

## Eicosapentaenoic acid and docosahexaenoic acid from fish oils: differential associations with lipid responses

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Fish-oil supplementation can reduce circulating triacylglycerol (TG) levels and cardiovascular risk. This study aimed to assess independent associations between changes in platelet eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) and fasting and postprandial (PP) lipoprotein concentrations and LDL oxidation status, following fish-oil intervention. Fifty-five mildly hypertriacylglycerolaemic (TG 1.5–4.0 mmol/l) men completed a double-blind placebo controlled cross over study, where individuals consumed 6 g fish oil (3 g EPA + DHA) or 6 g olive oil (placebo)/d for two 6-week intervention periods, with a 12-week wash-out period in between. Fish-oil intervention resulted in a significant increase in the platelet phospholipid EPA (+491 %,  $P < 0.001$ ) and DHA (+44 %,  $P < 0.001$ ) content and a significant decrease in the arachidonic acid (–10 %,  $P < 0.001$ ) and  $\gamma$ -linolenic acid (–24 %,  $P < 0.001$ ) levels. A 30 % increase in *ex vivo* LDL oxidation ( $P < 0.001$ ) was observed. In addition, fish oil resulted in a significant decrease in fasting and PP TG levels ( $P < 0.001$ ), PP non-esterified fatty acid (NEFA) levels, and in the percentage LDL as LDL-3 ( $P = 0.040$ ), and an increase in LDL-cholesterol ( $P = 0.027$ ). In multivariate analysis, changes in platelet phospholipid DHA emerged as being independently associated with the rise in LDL-cholesterol, accounting for 16 % of the variability in this outcome measure ( $P = 0.030$ ). In contrast, increases in platelet EPA were independently associated with the reductions in fasting ( $P = 0.046$ ) and PP TG ( $P = 0.023$ ), and PP NEFA ( $P = 0.015$ ), explaining 15–20 % and 25 % of the variability in response respectively. Increases in platelet EPA + DHA were independently and positively associated with the increase in LDL oxidation ( $P = 0.011$ ). EPA and DHA may have differential effects on plasma lipids in mildly hypertriacylglycerolaemic men.

**Eicosapentaenoic acid: Docosahexaenoic acid: Triacylglycerol: Plasma lipid: Low-density lipoprotein oxidation**

There is considerable evidence to support protective effects of the very-long-chain *n*-3 fatty acids against CHD (Burr *et al.* 1989; GISSI-Prevenzione Investigators, 1999). The dietary very-long-chain *n*-3 fatty acids eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), found mainly in marine oils, have a range of biological activities which may attenuate both atherosclerosis and thrombosis. These include: beneficial effects on circulating lipoproteins; platelet, endothelial, neutrophil and monocyte cell functions; vascular compliance and blood pressure (Buttriss, 1999). Although their mechanisms of action are

only partly understood, their influence on the degree of unsaturation of membrane phospholipids (and thereby membrane fluidity and function), together with regulatory effects on molecular events involved in lipid metabolism at the hepatic, adipose tissue and arterial intima level are now beginning to be elucidated. It was originally thought that both EPA and DHA had similar metabolic properties. However, recent evidence suggests that there are marked differences in the effects of the two fatty acids on the concentration and composition of plasma and membrane lipids.

**Abbreviations:** ALP, atherogenic lipoprotein phenotype; AUC, area under the curve; chol, cholesterol; DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; NEFA, non-esterified fatty acid; TG, triacylglycerol.

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The ability of fish oils to reduce fasting triacylglycerol (TG) levels is well established, with 25% and 34% reductions reported in normolipidaemic and hyperlipidaemic groups respectively (Harris, 1997). Suppression of hepatic lipogenesis together with an increased hepatic mitochondrial oxidation of fatty acids, which result in reduced endogenous VLDL synthesis and secretion, are thought to be the primary mechanisms involved (Nestel *et al.* 1984; Harris, 1989; Kendrick & Higgins, 1999). Animal (Willumsen *et al.* 1993; Berge *et al.* 1999) and *in vitro* (Wong *et al.* 1989) studies have suggested that EPA rather than DHA may be the hypotriacylglycerolaemic agent. However, divergent findings have been reported in studies of human subjects (Childs *et al.* 1990; Ågren *et al.* 1996; Rambjør *et al.* 1996; Davidson *et al.* 1997; Mori *et al.* 2000a,b).

In addition to an effect on TG, supplementation with fish oil also influences characteristics of LDL that have been linked with atherogenesis, including susceptibility of LDL to oxidation, LDL particle size, as well as circulating LDL concentration. The replacement of dietary saturated fat with *n*-6 polyunsaturated fatty acids has been shown to increase the susceptibility of LDL to oxidation (Abbey *et al.* 1993; Reaven *et al.* 1993). A number of studies have also examined the impact of supplementing with an EPA + DHA preparation on LDL oxidation, with some studies observing increased susceptibility to oxidation (Suzukawa *et al.* 1995; Hau *et al.* 1996; Oostenbrug *et al.* 1997) and others observing no effect (Bonanome *et al.* 1996; Brude *et al.* 1997; Higgins *et al.* 2000). The variability in these findings may be attributable to a number of factors, which include: the degree of EPA + DHA supplementation; the  $\alpha$ -tocopherol content of the fish oils, which is often unreported; and the methods used to assess oxidative status. Limited results indicate a modest increase in LDL particle size following fish-oil intervention (Suzukawa *et al.* 1995; Mori *et al.* 2000a,b), a response which would be considered beneficial with respect to CHD risk. In addition, *n*-3 polyunsaturated fatty acid supplementation has also been shown to impact on overall LDL-cholesterol (Chol) concentration, with a 5–10% increase commonly observed following fish-oil intervention (Harris, 1989, 1996; Suzukawa *et al.* 1995; Davidson *et al.* 1997). Davidson and co-workers reported that 0.0–2.5 g DHA/d increased LDL-cholesterol concentration in a dose-related manner (Davidson *et al.* 1997). However, other studies have failed to observe an increase in LDL-cholesterol following DHA feeding (Rambjør *et al.* 1996; Nelson *et al.* 1997).

