An ω-3 Polyunsaturated Fatty Acid Concentrate Increases Plasma High-Density Lipoprotein 2 Cholesterol and Paraoxonase Levels in Patients With Familial Combined Hyperlipidemia

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A remarkable reduction of plasma concentrations of high-density lipoproteins (HDL), especially of the HDL_2 subfraction, is one of the typical lipoprotein alterations found in patients with familial combined hyperlipidemia (FCHL). Fourteen FCHL patients received 4 capsules daily of Omacor (an ω-3 polyunsaturated fatty acid [ω3 FA] concentrate providing 1.88 g of eicosapentaenoic acid [EPA] and 1.48 g of docosahexaenoic acid [DHA] per day; Pronova Biocare, Oslo, Norway) or placebo for 8 weeks in a randomized, double-blind, crossover study. Plasma triglycerides were 44% lower, and LDL cholesterol and apoliporpotein (apo)B were 25% and 7% higher after Omacor than placebo. HDL cholesterol was higher (+8%) after Omacor than placebo, but this difference did not achieve statistical significance. Omacor caused a selective increase of the more buoyant HDL_2 subfraction; plasma HDL_2 cholesterol and total mass increased by 40% and 26%, respectively, whereas HDL_3 cholesterol and total mass decreased by 4% and 6%. Both HDL_2 and HDL_3 were enriched in cholesteryl esters and depleted of triglycerides after Omacor. No changes were observed in the plasma concentration of major HDL apolipoproteins, LpA-I and LpA-I:A-II particles, lecithin:cholesterol acyltransferase (LCAT), and cholesteryl ester transfer protein (CETP). The plasma concentration of the HDL-bound antioxidant enzyme paraoxonase increased by 10% after Omacor. Omacor may be helpful in correcting multiple lipoprotein abnormalities and reducing cardiovascular risk in FCHL patients.
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FAMILIAL combined hyperlipidemia (FCHL) is the most common inherited disorder of lipid metabolism among young survivors of myocardial infarction, with an estimated frequency of 0.3% to 2.0% in the general population.[1] FCHL patients present with elevated plasma cholesterol and/or triglycerides, predominance of small and dense low-density lipoprotein (LDL) particles, and low concentrations of high-density lipoprotein (HDL) cholesterol, particularly HDL_2 cholesterol.[2-4] Each of these lipid/lipoprotein abnormalities may contribute to the high prevalence of coronary heart disease (CHD) in FCHL patients.[5-7]

Because of the increased incidence of CHD in FCHL patients, diet and/or drug treatments aimed at lowering blood lipids and correcting the abnormal lipoprotein phenotype should be initiated early. Some early reports indicated that long-chain ω-3 fatty acids (ω-3 FAs) present in fish oils, mainly eicosapentaenoic and docosahexaenoic acid (EPA and DHA), may lower plasma triglycerides in FCHL patients, with little effects on plasma total, LDL, and HDL cholesterol.[8,9] We recently showed that a concentrate of EPA and DHA ethyl esters, which is used in Europe for the treatment of hypertriglyceridemia,[10] significantly lowers plasma triglycerides, and shifts LDL subclass distribution towards more buoyant particles, without affecting LDL size, in FCHL patients.[11] No changes were observed in plasma HDL cholesterol and apolipoprotein A-I levels.[11]

Despite the minor changes in plasma HDL cholesterol level, ω-3 FA administration to normolipidemic subjects[12,13] or mildly hypercholesterolemic patients with normal plasma HDL levels[14,15] has been shown to shift the distribution of HDL particle towards the more buoyant HDL_2 subfraction. The increase of HDL_2 was secondary to a reduced activity of the lecithin:cholesterol acyltransferase (LCAT) enzyme and of the cholesteryl ester transfer protein (CETP).[12,14] Since a low plasma HDL_2 concentration is a hallmark of FCHL, in the present study we investigated whether a concentrate of EPA and DHA ethyl esters may increase plasma HDL_2 levels in FCHL patients.

