Docosahexaenoic and Eicosapentaenoic Acids in Plasma Phospholipids Are Divergently Associated With High Density Lipoprotein in Humans

Kaare H. Bønaa, Kristian S. Bjerve, and Arne Nordøy

The effect of fish oil rich in eicosapentaenoic (EPA) and docosahexaenoic (DHA) acids on serum lipoprotein concentrations is not clear, and it is not known whether EPA and DHA are similarly related to serum lipids or lipoprotein levels. We conducted a randomized, 10-week, dietary supplementation trial in which the effects of 6 g per day of 85% EPA and DHA were compared with 6 g per day of corn oil in 156 men and women. Multivariate analyses were used to assess independent relations between plasma phospholipid EPA and DHA and serum lipoprotein levels. In the fish oil group triglycerides fell 21% (p<0.001) and high density lipoprotein cholesterol (HDL-C) rose 3.8% (p<0.05). In the corn oil group triglycerides did not change, but HDL-C rose 6.1% (p<0.01). Compared with fish oil, apolipoprotein A-I (apo A-I) rose 5.1% after corn oil intake (p<0.05). Plasma EPA and DHA levels rose after fish oil intake and fell after corn oil intake (all p<0.001). The change (A) in EPA was inversely correlated with Atriglycerides (p=0.035) and positively correlated with AHDL-C and Aapo A-I (both p<0.001) in the multivariate analyses. In contrast, ADHA was not correlated with Atriglycerides but was inversely correlated with AHDL-C and Aapo A-I (both p<0.001). Standardizing for DHA removed the difference in apo A-I levels between groups. This study suggests that EPA and DHA are divergently associated with HDL, possibly through different mechanisms.

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Serum high density lipoprotein (HDL) cholesterol and apolipoprotein A-I (apo A-I) concentrations are associated with atherogenesis and coronary heart disease.1-3 The risk factor status of triglycerides may be stronger than previously assumed.4,5 Dietary supplementation with fish oils rich in polyunsaturated fatty acids (PUFAs) of the n-3 family consistently lowers serum triglycerides, but the effects on HDL cholesterol and apo A-I have varied.6-10 High doses of fish oil may lower both HDL cholesterol and apo A-I levels.7 After moderate doses of n-3 PUFAs, however, an increase in the HDL cholesterol level was accompanied by a decrease in the apo A-I level.8-10

It is assumed that eicosapentaenoic (EPA; 20:5n-3) and docosahexaenoic (DHA; 22:6n-3) acids are the active agents mediating the metabolic effects of n-3 PUFAs.6 Significant associations between the fall in plasma triglyceride values and the increase in plasma levels of both EPA and DHA have been reported in human studies.11 However, in vitro studies with cultured cells12,13 and the perfused rat liver14 indicate that EPA and DHA may have different effects on the hepatic synthesis or release of triglycerides. It is presently not known whether HDL cholesterol and apo A-I are similarly associated with the dietary intake or the plasma phospholipid content of the different n-3 PUFAs.

In this article we examine the alterations in serum lipid, lipoprotein, and apolipoprotein levels after dietary supplementation with a concentrated formula containing EPA and DHA. We also examine the relation between the changes in serum lipid or lipoprotein concentrations and plasma phospholipid fatty acid levels by using multivariate analysis to assess the possible separate effects of EPA and DHA. We have previously reported that blood pressure in this study group was lowered after fish oil intake.15

Methods

Subjects and Study Design

Details about the selection of participants and the study design have been presented elsewhere.15 One
hundred fifty-six men and women aged 34–60 years were recruited from a population health survey conducted in Tromsø, Norway. The subjects were healthy as determined by medical history, clinical examination, laboratory tests, and electrocardiography. They had not previously used lipid-lowering or antihypertensive drugs. The study was approved by the regional board of research ethics, and each subject gave written consent. The study began with a 6-month observational period, during which the subjects were asked to continue their usual diet and living habits and to visit the clinic for measurement of various parameters on three occasions. All participants had a serum total cholesterol level between 5.2 and 10.0 mmol/l and a diastolic blood pressure between 85 and 110 mmHg on each occasion.