In the present study we have examined the impact of fish-oil intervention on LDL oxidation, particle density and concentration in subjects with an atherogenic lipoprotein phenotype (ALP). This common proatherogenic profile, which is defined by moderately elevated fasting and postprandial TG, low HDL-cholesterol and a predominance of the putatively atherogenic LDL-3, is associated with a 3–6-fold increase in CHD risk (Austin *et al.* 1988; Griffin *et al.* 1994). With platelet phospholipid fatty acid composition as a marker of responsiveness to EPA + DHA supplementation, we have used univariate and multivariate statistical analysis to assess the effects of increasing EPA

and DHA, together and separately, on circulating lipoproteins. We have previously reported the overall responsiveness of this group to fish-oil supplementation as well as the impact of apolipoprotein E genotype (Minihane *et al.* 2000a).

## Materials and methods

### Subjects

The methods of recruiting and screening our ALP group has been described elsewhere (Griffin *et al.* 1999; Minihane *et al.* 2000a). In brief, the study group were recruited on the basis of a health and lifestyle questionnaire and a screening blood sample. Only individuals with an ALP lipid profile (fasting TG 1.5–4.0 mmol/l, HDL-cholesterol <1.1 mmol/l, LDL-3 >40% total LDL) were recruited. Further exclusion criteria for participation were as follows: diagnosed cardiovascular disease, liver dysfunction, diabetes, smoking, blood pressure >160/95 mmHg, haemoglobin <130 g/l, BMI >35 kg/m<sup>2</sup>, or on hypolipidaemic therapy or any other medication known to interfere with lipid metabolism. The University of Reading and West Berkshire Health Authority Ethics Committees approved the study protocols and each individual gave their written consent.

### Study design

The study was a double-blind placebo controlled crossover study where participants were randomly assigned to consume 6 × 1 g capsules of fish oil (EPAX 5500TG; Pronova, Denmark) or 6 g olive oil (placebo; Pronova)/d for 6 weeks. The fish-oil supplement was approximately 50% EPA+DHA enriched, providing 279 mg EPA and 223 mg DHA/g oil. After a 12-week wash-out period individuals were crossed-over to receive the opposite supplementation regimen. Fasting blood samples were collected at 0, 3 and 6 weeks of each supplementation period, and a postprandial assessment was carried out at the end of each intervention arm (6 weeks).

### Postprandial assessment

Subjects underwent postprandial assessment following a 12 h overnight fast. No exercise or alcohol was allowed during the previous 24 h, and in order to standardize short-term fat intake, a set evening meal, which contained <20 g fat, was provided. On arrival at the Clinical Investigation Unit, an in-dwelling cannula was inserted into the antecubital vein of the forearm and a fasting blood sample taken. Following the test breakfast (0 min, 50 g fat) and lunch (330 min, 30 g fat), blood samples were collected at 0, 30, 60, 90, 150, 210, 270, 330, 360, 390, 420 and 480 min to assess postprandial TG, glucose, insulin and non-esterified fatty acid (NEFA) responses. At 480 min, 100 IU heparin/kg was administered intravenously, and a 5 ml blood sample collected 15 min later in order to determine post-heparin lipoprotein lipase activity.

### Biochemical analysis

All blood samples were collected into 10 ml potassium EDTA tubes, and 1 ml fluoride oxalate tubes for the measurement of blood glucose. The samples were centrifuged at 1600 *g* for 10 min and the plasma stored at  $-20^{\circ}\text{C}$  for TG, total chol, NEFA, glucose and insulin analysis and at  $-80^{\circ}\text{C}$  for plasma vitamin E analysis. For HDL-chol analysis a subsample of plasma was precipitated with dextran sulfate and magnesium chloride in order to remove apolipoprotein B-containing lipoproteins (McNamara *et al.* 1994), and the supernatant fraction stored at  $-20^{\circ}\text{C}$ . LDL-chol was determined using the Friedewald formula (Friedewald & Levi, 1972). Plasma collected for lipoprotein lipase activity was stored at  $-80^{\circ}\text{C}$  whilst the plasma (10 ml) collected for LDL subclass distribution was stored at  $4^{\circ}\text{C}$  and analysed within 24 h of collection.

As previously described (Minihane *et al.* 2000a), plasma samples were analysed for TG, total chol, HDL-chol, glucose and NEFA using the Monarch Automatic Analyser (Instrumentation Laboratories Ltd, Warrington, Ches., UK), for LDL subclasses using density gradient ultracentrifugation (Griffin *et al.* 1990), for lipoprotein lipase activity using the  $^3\text{H}$ -labelled triolein technique (Nilsson-Ehle *et al.* 1980), and for insulin using a specific commercial ELISA kit (Dako Ltd, High Wycombe, Bucks., UK). The inter- and intra-assay CV for these techniques have been reported previously (Minihane *et al.* 2000b).

Postprandial TG responses were expressed as area under the curve (AUC) (0–480 min) or incremental AUC (0–480 min), calculated using the trapezoidal rule. Due to the shape of the postprandial NEFA response, NEFA AUC (0–480 min) is difficult to interpret, with circulating NEFA concentrations dropping below baseline levels in the early postprandial period, therefore in the data analysis the NEFA postprandial response was represented as NEFA AUC (270–480 min).

Platelets were extracted from whole blood according to the method of Indu & Ghafoorunissa (1992) (300 *g* for 18 min followed by 1700 *g* for 10 min), and stored at  $-80^{\circ}\text{C}$  for platelet phospholipid fatty acid analysis. Butylated hydroxytoluene was added to all solvents at a concentration of 50 mg/l to minimize auto-oxidation during lipid extraction and analysis. Phosphatidylethanolamine diheptadecanoic (100  $\mu\text{l}$ ; 0.25 mg/ml chloroform) was added as internal standard to all samples. Lipids were extracted with chloroform–methanol (2:1, v/v) according to the method of Folch *et al.* (1957) and the phospholipid fraction isolated from the crude lipid using a Sep-Pack C18 column (Waters Associates, Milford, MA, USA) (Hamilton & Comai, 1988). The phospholipids were transmethylated using sulfuric acid (150 ml/l methanol), and the fatty acids quantified by GLC using a CPSil 88 column (Chrompak, Walton-on-Thames, Surrey, UK) (Indu & Ghafoorunissa, 1992).