MATERIALS AND METHODS

Patients and Experimental Design

Fourteen FCHL patients who had been followed as outpatients for several years at the E. Grossi Paoletti Lipid Clinic were recruited for the study. Patients characteristics and study design have been previously reported.[11] Patients were diagnosed as FCHL when they fulfilled the following criteria[16]: (1) primary hyperlipidemia, defined by a plasma cholesterol and/or triglyceride level exceeding the 90th percentile in the general population, adjusted for age and sex; (2) varying hyperlipidemia phenotype during at least 1 year of follow-up; (3) at least one first-degree relative with a hyperlipidemia phenotype different from the index patient; (4) presence of an LDL phenotype B, defined by a major LDL particle subpopulation with a diameter less than 25.5 nm by nondenaturing polyacrylamide gradient gel electrophoresis (GGE). All patients followed a standard low-fat (30% of calories) diet throughout the study. All participating subjects were fully informed of the

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modalities and end points of the study, which was approved by the Institutional Ethic Committee, and signed an informed consent form.

The study protocol was designed for a randomized, double-blind crossover trial. After a run-in period of 4 weeks, qualifying patients were randomly allocated to receive Omacor (4 g/d) and placebo capsules for 8 weeks in a different sequence. The Omacor capsules (Pronova Biocare, Oslo, Norway) contained 1 g of concentrated ω-3 FAs (92%; 44.4% EPA and 36.2% DHA), and 4 mg of α-tocopherol. The placebo capsules contained corn oil (56.3% linoleic acid), monounsaturated FA (26.8% oleic acid), saturated FA (2.3% stearic acid), and 2.4 mg vitamin E.

Laboratory Procedures

Fasting blood samples were collected at the end of each 8-week treatment phase. Both serum and plasma (Na_2-EDTA, 1 mg/mL) were prepared by low-speed centrifugation at 4°C. Serum aliquots were added immediately with EDTA (1 mg/dL) and NaBr (5.1 mol/L) before storage at 4°C for HDL subfraction separation by rate zonal ultracentrifugation. Plasma aliquots were stored at −80°C for immunoassays and cell cholesterol efflux determination. A serum aliquot was stored at −80°C for determination of paraoxonase (PON) concentration.

Plasma total cholesterol and triglyceride levels were determined with standard enzymatic techniques by using a Roche Diagnostics Cobas autoanalyzer (Indianapolis, IN). Plasma HDL cholesterol levels were routinely measured after precipitation of the apolipoprotein (apo)B-containing lipoproteins by dextran sulfate–$MgCl_2$. Levels of apoA-I and apoA-II were determined by immunoturbidimetry, using commercially available polyclonal antibodies (Boheringer Mannheim, Mannheim, Germany). The plasma concentration of lipoprotein particles containing only apoA-I (LpA-I) and of particles containing both apoA-I and apoA-II (LpA-I:A-II) was determined by electroimmunodiffusion in agarose gel.[17] Plasma concentrations of lecithin:cholesterol acyltransferase (LCAT), cholesteryl ester transfer protein (CETP), and paraoxonase were assayed by competitive enzyme-linked immunoassays.[18,19]

HDL subfractions were separated by rate zonal ultracentrifugation in a swinging bucket rotor, as previously described.[20] The lipid contents of the isolated HDL fractions were determined by enzyme techniques; the cholesteryl ester (CE) mass was calculated as (total cholesterol − unesterified cholesterol) × 1.68. Protein contents were measured by the method of Lowry et al[21] using bovine serum albumin as standard.

HDL particle size was analyzed by nondenaturing polyacrylamide gradient gel electrophoresis,[16] using precast 4% to 30% slab gels (Alamo Gels, San Antonio, TX). Aliquots of the $d < 1.21$ g/mL plasma fractions, which had been separated by ultracentrifugation at 100,000 rpm for 5½ hours at 4°C in a Beckman TL 100 ultracentrifuge (Fullerton, CA) equipped with a 100.3 rotor, were applied. Gels were run at 20 V for 15 minutes, then 70 V for 20 minutes, and 125 V for 24 hours at 4°C, stained with Comassie G-250 0.04 % for 24 hours, and scanned with a BioRad Model GS-690 Imaging Densitometer (Hercules, CA). Particle size was calculated with Multi-Analyst/PC Software (BioRad) using thyroglobulin (17.0 m), ferritin (12.2 nm), lactate dehydrogenase (8.15 nm), and bovine serum albumin (6.5 nm) as calibration proteins.