After the observational period the participants were randomly assigned to receive fish oil or corn oil and were entered into a 10-week, double-blind, dietary supplementation trial. The fish oil group was given 6 g 85% ethyl ester EPA and DHA (K85, Norsk Hydro, Oslo, Norway) per day, which provided 3.3 and 1.8 g per day of EPA and DHA, respectively. The corn oil group received 6 g corn oil per day with 3.4 g linoleic (18:2n-6) acid. The oils were given in indistinguishable soft gelatin capsules, each of which contained 1 g fatty acids and 3-4 IU α-tocopherol as an antioxidant. Either supplement provided approximately 2% of the mean daily energy intake and <6 mg cholesterol per day. The exact composition of the supplements has been previously reported.

Participants were questioned about side effects and intercurrent diseases. Compliance was assessed by capsule counts, measurements of plasma phospholipid fatty acid concentrations, and estimation of the intake of nutrients from unannounced 24-hour dietary recalls recorded by a registered dietitian.

Measurements
Fasting venous blood samples were obtained at the beginning and the end of the intervention period. Plasma and serum samples were stored at −80°C until the study was completed and were analyzed before the randomization code was broken. The concentrations of plasma phospholipid fatty acids were measured as previously described, and the results were quantified in weight percent (the fatty acid in question as a percentage of total phospholipid fatty acids). Serum total cholesterol was measured with an enzymatic colorimetric method (CHOD-PAP). HDL cholesterol was measured in a similar fashion after precipitation with phosphotungstic acid and magnesium chloride, and triglycerides (in fresh serum) were determined by enzymatic hydrolysis and subsequent measurement of the liberated glycerol by colorimetry (GPO-PAP). The kits were purchased from Boehringer-Mannheim, Mannheim, FRG. The methods were calibrated using Seronorm Lipid (Nycomed, Oslo, Norway). Low density lipoprotein (LDL) cholesterol was calculated using the formula

\[
\text{LDL cholesterol} = \text{total cholesterol} - \text{HDL cholesterol} - (\text{triglycerides} \times 0.47)
\]

when triglyceride is <4.5 mmol/l. Concentrations of apo A-I and apo B were measured by immunoturbidimetry on a Behring Nephelometer (Behringwerke AG, Marburg, FRG) using antibodies and standards from the manufacturer.

Statistical Analysis
Change was calculated as the value obtained at the end minus the value obtained at the beginning (baseline) of intervention and was tested for statistical significance by one-sample and two-sample t tests. Linear associations were evaluated by testing Pearson correlation coefficients and multiple linear regression analyses, with the change in serum lipid or lipoprotein concentration as the dependent variable and the changes in plasma phospholipid fatty acids as independent variables. Corresponding one-way analyses of covariance (ANCOVA) were run with quintiles of EPA or DHA as the grouping variable. The assumptions of the multivariate analyses were confirmed by residual analysis and computation of influence statistics. Neither the SEM nor other indexes indicated the presence of moderate or severe collinearity in the analysis. The tests are two tailed throughout. The SAS software package was used.

All 156 participants completed the study. Data for plasma fatty acids in five subjects were unavailable. Two subjects were treated with corticosteroids during the trial, and three subjects had febrile infectious diseases and were treated with antibiotics at baseline or during the study. These 10 subjects were not included in the analysis, which is therefore based on 146 subjects. Inclusion of all participants in the analysis did not notably alter the results. Two nonfasting participants were not included in the triglyceride or LDL cholesterol analyses. The number of observations ranged from 138 to 146 because of missing data.