LDL was isolated from the platelet-poor plasma derived from the platelet isolation method described earlier. The platelet-poor plasma (6 ml) was mixed by gentle inversion with 1.5 ml sucrose solution (500 g/l) and the mixture frozen at  $-80^{\circ}\text{C}$  awaiting analysis. Upon thawing, the

sucrose was removed by dialysing the sample using cellulose dialysis tubing (6 mm diameter, 1 mm width, 0.32 mm thickness; Fischer Scientific, Loughborough, Leicester, UK) against phosphate buffer (0.14 M-NaCl, 1.9 mM- $\text{NaH}_2\text{PO}_4$ , 8.1 mM- $\text{Na}_2\text{HPO}_4$ , 100  $\mu\text{M}$ - $\text{Na}_2\text{EDTA}$ ) for 12–15 h, changing the buffer on three occasions. LDL was isolated from the plasma using a method adapted from Vieira *et al.* (1996). In brief, the density of the plasma was adjusted to 1.21 g/ml with KBr. The density-adjusted plasma (3 ml) was overlaid with 1.006 g/ml density solution in 8.9 ml Beckman Optiseal polyallomer centrifuge tube and the solution centrifuged at 65000 rpm for 3 h at  $4^{\circ}\text{C}$  in the Beckman 70.1 rotor (Beckman Coulter (UK) Ltd., High Wycombe, Bucks, UK). The orange band of LDL was extracted and further purified by adjusting the density of the LDL layer to 1.15 g/ml. The solution was overlaid with a 1.063 g/ml density solution in a 13.5 ml polycarbonate thick-walled centrifuge tube and the tubes centrifuged at 46000 rpm for 16 h at  $4^{\circ}\text{C}$ . LDL, which formed the top layer, was dialysed against 1.5–2.0 litres phosphate buffer in the presence of Chelex 100 (Bio-rad Laboratories Ltd., Hemel Hempstead, Herts., UK) for 24 h, changing the buffer four times to remove the EDTA and KBr.

LDL oxidation was assessed using a conjugated diene formation assay (Esterbauer *et al.* 1989). In this assay, the formation of conjugated dienes (breakdown products of lipid peroxidation) are monitored at 234 nm. Lipid peroxidation in LDL (50  $\mu\text{g}$  LDL protein/ml) was induced by  $\text{CuSO}_4$ , and the lag phase used as the primary index of the susceptibility of the LDL particle to oxidation. The lag phase was taken as the intercept of the tangent of the lag and propagation phases.

For  $\alpha$ -tocopherol analysis, 0.5–1 ml sodium dodecyl sulfate (10 mM) and 1 ml ethanol, which containing  $\alpha$ -tocopherol acetate as internal standard, were added to 500  $\mu\text{l}$  plasma or 180  $\mu\text{g}$  LDL. Hexane (2 ml) was added, the tube mixed for 5 min on a rotator and centrifuged at 2500 rpm for 5 min. This step was repeated three times. The hexane extracts were combined and evaporated under  $\text{N}_2$  and redissolved in 2 ml hexane. The  $\alpha$ -tocopherol content of the extracts was analysed by HPLC using a Spherisorb 5  $\mu\text{m}$  analytical column (250  $\times$  4.60 mm; Phenomenex, Ches., UK), a hexane–isopropanol (99:25:0.75, v/v) mobile phase and fluorometric detection (excitation 290 nm, emission 330 nm) (American Oil Chemists' Society, 1990).

### Statistical analysis

Group results are expressed as mean values with their standard errors. The data was checked for normality and skewed variables were log transformed prior to statistical analysis. The percentage change in the lipid outcome measures following supplementation was determined as: % changes 0–6 week fish-oil – % change 0–6 week olive-oil. The impact of fish-oil supplementation on fasting lipid levels in the overall group (*n* 55) was determined using two-way repeated measures ANOVA with time (0, 3, 6 weeks) and oil (fish oil, olive oil) as the independent variables. Post-treatment postprandial AUC and

incremental AUC, and the fatty acid composition of platelet phospholipids at the end of each arm of the study were analysed using paired Student's *t* tests. Linear associations between outcome measures were evaluated by testing Spearman's correlation coefficients and multiple linear regression analysis, with the change in serum lipids or LDL characteristics as the dependent variable and the change in platelet membrane phospholipids fatty acids, BMI and age as independent variables. Dependent variables were corrected for baseline levels prior to inclusion in the model. The validity of the regression model was monitored by the study of residuals *v.* predicted values. A *P* value <0.05 was considered significant. All statistical analysis was performed using the SPSS statistical package (version 6.1; SPSS, Chicago, IL, USA).

## Results

All fifty-five individuals completed the study. The

responsiveness of a subgroup of these individuals for which apolipoprotein E genotype data was available has been previously reported (Minihane *et al.* 2000a). The baseline mean age, BMI, lipid and LPL activity of the group is shown in Table 1. No significant changes in body weight, background diet, alcohol consumption, or exercise or medication regimen was evident over the study period. The capsules, which provided 1.67 g EPA + 1.34 g DHA/d, were well tolerated. Levels of platelet phospholipid EPA, DHA and arachidonic acid at the beginning of each arm of the study, indicated that the 12 week wash-out period was sufficient to reverse the metabolic effects of supplementation (results reported previously: Minihane *et al.* 2000a).

### Plasma lipids, glucose, insulin

Fish-oil supplementation resulted in significant reductions of 33 % ( $P < 0.001$ ), 23 % ( $P < 0.001$ ) and 9 % ( $P = 0.002$ )

**Table 1.** The baseline characteristics of the study group and the responsiveness to fish-oil intervention for 6 weeks\*  
(Mean values with their standard errors for fifty-five subjects)

