021 | 2.53 (2.39,2.68) | 2.37 (2.27,2.47) | 0.023 | 2.54 (2.39,2.69) | 2.37 (2.28,2.47) | 0.030 |

Data are presented for subjects for whom DNA samples and plasma fatty acid measurements were available ($n = 310$). Data show mean (95 % CI) for each LC-PUFA as a percentage of total fatty acids measured at baseline after 4-week run-in on background diet with placebo supplement

*EPA* eicosapentaenoic acid; *DPA* docosapentaenoic acid; *DHA* docosahexaenoic acid

\* Significance of genotype association with proportions of fatty acids with respect to SNP genotype based on a dominant model was tested by multivariate ANOVA. *P*-values were adjusted for BMI, age, gender and ethnicity

genotype frequencies in all subjects who completed the study ($n = 310$) are shown in online resource 2. Genotype distributions did not deviate from Hardy–Weinberg expectations and minor allele frequencies were in close agreement with those listed for Europeans on the NCBI SNP database (2012).

*ELOVL2* SNP genotype associations with plasma LC-PUFA proportions at baseline

Table 2 shows plasma proportions of ELOVL2 substrate EPA, product DPA and downstream derivative DHA, after a 4-week run-in on normal diet with placebo supplement, stratified by rs3734398, rs2236212 and rs953413 genotypes. Based on a dominant model, only the associations with DHA were significant after adjustment for BMI, age,

gender and ethnicity ($P < 0.05$). Carriers of the minor alleles of each *ELOVL2* SNP had a lower mean proportion of plasma DHA than non-carriers.

*ELOVL2* SNP haplotype reconstruction

Any one of a set of SNPs in LD associated with a phenotype may be potentially functional; alternatively, all SNPs could be in LD with the functional site; or observed SNP associations could reflect that of another unknown marker on the same haplotype. The latter case would be indicated by a stronger association with haplotype than with individual SNPs. If haplotype and individual SNP associations have similar levels of significance, SNPs in LD are unlikely to exert independent effects. To attempt to differentiate between these possibilities, we reconstructed haplotypes

and compared associations with plasma n-3 LC-PUFA proportions at baseline with those of individual SNPs.

Statistical reconstruction of haplotypes in unrelated individuals, for whom phase is unknown, is possible when SNPs are in strong LD. We confirmed this by determining pairwise LD, based on diplotypes available for 282 MARINA subjects. The pairwise squared correlations were as follows: rs3734398 versus rs2236212, $r^2 = 0.872$; rs3734398 versus rs953413, $r^2 = 0.922$; rs2236212 versus rs953413, $r^2 = 0.813$. Reconstruction of haplotypes of the SNPs (rs3734398–rs2236212–rs953413) was based on participants for whom all genotypes were available, to avoid errors resulting from missing data. Plasma EPA, DPA and DHA proportions were available for 255 of these subjects. Five of the eight possible haplotypes were represented, with frequencies ranging from 0.2 to 50.4 % (shown in online resource 3). Two haplotypes were represented at frequency >5 %: the most common haplotype 1 carried the major alleles T–G–G at all loci (frequency 50.4 %) and haplotype 2 carried the minor alleles C–C–A at all loci (frequency 45.1 %).

### ELOVL2 SNP haplotype associations with plasma LC-PUFA proportions at baseline

The associations between haplotype and plasma proportions of EPA, DPA and DHA were not significant ($n = 255$; $P = 0.425$; $P = 0.418$; $P = 0.108$, respectively). $P$-values for haplotype associations were not substantially more significant than those for the single SNP genotypes (Table 2). There were no significant differences in the effects of a single copy of haplotype 2 compared with reference haplotype 1 on plasma EPA, DPA and DHA proportions (data not shown).

### Interaction between ELOVL2 SNP genotypes and treatment

We previously reported significant increases compared with baseline in the proportions of plasma EPA, DPA and DHA, after treatment of MARINA subjects with three doses of EPA and DHA for 6 months (Al-Hilal et al. 2013). We aimed to first discover whether interaction between treatment and genotype of each SNP was a significant determinant of plasma n-3 LC-PUFA proportions and, if confirmed, to test the significance of differences between non-carriers and carriers of the minor alleles of the ELOVL2 SNPs after each dose.