Results
Sixty-one percent of the participants were men, and 37% were smokers. The age was 49±7 years (mean±SD), and the body mass index was 26.0±3.3 kg/m². Systolic and diastolic blood pressures were 144.3±12.7 and 94.9±6.6 mmHg, respectively. The groups assigned to receive fish oil or corn oil were well balanced at baseline (Tables 1 and 2). Overall adherence to the study protocol was satisfactory. Ten and seven subjects in the fish oil and corn oil groups, respectively, reported that they did not take the prescribed number of capsules, frequently because of self-limited mild or moderate abdominal discomfort. In the fish oil group the mean dietary intake of EPA and DHA increased from 0.37 to 3.35 g per day and from 0.65 to 2.32 g per day, respectively, whereas dietary linoleic acid (9.3 g per day) did not change. In the corn oil group the intake of linoleic acid increased from 8.7 to 11.3 g per day, whereas the intake of EPA and DHA fell from 0.35 to 0.16 g per day (p=0.013) and from 0.63 to 0.35 g per day (p=0.019), respectively. Dietary intake of saturated or monounsaturated fat and protein and alcohol consumption did not change in either group. Dietary carbohydrate intake decreased in the corn oil group (−11%; p=0.002) but not in the fish oil group. Body weight increased by 0.70 kg in the fish oil group and 0.56 kg in the corn oil group (both p<0.01); the difference was not significant.
Serum Lipids and Lipoproteins

Serum triglyceride values fell by 0.30 mmol/l (95% confidence interval [CI] , -0.41 to -0.19) in the fish oil group in contrast with no change in the corn oil group (Table 1). HDL cholesterol levels rose by 0.05 mmol/l (95% CI, 0.01–0.09) after fish oil intake and by 0.08 mmol/l (95% CI, 0.03–0.12) after corn oil intake. Apo A-I levels did not change during fish oil or corn oil supplementation. In the corn oil group, however, apo A-I levels rose by 0.06 g/l (95% CI, 0.02–0.12) after corn oil intake. Apo B levels fell by 0.11 and r=-0.08 (both p<0.05) in the fish oil group between the fall in serum triglyceride levels and the increase in the plasma phospholipid EPA and DHA content in the fish oil or the corn oil group (data not shown).

Relation Between Changes in Plasma Phospholipid Eicosapentaenoic or Docosahexaenoic Acid and Serum Lipid and Lipoprotein Levels

Triglycerides. There were significant correlations in the fish oil group between the fall in serum triglyceride level and the increase in the plasma phospholipid content of both EPA (r=-0.30) and DHA (r=-0.27) (both p<0.05). The corresponding coefficients in the corn oil group were r=-0.11 and r=-0.08 (both p=NS). The change in EPA (p=0.035) but not DHA (p=0.5) was inversely associated with a change in serum triglycerides when both fatty acids were included simul-
The multivariate analysis gave a notable increment in glyceride levels during the study did not notably alter HDL cholesterol were inversely correlated (Table 3). There was a strong inverse relation between the change in HDL cholesterol and plasma phospholipid DHA levels did not correlate significantly (p<0.01) (Figure 1). In contrast, apo A-I and plasma phospholipid EPA levels were not bivariately associated. The multiple regression analysis strengthened the inverse relation between DHA and apo A-I, whereas EPA showed a consistent positive relation to apo A-I (Table 3). The multivariate analysis gave a better fit to the data than the bivariate analysis (see "Appendix"), as indicated by the increment in R². The slope for the change in DHA versus the change in HDL cholesterol was significantly (p<0.01) steeper in the corn oil than the fish oil group.

The slope for the change in DHA versus the change in HDL cholesterol was significantly (p=0.006) steeper in the fish oil group than in the corn oil group (r=-0.51, p=0.0001) (Figure 1). In contrast, apo A-I and plasma phospholipid EPA levels were not bivariately associated. The multiple regression analysis strengthened the inverse relation between DHA and apo A-I, whereas EPA showed a consistent positive relation to apo A-I (Table 3).

The inverse relation between the changes in DHA and apo A-I still remained significant after accounting for the variability in HDL cholesterol level (by ANCOVA with the change in HDL cholesterol as the covariate) (Figure 3). In contrast, the change in EPA did not correlate with apo A-I when controlling for HDL cholesterol.