|                              | Baseline |      | Change (%) |      | <i>P</i> † |
|------------------------------|----------|------|------------|------|------------|
|                              | Mean     | SEM  | Mean       | SEM  |            |
| Age (years)                  | 55       | 1    | NA         |      | NA         |
| BMI (kg/m <sup>2</sup> )     | 28.0     | 0.5  | 0.2        | 0.4  | 0.226      |
| Fasting                      |          |      |            |      |            |
| Total chol (mmol/l)          | 6.60     | 0.11 | -0.7       | 2.0  | 0.392      |
| TG (mmol/l)                  | 2.50     | 0.11 | -33.3      | 5.0  | 0.000      |
| HDL-chol (mmol/l)            | 0.95     | 0.02 | -0.3       | 3.2  | 0.905      |
| LDL-chol (mmol/l)            | 4.53     | 0.12 | 7.6        | 3.1  | 0.027      |
| LDL-chol:HDL-chol            | 4.86     | 0.18 | 10.3       | 5.4  | 0.077      |
| LDL-3 (%)‡                   | 62       | 3    | -21.1      | 9.3  | 0.040      |
| NEFA (μmol/l)                | 459      | 16   | -5.5       | 5.0  | 0.070      |
| Glucose (mmol/l)             | 5.49     | 0.09 | 3.3        | 2.2  | 0.233      |
| Insulin (pmol/l)             | 71.7     | 7.0  | -0.8       | 12.0 | 0.986      |
| Postprandial                 |          |      |            |      |            |
| TG AUC§                      | 1823     | 73   | -22.5      | 2.8  | 0.000      |
| TG incremental AUC           | 525      | 33   | -9.0       | 5.3  | 0.002      |
| Peak TG¶                     | 5.13     | 0.22 | -20.9      | 2.9  | 0.000      |
| Glucose AUC§**               | 3277     | 48   | -1.6       | 0.9  | 0.023      |
| Glucose incremental AUC  **  | 580      | 39   | -4.5       | 10.6 | 0.004      |
| NEFA AUC (270–480 min)§††    | 111026   | 3704 | -5.4       | 2.6  | 0.023      |
| LPL (nmol FA/ml per min) ‡‡  | 63       | 8    | 12.8       | 12.4 | 0.094      |
| Fatty acids (mol/100 mol) §§ |          |      |            |      |            |
| 18:2                         | 5.78     | 0.19 |            |      |            |
| 20:4                         | 24.31    | 0.57 |            |      |            |
| 20:5                         | 0.65     | 0.13 |            |      |            |
| 22:6                         | 2.64     | 0.14 |            |      |            |

NA, not applicable; chol, cholesterol; TG, triacylglycerol; NEFA, non-esterified fatty acids; AUC, area under the curve; LPL, lipoprotein lipase; FA, fatty acid; 18:2, linoleic acid; 20:4, arachidonic acid; 20:5, eicosapentaenoic acid; 22:6, docosahexaenoic acid.

\* For details of subjects, supplements and procedures, see p. 436.

† The statistical significance of the changes in fasting outcome variables were calculated using repeated-measures ANOVA with time and oil supplement (fish oil or olive oil) as the independent variables. The significance of changes in postprandial measurements were assessed using paired *t* tests, or Wilcoxon signed rank test for non-normally distributed data.

‡ Small dense LDL.

§ Calculated by the trapezoidal rule.

|| Calculated as AUC minus the baseline value.

¶ Peak postprandial TG value (0–480 min).

\*\* mmol/l per 480 min.

†† μmol/l per 210 min.

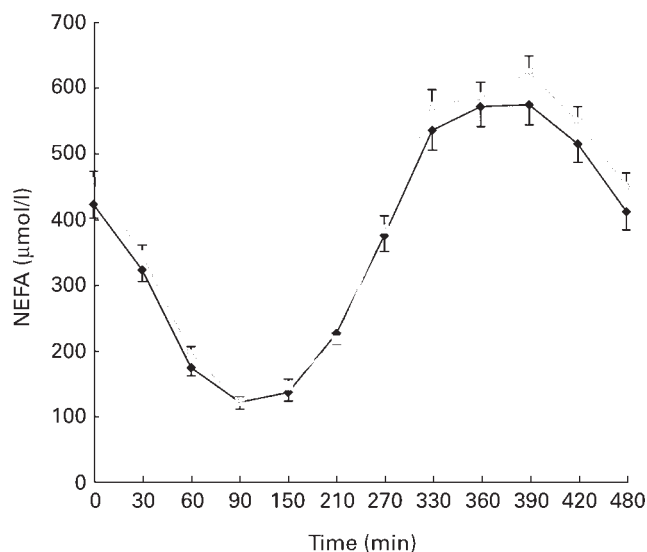
‡‡ 15 min post-heparin LPL activity.

§§ Platelet membrane phospholipid fatty acid composition.

in fasting TG, TG AUC and TG incremental AUC respectively (Table 1). For the group as a whole, little change in total chol or HDL-chol levels was evident following supplementation. However, there was a significant increase in circulating LDL-chol (+8 %,  $P=0.027$ ) but a significant reduction in the percentage of LDL as LDL-3 (-21 %,  $P=0.040$ ) was also evident. The 10 % increase in LDL-chol:HDL-chol reached borderline significance ( $P=0.077$ ). The intervention regimen had little impact on fasting or postprandial (results not shown) insulin or fasting glucose levels. However, significant decreases in glucose AUC ( $P=0.023$ ) and glucose incremental AUC ( $P=0.004$ ) were observed, suggesting improvements in insulin sensitivity. There was a trend towards a reduction in circulating NEFA levels by fish oils throughout the postprandial period, with a 5 % ( $P=0.023$ ) decrease in the late postprandial period (270–480 min) (Fig. 1). Due to large inter-individual variability in responsiveness, the 13 % increase in mean post-heparin lipoprotein lipase activity failed to reach significance ( $P=0.094$ ).

#### Platelet membrane phospholipid fatty acid composition

Figure 2 shows the change mol/100 mol in platelet phospholipid fatty acid composition from 0–6 weeks on the placebo (olive oil) and fish-oil supplements ( $n=21$ ). No significant changes in linoleic acid (LA) ( $P=0.663$ ),  $\gamma$ -LA ( $P=0.793$ ), arachidonic acid ( $P=0.490$ ), EPA ( $P=0.207$ ), or DHA ( $P=0.394$ ) levels were evident following olive-oil supplementation. Fish-oil supplementation resulted in a significant 24 % ( $P<0.001$ ) decrease in the  $\gamma$ -LA content from 1.92 to 1.45 mol/100 mol, and a 10 % ( $P<0.001$ ) decrease in arachidonic acid from 24.10 to 21.71 mol/100 mol ( $P<0.001$ ). Highly significant increases in the EPA and DHA content of the platelet membrane were



**Fig. 1.** Responsiveness of non-esterified fatty acids (NEFA) to test meals following 6 g fish-oil or olive-oil/d for 6 weeks.  $\blacklozenge$ , Fish oil;  $\blacksquare$ , placebo. For details of subjects, supplements and procedures, see p. 436. Breakfast was given at 0 min and lunch at 330 min. Values are means for fifty-five subjects with standard errors shown by vertical bars.