There were no significant differences in SNP genotype frequencies between the treatment groups: rs3734398, $\chi^2 = 3.42$, 6 $df$, $P = 0.76$; rs2236212 $\chi^2 = 5.97$, 6 $df$, $P = 0.43$; rs953413 $\chi^2 = 4.24$, 6 $df$, $P = 0.64$. Two-way interaction between treatment and genotype of all three

ELOVL2 SNPs was a highly significant determinant of plasma EPA proportions ($P < 0.0001$). Interaction also significantly influenced DHA proportions, as follows: rs3734398, $P = 0.008$; rs2236212, $P = 0.004$; rs953413, $P = 0.032$. Interaction between treatment and genotypes of all SNPs was not a significant determinant of plasma DPA. Therefore, we examined differences only in plasma EPA and DHA between genotype groups with respect to dose. Significant differences in proportions of EPA and DHA between non-carriers and carriers of all three ELOVL2 SNP minor alleles were seen only after the highest dose of 1.8 g/day EPA and DHA. In carriers of the minor allele of rs3734398, the plasma proportion of EPA was 25.7 % higher and DHA 8.7 % higher than the proportion in non-carriers (Table 3). In carriers of the minor allele of rs2236212, plasma EPA was 25.7 % higher and DHA 9.0 % higher (Table 4), and in rs953413 minor allele carriers, EPA was 29.7 % higher and DHA 8.2 % higher than in non-carriers (Table 5).

## Discussion

We investigated associations of genotypes of three SNPs in the ELOVL2 gene with proportions of plasma n-3 LC-PUFA before and after dietary supplementation with EPA and DHA. All minor alleles were significantly associated with lower proportions of DHA at baseline. Haplotype analysis infers that these SNPs in strong LD do not have independent effects on n-3 PUFA proportions. Interaction between genotype and treatment was a significant determinant of plasma EPA and DHA. After treatment with the highest dose of 1.8 g/day, proportions of EPA and DHA were significantly higher in carriers of the ELOVL2 SNP minor alleles than in non-carriers.

The object of the MARINA study was to ascertain in healthy men and women at moderate risk of developing CVD whether intakes of EPA and DHA of 0.45, 0.9 and 1.8 g/day, equivalent to those provided by 1, 2 and 4 portions of oily fish per week, affected predictors of CVD risk. Intakes in the range of 0.7–1.5 g/day provided either as oily fish (Griffin et al. 2006) or as purified lipids (Caslake et al. 2008) in healthy subjects have shown a small effect on lowering TAG (8–11 %) and a 2–3 % increase in LDL—cholesterol concentration. With regard to the plasma lipid profile, we previously found a 15 % reduction in TAG concentration in women after 1.8 g/day, showing a clear dose–response relationship with increasing intakes ($P = 0.002$), but no significant effect on lipoprotein-cholesterol (Sanders et al. 2011). We also found significant increases in the plasma proportions of EPA, DPA and DHA (Al-Hilal et al. 2013). After 1.8 g/day, the increase with respect to baseline was greatest in EPA (2.75 %),

**Table 3** Proportions of plasma EPA and DHA after treatment, stratified by *ELOVL2* rs3734398 genotype

| Treatment | $n$ (TT/TC + CC)[a] | EPA | | | DHA | | |
|---|---|---|---|---|---|---|---|
| | | TT | TC + CC | $P$* | TT | TC + CC | $P$* |
| Placebo | 22/44 | 1.00 (0.86,1.13) | 0.96 (0.86,1.06) | 0.941 | 2.23 (2.08,2.37) | 2.22 (2.12,2.32) | 0.714 |
| 0.45 g/day | 19/50 | 2.01 (1.65,2.38) | 1.78 (1.55,2.00) | 0.180 | 3.02 (2.79,3.24) | 2.83 (2.70,2.97) | 0.035 |
| 0.90 g/day | 26/48 | 2.52 (2.25,2.78) | 2.25 (2.06,2.44) | 0.018 | 3.36 (3.17,3.55) | 3.34 (3.20,3.47) | 0.282 |
| 1.80 g/day | 22/47 | 3.34 (2.87,3.82) | 4.20 (3.88,4.52) | 0.003 | 3.90 (3.68,4.13) | 4.24 (4.08,4.39) | 0.016 |

Data show mean (95 % CI) follow-up values for EPA and DHA as a percentage of total fatty acids, adjusted for baseline. Subjects received supplements of EPA and DHA (1.51:1) at the daily doses shown for 6 month