**Table 3. Results of Multiple Linear Regression Analysis of Change in Serum High Density Lipoprotein Cholesterol or Apolipoprotein A-I Level as Dependent Variable With Changes in Plasma Phospholipid Eicosapentaenoic and Docosahexaenoic Acid Levels as Predictor Variables.**

<table>
<thead>
<tr>
<th>Group/predictor variable (weight %)</th>
<th>ΔHDL-C (mmol/l)</th>
<th>ΔApo A-I (g/l)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fish oil</td>
<td>n=70</td>
<td>n=71</td>
</tr>
<tr>
<td>ΔEPA</td>
<td>0.29±0.08*</td>
<td>0.28±0.08†</td>
</tr>
<tr>
<td>ΔDHA</td>
<td>-0.27±0.13‡</td>
<td>-0.53±0.13*</td>
</tr>
<tr>
<td>Model R²</td>
<td>0.161</td>
<td>0.205</td>
</tr>
<tr>
<td>Corn oil</td>
<td>n=69</td>
<td>n=74</td>
</tr>
<tr>
<td>ΔEPA</td>
<td>0.27±0.14</td>
<td>0.23±0.11‡</td>
</tr>
<tr>
<td>ΔDHA</td>
<td>-0.76±0.17*</td>
<td>-0.73±0.13*</td>
</tr>
<tr>
<td>Model R²</td>
<td>0.227</td>
<td>0.306</td>
</tr>
<tr>
<td>Both groups</td>
<td>n=139</td>
<td>n=145</td>
</tr>
<tr>
<td>ΔEPA</td>
<td>0.20±0.06*</td>
<td>0.17±0.05*</td>
</tr>
<tr>
<td>ΔDHA</td>
<td>-0.63±0.11*</td>
<td>-0.65±0.10*</td>
</tr>
<tr>
<td>(ΔDHA)²</td>
<td>0.08±0.02*</td>
<td>0.04±0.02</td>
</tr>
<tr>
<td>Model R²</td>
<td>0.198</td>
<td>0.264</td>
</tr>
</tbody>
</table>

Δ, Change; HDL-C, high density lipoprotein cholesterol; apo, apolipoprotein; EPA, eicosapentaenoic acid; DHA, docosahexaenoic acid. Values are 10(slope±SEM).

*p<0.001, †p<0.01, ‡p<0.05.
TABLE 4. Cumulative Amount of Variability in ΔSerum High Density Lipoprotein Cholesterol and Apolipoprotein A-I Explained by ΔPlasma Phospholipid Fatty Acids (Pooled Analysis)

<table>
<thead>
<tr>
<th>Fatty acid (weight %)</th>
<th>ΔHDL-C (mmol/l) cumulative R² (n = 139)</th>
<th>ΔApo A-I (g/l) cumulative R² (n = 145)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ADHA22:6n-3</td>
<td>0.046*</td>
<td>0.178*</td>
</tr>
<tr>
<td>AEPA20:5n-3</td>
<td>0.129*</td>
<td>0.246*</td>
</tr>
<tr>
<td>(ΔDHA)²</td>
<td>0.198*</td>
<td>—</td>
</tr>
<tr>
<td>ΔOleic 18:1n-9</td>
<td>—</td>
<td>0.283†</td>
</tr>
<tr>
<td>ΔNervonic 24:1n-9</td>
<td>—</td>
<td>0.309‡</td>
</tr>
<tr>
<td>ΔArachidonic 20:4n-6</td>
<td>—</td>
<td>0.329‡</td>
</tr>
</tbody>
</table>

Results of stepwise multiple linear regression analysis with plasma phospholipid fatty acids shown in Table 2 as potential predictor variables. "—" denotes variables not contributing significantly to R² (cumulative amount of variability). Δ, Change; HDL-C, high density lipoprotein cholesterol; apo, apolipoprotein; DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid.

*p<0.001, †p<0.01, ‡p<0.05, for increment in R².

Changes in the levels of plasma phospholipid oleic (18:1n-9) (positively), nervonic (24:1n-9), and arachidonic (20:4n-6) (both inversely) acids were also related to serum apo A-I concentrations (Table 4), but these fatty acids did not much alter the slope for the change in DHA or EPA versus apo A-I (data not shown). When introduced in the regression as the last variable, DHA and EPA accounted for 44% and 17%, respectively, of the explained variability in apo A-I.