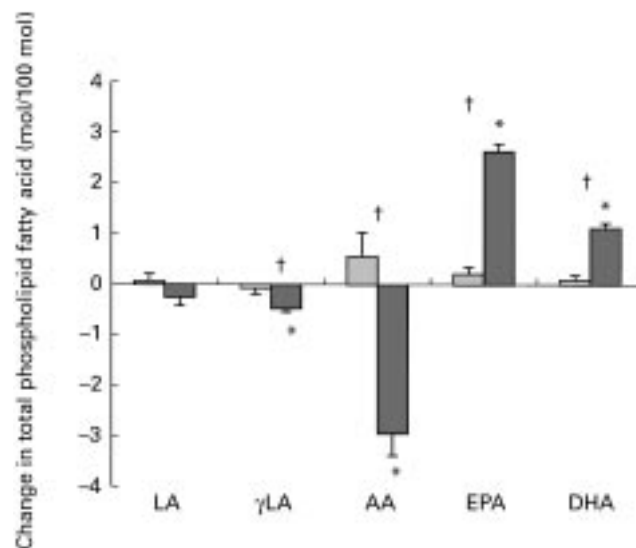
observed following fish-oil intervention. EPA levels increased 5-fold ( $P<0.001$ ), from 0.53 to 3.13 mol/100 mol, whilst DHA levels increased by 44 % from 2.50 to 3.61 mol/100 mol ( $P<0.001$ ).

#### LDL oxidation

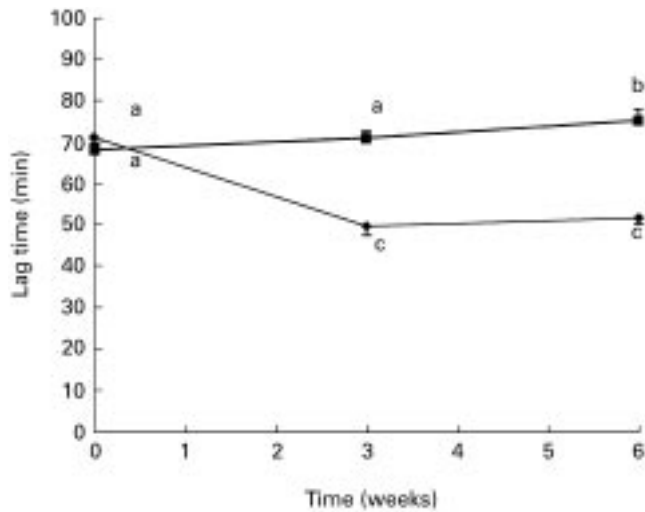
Mean values for the lag times before initiation of conjugated diene formation and LDL oxidation for the placebo and fish-oil periods are shown in Fig. 3. A 30 % reduction of the lag phase occurred after 3 weeks of fish-oil supplementation (71.2 (SEM 2.2) to 49.7 (SEM 2.2) min), which was sustained at 6 weeks (51.6 (SEM 1.5) min) ( $P<0.001$ ). The lag phase after 3 weeks on the placebo phase was similar to that at time zero (68.2 (SEM 1.6) min *v.* 71.1 (SEM 2.2) min respectively, ( $P=0.064$ )), but the mean increase of 6.9 min by week 6 was statistically significant ( $P=0.008$ ).

#### Plasma and LDL Vitamin E composition

Figure 4 gives the group mean changes in plasma and LDL  $\alpha$ -tocopherol levels. An increase in plasma  $\alpha$ -tocopherol from 15.5 (SEM 2.6) to 19.5 (SEM 2.3)  $\mu$ mol/l ( $P=0.027$ ) and LDL  $\alpha$ -tocopherol from 8.3 (SEM 0.8) to 9.4 (SEM 1.2) nmol/mg LDL protein (NS) was evident following olive-oil supplementation. On the fish-oil treatment the changes in plasma  $\alpha$ -tocopherol from 19.8 (SEM 1.0) to 19.6 (SEM 1.0)  $\mu$ mol/l and LDL  $\alpha$ -tocopherol from 10.1 (SEM 1.4) to 9.1 (SEM 1.1) nmol/mg LDL following 6 weeks of supplementation were not significant. Using repeated-measures ANOVA no treatment effect over time on plasma or LDL  $\alpha$ -tocopherol levels was evident.



**Fig. 2.** Changes in platelet phospholipid fatty acid from baseline to the end of each intervention period (6 weeks) in the fish-oil and olive-oil phases ( $n=21$ ).  $\square$ , Olive oil;  $\blacksquare$ , fish oil. LA, linoleic acid;  $\gamma$ -LA, gamma-linoleic acid; AA, arachidonic acid; EPA, eicosapentaenoic acid; DHA, docosahexaenoic acid. For details of subjects, supplements and procedures, see p. 436. Values are means with standard errors shown by vertical bars. Mean values were significantly different from those of the control group: \* $P<0.05$  Overall treatment effects were significant; † $P<0.05$  (repeated-measures ANOVA).



**Fig. 3.** The effect of dietary supplementation of 6g fish oil or olive oil/d for 6 weeks on LDL oxidation, measured as the lag phase of conjugated diene formation by copper. ♦, Fish oil, ■, olive oil. For details of subjects, supplements and procedures, see p. 436. Values are means for fifty-five subjects with standard errors shown by vertical bars. Statistical significance of the group changes was calculated using repeated-measures ANOVA, with time and oil supplement as the independent variables. <sup>a,b,c</sup>Mean values with unlike superscript letters were significantly different ( $P < 0.05$ ).

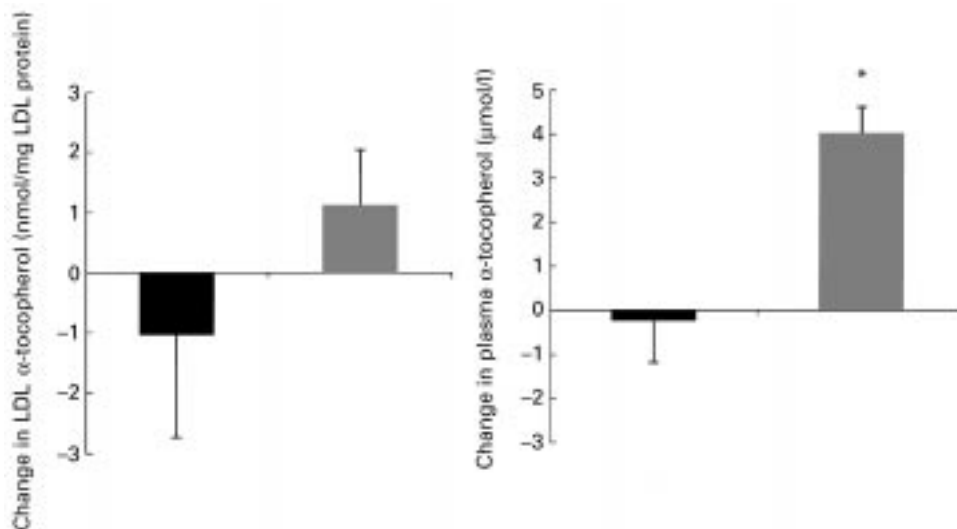
#### Correlation between changes in platelet phospholipid fatty acid composition and plasma lipid and lipoprotein levels