*EPA* eicosapentaenoic acid; *DHA* docosahexaenoic acid

* Significance of genotype association with proportions of fatty acids with respect to SNP genotype based on a dominant model was tested by multivariate ANOVA. Interaction between genotype and treatment tested by univariate ANOVA was a significant determinant of EPA ($P < 0.0001$) and DHA ($P = 0.008$). All $P$-values were adjusted for baseline values, BMI, age, gender and ethnicity

[a] $n$ = number of subjects in genotype groups TT and TC + CC

**Table 4** Proportions of plasma EPA and DHA after treatment, stratified by *ELOVL2* rs2236212 genotype

| Treatment | $n$ (GG/GC + CC)[a] | EPA | | | DHA | | |
|---|---|---|---|---|---|---|---|
| | | GG | GC + CC | $P$* | GG | GC + CC | $P$* |
| Placebo | 23/42 | 0.98 (0.84,1.11) | 0.98 (0.88,1.08) | 0.727 | 2.22 (2.08,2.36) | 2.23 (2.13,2.34) | 0.553 |
| 0.45 g/day | 18/50 | 2.04 (1.67,2.42) | 1.76 (1.54,1.99) | 0.410 | 3.05 (2.82,3.28) | 2.82 (2.69,2.96) | 0.027 |
| 0.90 g/day | 29/49 | 2.52 (2.26,2.78) | 2.27 (2.08,2.46) | 0.131 | 3.36 (3.17,3.55) | 3.34 (3.20,3.48) | 0.281 |
| 1.80 g/day | 23/47 | 3.34 (2.88,3.80) | 4.20 (3.88,4.52) | 0.002 | 3.89 (3.67,4.11) | 4.24 (4.08,4.39) | 0.013 |

Data show mean (95 % CI) follow-up values for EPA and DHA as a percentage of total fatty acids, adjusted for baseline. Subjects received supplements of EPA and DHA (1.51:1) at the daily doses shown for 6 month

*EPA* eicosapentaenoic acid; *DHA* docosahexaenoic acid

* Significance of genotype association with proportions of fatty acids with respect to SNP genotype based on a dominant model was tested by multivariate ANOVA. Interaction between genotype and treatment was a significant determinant of EPA ($P < 0.0001$) and DHA ($P = 0.004$). All $P$-values were adjusted for baseline values, BMI, age, gender and ethnicity

[a] $n$ = number of subjects in genotype groups GG and GC + CC

**Table 5** Proportions of plasma EPA and DHA after treatment, stratified by *ELOVL2* rs953413 genotype

| Treatment | $n$ (GG/GA + AA)[a] | EPA | | | DHA | | |
|---|---|---|---|---|---|---|---|
| | | GG | GA + AA | $P$* | GG | GA + AA | $P$* |
| Placebo | 21/45 | 1.00 (0.86,1.14) | 0.96 (0.87,1.06) | 0.934 | 2.22 (2.07,2.37) | 2.22 (2.12,2.32) | 0.544 |
| 0.45 g/day | 15/54 | 2.10 (1.70,2.51) | 1.77 (1.55,1.98) | 0.199 | 3.00 (2.75,3.25) | 2.85 (2.72,2.98) | 0.189 |
| 0.90 g/day | 23/52 | 2.55 (2.27,2.83) | 2.27 (2.09,2.46) | 0.050 | 3.35 (3.15,3.56) | 3.34 (3.21,3.48) | 0.671 |
| 1.80 g/day | 20/50 | 3.23 (2.75,3.72) | 4.19 (3.88,4.50) | 0.004 | 3.89 (3.65,4.13) | 4.21 (4.06,4.36) | 0.017 |

Data show mean (95 % CI) follow-up values for EPA and DHA as a percentage of total fatty acids, adjusted for baseline. Subjects received supplements of EPA and DHA (1.51:1) at the daily doses shown for 6 month

*EPA* eicosapentaenoic acid; *DHA* docosahexaenoic acid

* Significance of genotype association with proportions of fatty acids with respect to SNP genotype based on a dominant model was tested by multivariate ANOVA. Interaction between genotype andtreatment was a significant determinant of EPA ($P < 0.0001$) and DHA ($P = 0.032$). All $P$-values were adjusted for baseline values, BMI, age, gender and ethnicity

[a] $n$ = number of subjects in genotype groups GG and GA + AA

accompanied by a smaller change in DPA, the product of the first elongase reaction (0.28 %). 24:5n-3, product of the second elongase reaction was not detected, presumably because of rapid conversion via 24:6n-3 to DHA, which increased by 1.6 %. Therefore, at the highest dose, availability of EPA seemed not to limit the rate of formation of downstream products DPA and DHA. Minimal changes in EPA, DPA and DHA seen in the placebo group at the end

point excluded the possibility that stimulation by MUFA in placebo may have diminished the apparent effects of treatment.