The observed difference in serum apo A-I concentration during corn oil and fish oil supplementation (0.08 g/l; p=0.014) (Table 1) was completely removed when controlling for the difference in plasma phospholipid DHA contents between groups (by ANCOVA with the change in DHA as the covariate). In contrast, controlling for the variability in plasma phospholipid EPA did not alter the observed difference in apo A-I between the corn oil and the fish oil groups (data not shown).

Apolipoprotein B and low density lipoprotein cholesterol. Changes in plasma phospholipid EPA or DHA content were not associated with changes in apo B or LDL cholesterol levels in either group (data not shown). In the pooled data, changes in EPA (r=0.17, p=0.054) and DHA (r=0.19, p=0.028) were marginally correlated with changes in LDL cholesterol concentrations.

Discussion

Participants in the present study consumed a relatively high amount of n-3 PUFAs from fish in their usual diet. It is likely that the fall in the level of plasma phospholipid n-3 PUFAs in the corn oil group reflected both the decrease in the dietary intake of fish as well as the competition between linoleic acid and n-3 PUFAs for the 2 position in the phospholipid molecules. The fall in plasma phospholipid erucic acid also suggests that fish consumption was lowered during corn oil supplementation. The opposite trends for n-3 PUFAs in the corn oil and fish oil groups, therefore, made it possible to study serum lipoprotein levels in relation to changes in plasma phospholipid EPA or DHA levels over a wide range of values in a randomized study.

The major new observation reported here was the divergent association between the change in plasma phospholipid DHA or EPA content and the change in serum HDL cholesterol and apo A-I levels. It is unlikely
that the results can be explained by the play of chance. The validity of the findings is further supported by the similar trends observed in the fish oil and corn oil groups as well as the subgroups defined by gender or smoking status. It is unlikely that alcohol consumption or body weight could have confounded the results because EPA or DHA was not associated with these variables. The fish oil supplement provided more EPA than DHA, but the results obtained in the corn oil group indicate that the effects were not caused by different dosages of the two fatty acids.

The present data do not establish the mechanism(s) for the difference between EPA and DHA. At least two categories of mechanisms may be considered. In the first case, phospholipid molecular species containing EPA or DHA may be differently enriched in the various blood lipids or lipoprotein fractions without being causally related to the cholesterol or protein content of the lipoproteins. In the second case, EPA and DHA may have disparate causal effects on the HDL cholesterol and/or the apo A-I level.

In this study DHA appeared to be more closely related than EPA to apo A-I. DHA accounted for a greater amount of the variability in apo A-I than did EPA, and the observed difference in the apo A-I level between the fish oil and corn oil groups was removed after standardizing for the variability in plasma phospholipid DHA. It may be suggested that DHA itself or a factor associated with DHA influences synthesis or catabolism of apo A-I. Dietary n-3 fatty acids have been reported to suppress the synthesis of apo A-I in the rat liver and intestine, but that study did not report the results for individual n-3 fatty acids. Apo A-I, the major protein of the HDL particle, is believed to play an important role in plasma cholesterol transport by acting as an activator of lecithin:cholesterol acyltransferase (LCAT). HDL may originate as “nascent HDL” particles containing apo A-I and phospholipids as bilayer discs formed in the liver and the intestine or may originate from the surface coat of lipolyzed triglyceride-rich lipoproteins. Nascent HDL is thought to grow into mature spherical HDL particles by incorporating into a hydrophobic core the cholesteryl esters derived from the LCAT reaction. LCAT is apparently also involved in the interconversion of HDL2 and HDL3. Fish oil may lower plasma LCAT activity.

There is evidence that LCAT shows substrate specificity for fatty acid molecular species and that phospholipids containing DHA may be a poorer acyl-chain donor to the LCAT reaction than are phospholipid molecules with EPA, linoleic acid, or oleic acid in the 2 position. It is suggested that higher amounts of phospholipid DHA may interfere with the transformation of nascent HDL to spherical HDL and/or that they may alter the distribution of HDL2 and HDL3, possibly by modulating the LCAT reaction. Phospholipid-apo A-I bilayer discs and nascent HDL may be more easily cleared from the plasma than are mature HDL particles, which could lead to a depletion of plasma apo A-I at higher levels of phospholipid DHA. Fish oil has been reported to increase the larger, less dense HDL, and changes in HDL size or core lipid composition may also influence apo A-I catabolism.