A significant correlation between the fall in fasting TG ( $r = -0.462$ ,  $P = 0.035$ ) and TG AUC ( $r = -0.453$ ,  $P = 0.045$ ) and the increase in the platelet content of EPA was observed (Table 2). Little association between changes in LA,  $\gamma$ -LA, arachidonic acid or DHA and fasting or postprandial TG responses were evident. However, the change in platelet DHA was positively associated with the observed increases in LDL-chol ( $r = 0.699$ ,  $P < 0.001$ ).

No significant associations between the modest changes in total chol ( $-0.7\%$ , Table 2) and HDL-chol ( $-0.3\%$ , Table 2) and any post-supplementation fatty acid changes were observed. The decrease in fasting NEFA levels was modestly, but not significantly correlated with the increase in platelet DHA ( $r = -0.391$ ,  $P = 0.088$ ), whilst the decrease in NEFA levels in the late postprandial period (NEFA AUC 270–480 min) was negatively correlated with the changes in platelet EPA ( $P = -0.570$ ,  $P = 0.009$ ). No significant association between EPA or DHA changes and glucose responsiveness was evident. Significant univariate correlation's between the increases in both EPA ( $P = 0.042$ ) and DHA ( $P = 0.027$ ) and the increase in LDL oxidizability were evident.

#### Multiple regression analysis

Multiple regression analysis was used to establish the independence of the associations observed in the univariate correlation analysis. The results are given in Table 3. The change in DHA ( $P = 0.030$ ) but not EPA ( $P = 0.293$ ), emerged as an independent determinant of the rise in LDL-chol, accounting for 16% of the variability in this outcome measure. Changes in platelet phospholipid EPA were independently associated with the decrease in fasting TG ( $P = 0.046$ ), TG AUC ( $P = 0.023$ ) and peak postprandial TG ( $P = 0.027$ ), and explained 15–20% of the variability of the changes in these variables. The enrichment of platelet EPA was also an independent determinant of the reductions in circulating NEFA levels in the late postprandial period (NEFA AUC (270–480 min),  $P = 0.015$ ), with the increase in platelet EPA being associated with a reduction in circulating NEFA. Neither EPA nor DHA emerged as independent determinants of the post-supplementation increase in LDL oxidizability. Due to multicollinearity of the variables EPA, DHA and total  $n-3$  (EPA + DHA), changes cannot be included in the same



**Fig. 4.** Changes in the  $\alpha$ -tocopherol content of LDL and plasma following the consumption of 6g olive oil or 6g fish oil/d for 6 weeks. ■, Olive oil; □, fish oil. For details of subjects, supplements and procedures, see p. 436. Values are means with standard errors shown by vertical bars. Mean value was significantly different from baseline value: \* $P < 0.027$ .

**Table 2.** Correlation coefficients (*r*) between absolute changes in fatty acid composition and changes in lipid outcome measures following supplementation of fifty-five subjects, with fish oil for 6 weeks§|| (spearmans bivariate correlation coefficients)

| Change in...              | Fatty acid |        |        |          |          |
|---------------------------|------------|--------|--------|----------|----------|
|                           | c18:2      | c20:3  | c20:4  | c20:5    | C22:6    |
| Total chol                | 0.142      | 0.112  | -0.029 | -0.235   | 0.365    |
| LDL-chol                  | 0.327      | 0.207  | -0.144 | 0.083    | 0.699*** |
| HDL-chol                  | -0.044     | -0.107 | 0.221  | -0.048   | -0.099   |
| Fasting TG                | -0.062     | -0.114 | 0.134  | -0.462*  | -0.215   |
| TG AUC¶                   | 0.084      | -0.055 | 0.029  | -0.453*  | -0.149   |
| TG incremental AUC**      | 0.232      | 0.187  | -0.004 | -0.317   | -0.027   |
| Peak TG††                 | 0.199      | 0.005  | 0.067  | -0.403§  | -0.071   |
| LDL-3 (%) ‡‡              | 0.177      | 0.138  | 0.120  | 0.037    | 0.116    |
| LDLox                     | -0.212     | 0.112  | 0.068  | -0.447*  | -0.483*  |
| Fasting NEFA              | -0.464*    | -0.284 | 0.023  | -0.147   | -0.391‡  |
| NEFA AUC (270–480 min)¶§§ | 0.021      | 0.202  | 0.114  | -0.570** | -0.152   |

18:2, linoleic acid; 20:3,  $\gamma$ -linolenic acid; 20:4, arachidonic acid; 20:5, eicosapentaenoic acid; 22:6, docosahexaenoic acid; chol, cholesterol; TG, triacylglycerol; AUC, area under the curve; LDLox, Cu-induced oxidation of LDL; NEFA, non-esterified fatty acids.

\* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$ .

† $P = 0.078$ , ‡ $P = 0.088$ .

§ For details of subjects, supplements and procedures, see p. 436.

|| Absolute changes = fish oil (week 6) – olive oil (week 6).

¶ Calculated by the trapezoidal rule.

\*\* Calculated as AUC minus the baseline value.

†† Peak postprandial TG value (0–480 min).

‡‡ Small dense LDL.

§§  $\mu\text{mol/l}$  per 210 min.

regression model. However when total *n*-3 was added as a variable as opposed to EPA or DHA, it emerged as an independent determinant of the increases in LDL oxidizability ( $P = 0.011$ , results not shown)

## Discussion

The purpose of the present study was to examine the effects of fish-oil supplementation on LDL oxidizability, density and concentration, as each of these variables are key determinants of atherogenesis. In addition, using statistical analysis, associations between EPA and DHA changes following fish-oil supplementation and lipid and lipoprotein changes were examined in order to establish which of the two fatty acids is associated with alterations in lipoprotein metabolism.