Genome-wide association studies have identified a number of SNPs in the *ELOVL2* gene to be strongly associated with plasma EPA, DPA and DHA proportions. In the InCHIANTI study ($n = 1,075$), the *ELOVL2* SNP rs953413 minor allele was associated with higher EPA and lower DHA, and in the replication cohort GOLDN ($n = 1,076$) with higher DPA and lower DHA (Tanaka et al. 2009). In a meta-analysis ($n = 8,866$), the rs2236212 minor allele was most strongly associated with increased substrates EPA ($P = 1.97 \times 10^{-12}$) and DPA ($P = 2.87 \times 10^{-43}$) and decreased downstream product DHA ($P = 1.26 \times 10^{-15}$) (Lemaitre et al. 2011). Associations of *ELOVL2* SNP rs3734398, rs2236212 and rs953413 minor alleles with increased plasma EPA and DPA and reduced DHA proportions are suggestive of reduced enzyme activity. In MARINA subjects at baseline, we confirmed a significantly lower proportion of DHA in *ELOVL2* minor allele carriers compared with non-carriers but found no significant differences in EPA and DPA.

Location of the three *ELOVL2* SNPs within a strong LD block makes identification of a causal SNP difficult. To investigate whether the multiple markers had independent effects on the n-3 PUFA proportions or were simply in LD with each other, genotypes were phased into haplotypes and used as the unit of analysis. It is impossible to define the combination of haplotypes carried by any one individual, but all possible combinations can be computed and techniques like the SEM algorithm (Tregouet et al. 2004) incorporated in the THESIAS program (Tregouet and Garelle 2007) can be used to assign a probability to each haplotype pair. Haplotype associations with n-3 PUFAs at baseline were not substantially more significant than those for the single SNP genotypes. The DHA data suggest that the three SNPs analysed are potential LD markers of a site influencing ELOVL2 activity. Absence of any stronger associations between EPA, DPA or DHA and haplotype than with single SNPs suggests that these multiple markers in LD do not have independent effects on the n-3 PUFA proportions.

A limited number of interactions between gene variants and fish oils, EPA, DHA and total n-3 LC-PUFA treatment that influence phenotype have been reported (Minihane 2010; Olano-Martin et al. 2010; Grimble et al. 2002; Pishva et al. 2010). However, few gene × treatment interactions have been followed up in other cohorts or confirmed in independent samples (Madden et al. 2011). In general, the rate of bioconversion of n-3 LC-PUFA is relatively low in humans. Conversion of α-linolenic acid (ALA, 18:3n-3) to EPA is estimated at 0.2–6 % and to DHA at 0–0.05 % (Burdge 2006). Even so, highly

significant associations between SNPs in both desaturase and elongase genes and plasma n-3 LC-PUFA have been observed in GWAS (Lemaitre et al. 2011). In MARINA subjects, we found that minor allele carriage associated with decreased activities of delta 5 (FADS1) and delta 6 (FADS2) desaturases was predictably accompanied by increased substrate and decreased product proportions. Increasing EPA and DHA intake significantly increased FADS1 and decreased FADS2 activity (Al-Hilal et al. 2013). The efficiency of the FADS1 and FADS2 enzymes together with relative substrate availability may contribute to inter-individual variation in response to n-3 LC-PUFA supplementation. Common SNPs in desaturase or elongase genes altering endogenous fatty acid metabolism are likely to contribute to variability in response to n-3 LC-PUFA intake; however, the impact of individual SNPs on CVD risk markers remains to be established.