Decreased synthesis or increased catabolism of apo A-I would be expected to lower HDL cholesterol levels and could account for the inverse correlation between plasma phospholipid DHA and HDL cholesterol levels in the present study. Although the majority of plasma apo A-I is associated with HDL, other lipoproteins also contain some apo A-I, and there is also a “free” plasma pool of apo A-I. We observed that DHA and apo A-I remained correlated after statistical control for HDL cholesterol, whereas EPA was not related to apo A-I after standardizing for HDL cholesterol. The present data are consistent with the hypothesis that the relation between DHA and HDL cholesterol is a consequence of a relation between DHA and apo A-I and that EPA and HDL cholesterol may be related through other mechanisms.

The fall in serum triglyceride concentrations after fish oil intake probably depends on decreased hepatic very low density lipoprotein (VLDL) triglyceride synthesis and/or secretion. Experiments with cultured liver cells and the perfused rat liver indicate that EPA lowers hepatic secretion of triglycerides to a greater extent than does DHA. The present data support these results, as EPA but not DHA remained associated with triglycerides in the multivariate analysis. Could the association between EPA and HDL cholesterol be secondary to an effect of EPA on triglyceride production? Measurements of the production rates of VLDL triglyceride have provided no evidence that HDL cho-

### Simple Linear Regression Coefficients (Slopes) From Fish Oil and Corn Oil Groups

<table>
<thead>
<tr>
<th>Predictor variable</th>
<th>Dependent variable (fish oil group)</th>
<th>Dependent variable (corn oil group)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>ΔHDL-C (n=70)</td>
<td>ΔApo A-I (n=71)</td>
</tr>
<tr>
<td>EPA</td>
<td>0.17±0.06*</td>
<td>0.04±0.07</td>
</tr>
<tr>
<td>Weight percent</td>
<td>0.04±0.07</td>
<td>-0.04±0.14</td>
</tr>
<tr>
<td>Model R²</td>
<td>0.006</td>
<td>0.001</td>
</tr>
<tr>
<td></td>
<td>0.001</td>
<td>0.005</td>
</tr>
<tr>
<td>ΔDHA</td>
<td>-0.07±0.11</td>
<td>-0.60±0.15</td>
</tr>
<tr>
<td>Weight percent</td>
<td>-0.25±0.10†</td>
<td>-0.59±0.12‡</td>
</tr>
<tr>
<td>Model R²</td>
<td>0.003</td>
<td>0.186</td>
</tr>
<tr>
<td></td>
<td>0.080</td>
<td>0.263</td>
</tr>
</tbody>
</table>

Δ, Change; HDL-C, high density lipoprotein cholesterol (mmol/L); apo A-I, apolipoprotein A-I (g/L); EPA and DHA, plasma phospholipid eicosapentaenoic and docosahexaenoic acids, respectively.

*p<0.01, *p<0.05, †p<0.001.
lesterol is influenced by VLDL synthesis in humans.32 In the present data there was no correlation between the changes in triglyceride and HDL cholesterol levels, and controlling for the variability in triglycerides failed to modify the relation between EPA (or DHA) and HDL. These results suggest that the association between n-3 PUFAs and HDL is not a consequence of their hypotriglyceridemic effects. Accelerated exchange of HDL core cholesteryl ester for VLDL and/or chylomicron core triglyceride via lipid transfer protein has been proposed as a mechanism for the low plasma HDL cholesterol levels in hypertriglyceridemia.33 Fish oil may lower lipid transfer protein activity,11 thereby retaining more cholesteryl esters within HDL. Lipid transfer protein may show specificity for fatty acid molecular species,34 but we are unaware of any studies that have examined whether EPA and DHA modulate lipid transfer protein activity differently.

In summary, the present data indicate a major difference between EPA and DHA in relation to both the cholesterol and the apolipoprotein content of the HDL particle. Further studies using purified fatty acid formulas are needed to determine whether these relations represent causality and to define the underlying mechanisms.

References
Docosahexaenoic and eicosapentaenoic acids in plasma phospholipids are divergently associated with high density lipoprotein in humans.

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