In the current study, fish oils, providing 1.67 g EPA + 1.34 g DHA/d, resulted in 491 and 44 % increases in the platelet phospholipid content of EPA and DHA respectively. Other investigators have reported comparable changes in fatty acid composition (Sanders & Roshanai, 1983). As has been previously observed, greater increases in membrane phospholipid levels relative to intake were evident for EPA compared with DHA (Sanders & Roshanai, 1983; Vidgren *et al.* 1997; Zuidgeest-van Leeuwen *et al.* 1999). This may be attributable to a number of factors including: higher baseline DHA levels, selective post-absorptive oxidation of DHA; selective release of DHA from membranes; retroconversion of DHA to EPA; or reduced conversion of EPA to DHA due to the known inhibitory effect of EPA on  $\Delta^6$ -desaturase (Von Schacky & Weber, 1985; Voss *et al.* 1991; Conquer & Holub, 1997).

The efficacy of EPA + DHA intervention in counteract-

ing the dyslipidaemia of the ALP and the possible mechanisms involved has been discussed previously (Minihane *et al.* 2000a). In brief, supplementation with 6 g fish-oil/d providing 3 g EPA + DHA resulted in significant reductions in fasting and postprandial TG responses, and in the percentage of LDL as LDL-3, effects which could be considered as beneficial with respect to CHD risk. However, a significant increase in LDL-chol and LDL oxidizability was also evident, both of which can be considered as potential adverse consequences of fish-oil intervention in terms of cardiovascular risk.

Although it was previously thought that EPA and DHA have comparable effects on lipid metabolism, it is now recognized that there may be discernible differences in the effects of these fatty acids on plasma and membrane lipids. In addition, they have also been shown to have different effects on endothelial function (Mori *et al.* 2000b). Knowledge of the relative actions and potencies of these fatty acids has a wide practical importance, as such information could be used to design mixtures of variable EPA + DHA content, tailored according to the baseline lipid status of the individual, and use selectively in order to ensure maximum benefit.

In agreement with previous studies, an 8 % increase in circulating LDL-chol levels was evident following fish-oil supplementation (Hughes *et al.* 1990; Harris, 1996). Using correlative ( $r = 0.699$ ,  $P < 0.001$ ) and multivariate statistics ( $P = 0.030$ ), it was observed that changes in membrane phospholipid DHA content were independently associated with the increase in the LDL-chol fraction in the present study. Davidson and co-workers observed increases in LDL-chol of 9 % (NS) and 14 % ( $P < 0.001$ ) following supplementation with 1.25 g and 2.5 g DHA/d

**Table 3.** Multiple linear regression analysis of changes in platelet lipids as dependent variables with changes in plasma phospholipid eicosapentaenoic and docosahexaenoic acid as independent variables\*†

|                           | Δ EPA          | Δ DHA          |
|---------------------------|----------------|----------------|
| Δ LDL-chol                |                |                |
| Coefficient (SEM)         | -0.230 (0.211) | 0.754 (0.319)  |
| Adjusted model $R^2$      |                | 0.159          |
| $P$                       | 0.293          | 0.030          |
| Δ LDLox                   |                |                |
| Coefficient (SEM)         | -5.2 (-0.3)    | -9.5 (5.8)     |
| Adjusted model $R^2$      |                |                |
| $P$                       | 0.188          | 0.116          |
| Δ Fasting TG              |                |                |
| Coefficient (SEM)         | -0.748 (0.349) | 0.014 (0.531)  |
| Adjusted model $R^2$      | 0.150          |                |
| $P$                       | 0.046          | 0.978          |
| Δ TG AUC‡                 |                |                |
| Coefficient (SEM)         | -498 (199)     | 157 (301)      |
| Adjusted model $R^2$      | 0.194          |                |
| $P$                       | 0.023          | 0.609          |
| Δ TG incremental AUC§     |                |                |
| Coefficient (SEM)         | -131 (88)      | 140 (133)      |
| Adjusted model $R^2$      |                |                |
| $P$                       | 0.155          | 0.304          |
| Δ Peak TG (0–480 min)     |                |                |
| Coefficient (SEM)         | -1.38 (0.57)   | 0.634 (0.861)  |
| Adjusted model $R^2$      | 0.171          |                |
| $P$                       | 0.027          | 0.471          |
| Δ Fasting NEFA            |                |                |
| Coefficient (SEM)         | -7.36 (31.81)  | -86.99 (48.01) |
| Adjusted model $R^2$      |                |                |
| $P$                       | 0.820          | 0.088          |
| Δ NEFA AUC (270–480 min)‡ |                |                |
| Coefficient (SEM)         | -29512 (10890) | 5175 (16434)   |
| Adjusted model $R^2$      | 0.246          |                |
| $P$                       | 0.015          | 0.757          |

EPA, eicosapentaenoic acid; DHA, docosahexaenoic acid; chol, cholesterol; LDLox, Cu-induced oxidation of LDL; TG, triacylglycerol; AUC, area under the curve; NEFA, non-esterified fatty acid.

\* For details of subject and procedures, see p. 436.

† A negative coefficient indicates an inverse relation.

‡ Calculated by the trapezoidal rule.

§ Calculated as AUC minus the baseline value.

||  $\mu\text{mol/l}$  per 210 min.

respectively for a 6-week period to patients with combined hyperlipidaemia (Davidson *et al.* 1997). The LDL-chol changes were highly correlated ( $r$  0.94,  $P$ <0.001) with the increase in plasma phospholipid DHA (Davidson *et al.* 1997; Hughes *et al.* 1990). These findings were confirmed by Mori *et al.* (2000a) who recorded an 8% increase in LDL-chol following supplementation with 4 g purified DHA for 6 weeks. This increase in LDL-chol associated with increased DHA intake may result from a number of mechanisms including: greater conversion of DHA-enriched VLDL to LDL; an impact of DHA enrichment of the hepatocyte on the orientation of the LDL receptor; or an impact of DHA enrichment of LDL on its physio-chemical properties and interaction with the LDL receptor. In addition DHA may impact on LDL-chol at the nuclear level by influencing the expression of key genes involved in LDL metabolism. Further work is needed to examine these possibilities. Despite the apparent concordance of our own findings with those of others, an increase in LDL-chol following DHA intervention is not a universal finding (Rambjør *et al.* 1996; Nelson *et al.* 1997).