Differences in plasma proportions of EPA and DHA between non-carriers and carriers of all *ELOVL2* minor alleles were significant only at the highest dose of 1.8 g/day. In carriers, the proportions of EPA were approximately 26–30 % higher and DHA 8–9 % higher than proportions in non-carriers. At baseline, carriers had lower plasma proportions of both n-3 LC-PUFA. Increased supply of the elongase substrate EPA following treatment was accompanied by its accumulation in carriers of variants associated with reduced enzyme activity. Accumulation of EPA seems to have enhanced enzyme activity in minor allele carriers, enabling the increase in DHA. DPA did not accumulate and other downstream products were not detected, suggesting that the second elongase did not limit the appearance of the downstream product. *ELOVL2* minor allele carriers, representing almost 70 % of the population, appeared to account for the significant increases in plasma EPA and DHA with dosage that we observed in the total sample (Al-Hilal et al. 2013).

Demonstration of significant effects of n-3 LC-PUFA treatment on clinical outcomes is limited by the time of exposure, the statistical power of the studies and the dosage supplied. Few attempts have distinguished differences in endpoints in less than 6 months, suggesting that any changes take place over much longer time scales. In addition, studies using doses lower than 0.5 g EPA/DHA may not have allowed an effective plasma concentration of the n-3 LC-PUFA to be reached. The main strength of our study lies in the use of controlled doses of EPA and DHA up to 1.8 g/day over a 6-month period, rather than reliance on estimates of n-3 LC-PUFA consumption from food frequency questionnaires or similar. Intake in subjects randomised to receive fish oil or olive oil placebo was verified by capsule counts and long-term compliance was established by measurement of fatty acids in erythrocyte membranes.

The main limitation of our study was the relatively small sample for post-hoc genetic analysis. Dietary intervention studies are by their nature relatively small scale. Most genes contributing to quantitative phenotypes confer only very modest effects, requiring large sample sizes for detection with high power. Our finding of highly significant gene × diet interaction as a determinant of the plasma proportions of both EPA ($P < 0.0001$) and DHA ($P = 0.004–0.032$) in 310 subjects must therefore be regarded with caution. In addition to power issues, studies based on small samples carry the risk of a false-positive finding, which can arise by chance or systematic bias, for example population stratification and genotyping errors or failure to correct for multiple testing across the number of SNPs or phenotypes tested. However, our genotyping success rate was good and genotypes were in Hardy–Weinberg proportions. For independent tests (e.g. multiple genes that are not in LD), a Bonferroni correction may be applied to the $P$-values; however, this is too stringent a measure to apply here. The SNPs are in strong LD and we showed that they do not have independent effects. In addition, the plasma proportions of the three LC-PUFAs are inter-related. We used multivariate ANOVA to allow for multiple testing of genotype associations with phenotypes. After this procedure, a dose of 1.8 g/day was shown to significantly increase plasma proportions of both EPA and DHA in carriers of all 3 minor alleles; however, we also observed significant decreases in EPA after the 0.9 g/day dose and a decrease in DHA after the 0.45 g/day dose in some carriers. A Bonferroni correction to take in account of testing three SNPs and three phenotypes would have raised the significance threshold from $P = 0.05$ to $P = 0.0056$. Were this to have been applied, the proportion of plasma EPA in carriers compared with non-carriers after 1.8 g/day would remain significantly higher, but not the lower proportion in carriers of two SNPs after 0.9 g/day. A similar correction for DHA data would remove all significance for the higher proportion in carriers compared with non-carriers after 1.8 g/day, as well as the lower level in carriers of two SNPs after 0.45 g/day. The effect of the 1.8 g/day dose on plasma DHA proportions is therefore probably of borderline significance, but the effect on EPA with respect to genotype would remain significant even after an over-stringent Bonferroni correction. Replication of the observed effects of treatment following prospective recruitment of larger ELOVL2 genotype groups would offer the most reliable confirmation of our results. However, heterogeneity between studies including the criteria used in selection of subjects, the study protocol and laboratory analytical methods may lead to different, valid conclusions about the role of a SNP in determining phenotype. Population heterogeneity across studies such as variations in SNP frequencies across the major population groups may also lead to differences in study outcomes.

In conclusion, ELOVL2 minor allele carriers, who have significantly lower plasma DHA than non-carriers on habitual diet, could be major beneficiaries of supplements. If our results are substantiated, advantageous effects of EPA and DHA supplemented at 1.8 g/day as a primary preventive measure would accrue to the 68–71 % of the population who carry the variants. Intervention trials powered to detect significant nutrient interaction with SNPs in determination of CVD biomarkers should eventually enable personalised dietary recommendations to reduce risk based on genotype.

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