This apparently adverse effect of fish-oil intervention on LDL-chol may be counteracted by the 21% decrease in the percentage of LDL as the small dense LDL-3, which represents a shift towards a larger, less atherogenic LDL particle. However, in contrast to the findings of Mori *et al.* (2000a), little association between membrane fatty acid changes and changes in LDL density were evident in the present study. Results from our own (Minihane *et al.* 2000b) and other studies (Suzukawa *et al.* 1995) have indicated that circulating TG are a primary determinant of LDL-3 levels, with fasting TG shown to explain 47% of the variability in the percentage of LDL as LDL-3 (Minihane *et al.* 2000b). In the present study it is likely, therefore, that it is the reduction in TG levels rather than any fatty acid changes which results in the shift in LDL subclass distribution.

Uncertainties regarding the possible adverse effects of fish-oil intervention on the susceptibility of the LDL particle to oxidation is a major concern when recommending the use of fish oils as an anti-atherogenic agent. This is of particular concern in individuals with an ALP, in whom a predominance of the small dense LDL-3 particle,



which is putatively more susceptible to oxidation (DeGraaf *et al.* 1991), is a defining feature. Although it remains controversial whether or not LDL oxidizability is a significant determinant of CHD risk (Regnstrom *et al.* 1992; Andrews *et al.* 1995; Toshima *et al.* 2000), many consider oxidized LDL to be important in the initiation and progression of atherogenesis (Berliner *et al.* 1990; Weis *et al.* 1991; Steinberg, 1997; Holvoet & Collen, 1998). Several studies have investigated the effect of altering the fat composition of the diet on LDL oxidation. An increase in linoleic acid (*n*-6 polyunsaturated fatty acid) has repeatedly been shown to result in an increase in *ex vivo* oxidation and macrophage degradation of LDL, relative to a comparable oleic acid-rich diet (Abbey *et al.* 1993; Reaven *et al.* 1993). In addition, and in agreement with the findings of the current study, a number of investigators have reported an adverse affect of fish oil (*n*-3 polyunsaturated fatty acid) supplementation on LDL oxidation (Harats *et al.* 1991; Abbey *et al.* 1993; Reaven *et al.* 1993; Suzukawa *et al.* 1995; Mata *et al.* 1996). Following consumption of fish oils providing 3.4 g EPA + DHA/d for 6 weeks, Suzukawa *et al.* (1995) observed a significant 26% reduction in the lag time before rapid oxidation of LDL by Cu commenced. In the present study, where fish-oil intervention provided 3 g EPA + DHA/d for 6 weeks, a comparable 28% reduction in lag time was observed. Correlation and regression analysis indicated that an increase in both the EPA and DHA status was associated with the observed increase in LDL oxidation.

The level of vitamin E in plasma, and in particular in the LDL particles, is thought to be an important determinant of the susceptibility of LDL to oxidation. A number of investigators have studied the effect of vitamin E supplementation on oxidation status, with the levels of supplemented tocopherol ranging from 17 to 1450 mg/d (25 to 1500 IU/d) (Jessup *et al.* 1990; Dieber-Rotheneder *et al.* 1991; Reaven *et al.* 1994; Jialal *et al.* 1995; Li *et al.* 1996). In the present study, the fish-oil and olive-oil supplements provided a total of 21 mg total tocopherol/d of which 16 mg was  $\alpha$ -tocopherol. A 9% and 13% increase in plasma and LDL  $\alpha$ -tocopherol occurred following olive-oil intervention. Although the level of supplementation was relatively low, it may have contributed to the increased resistance of LDL to oxidation, which was evident after 6 weeks of supplementation. In addition, an increased LDL content of oleic acid, replacing polyunsaturated fatty acids in the particle, which has been observed by other investigators (Reaven *et al.* 1994; Mata *et al.* 1996) may have been responsible in part. No significant changes in plasma or LDL  $\alpha$ -tocopherol were observed following fish-oil intervention and this level of supplementation (16 mg/d), which would result in a doubling in  $\alpha$ -tocopherol intake for the population at large, was not effective in protecting the LDL particles against the increase in oxidative susceptibility following fish-oil intervention. Other investigators have also observed that much larger doses of  $\alpha$ -tocopherol are needed to impact positively on LDL oxidation. For example, in a study by Jialal *et al.* (1995) it was found that 268 mg (400 IU)  $\alpha$ -tocopherol/d was required to decrease the oxidizability of LDL and that lower doses of 40 (60 IU) or 134 (200 IU) mg/d were ineffective.

Fasting and postprandial TG responses were negatively and independently associated with the increase in platelet EPA. No significant associations with DHA changes were evident. Our results are concurrent with earlier *in vitro* and animal studies, which indicated that EPA is the hypotriacylglycerolaemia component of fish oil, by virtue of its impact on hepatic fatty acid oxidation and its direct impact on TG synthesis, and VLDL assembly and secretion (Willumsen *et al.* 1993; Berge *et al.* 1999). Previous studies in human subjects linking fatty acid and lipaemic changes have produced conflicting findings (Childs *et al.* 1990; Ågren *et al.* 1996; Rambjør *et al.* 1996; Davidson *et al.* 1997; Mori *et al.* 2000a). Further research is merited, in particular a closer examination of the individual impact of EPA and DHA supplementation on postprandial lipaemia. In addition, increased EPA status was associated with lower postprandial NEFA levels. As NEFA supply to the liver influences VLDL synthesis and circulating TG levels (Frayn, 1998), lower circulating NEFA may have contributed to the lower lipaemic response. A greater tissue oxidation of NEFA, a reduction in hormone-sensitive lipase-catalysed release of NEFA from adipocytes, or a greater tissue accumulation of NEFA produced at the capillary endothelium by the lipoprotein lipase-mediated hydrolysis of circulating TG may be responsible for the EPA-mediated improved NEFA status (Frayn *et al.* 1996). This latter lipoprotein lipase-mediated release of NEFA following the breakfast meal is thought to be responsible for the 'apparent' lack of inhibition of circulating NEFA levels post-lunch.

The findings of the present study indicate that, despite an apparent increase in LDL oxidation and LDL-chol concentration following fish-oil intervention, there is an increase in LDL density, which may counteract these proatherogenic trends. In addition, the current study may help clarify which of the *n*-3 fatty acids is responsible for the beneficial and adverse lipid changes following fish-oil intervention. However, further work is needed in order to elucidate the mechanisms by which DHA and EPA impact on lipid metabolism at the hepatic and systemic level.

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