Cell cholesterol efflux to whole plasma was assayed as previously described.[22] Briefly, diluted plasma was incubated with ^{3}H-cholesterol-labeled Fu5AH rat hepatoma cells for 4 hours at 37°C. At the end of this period, the medium was removed, collected into tubes and centrifuged for 5 minutes at 2,000 rpm to remove any floating cells. An aliquot of the medium was then counted for [^{3}H]cholesterol radioactivity (Formula 989, Packard, Groningen, The Netherlands). Cellular lipids were extracted with 2-propanol by overnight incubation at room temperature and radioactivity was measured in an aliquot of the extract (Insta-Fluor, Packard). Cholesterol efflux was calculated as the percentage of total label in each well released to the medium, normalized to the cholesterol efflux obtained with a pool of normolipidemic sera tested in each experiment.

Table 1. Plasma Lipid and Lipoprotein Levels (mg/dL) at Baseline, and During Placebo and Omacor Treatment

	Baseline	Placebo	Omacor
Total cholesterol	270.7 ± 16.1	266.0 ± 10.4	282.5 ± 11.8
Triglycerides	378.1 ± 141.8	375.7 ± 104.5	210.1 ± 29.7*
LDL cholesterol	167.1 ± 12.7	161.5 ± 13.5	202.7 ± 12.2*
HDL cholesterol	39.6 ± 4.0	38.0 ± 3.6	41.2 ± 4.1
HDL_2 cholesterol	6.8 ± 1.9	6.3 ± 1.6	9.5 ± 2.9*
HDL_3 cholesterol	32.9 ± 2.5	32.0 ± 2.3	31.7 ± 2.1
Apolipoprotein A-I	112.9 ± 6.3	113.0 ± 5.8	111.7 ± 7.5
Apolipoprotein A-II	35.6 ± 2.0	35.1 ± 1.7	36.0 ± 1.8
LpA-I	38.4 ± 5.1	37.9 ± 5.0	36.3 ± 4.2
LpA-I:A-II	74.4 ± 4.8	75.1 ± 4.4	75.4 ± 5.2
Apolipoprotein B	135.2 ± 5.6	134.1 ± 7.0	143.8 ± 6.3*

NOTE. Results are expressed as means ± SEM, N = 14.
*Significantly different from baseline and placebo.

Statistical Analyses

The number of subjects needed to detect a difference in HDL_2-C between the 2 treatments of 2.5 mg/dL with a standard deviation of 3.0 mg/dL, a power of 80%, and $\alpha = 0.05$ is 14. Therefore, the study is adequately powered to disprove multiple null hypotheses.

Results are given as means ± SEM, if not otherwise stated. All statistical tests were performed using the SigmaStat computer software (Jandel, San Rafael, CA). Changes caused by treatments were analyzed by using repeated-measures analysis of variance (ANOVA) with post hoc evaluation by the Neuman-Keuls test. A probability value of less than .05 was considered significant.

RESULTS

Omacor was well tolerated; monitoring of drug intake by capsule counting and measurement of FAs profile in plasma phospholipids[11] indicated that compliance to treatment was satisfactory. Plasma total cholesterol did not change after placebo or Omacor treatment; plasma triglycerides were significantly lower after Omacor compared to baseline and placebo (Table 1). Plasma LDL cholesterol and apoB concentrations were 25% and 7% higher after Omacor than placebo.[11]

HDL cholesterol was higher after Omacor (+4% and +8% compared to baseline and placebo), but neither of these differences achieved statistical significance. No changes were observed in the plasma levels of apoA-I and apoA-II, and of LpA-I and LpA-I:A-II particles (Table 1).

Although the HDL cholesterol level did not change, a marked effect was observed on the cholesterol distribution between the major HDL subfractions: HDL_2 cholesterol increased by 40% after Omacor and decreased by 7% after placebo; minor, nonsignificant changes were found in plasma HDL_3 cholesterol (Table 1). Similar changes were observed in total lipoprotein mass; HDL_2 increased from 42.5 ± 14.0 mg/dL (baseline) and 40.1 ± 11.7 mg/dL (placebo) to 53.7 ± 21.3 mg/dL (Omacor), and HDL_3 decreased from 302.7 ± 18.9 mg/dL (baseline) and 302.5 ± 19.0 mg/dL (placebo) to 285.3 ± 15.4 mg/dL (Omacor). Due to these changes, the HDL_2/HDL_3 cholesterol and mass ratios increased from

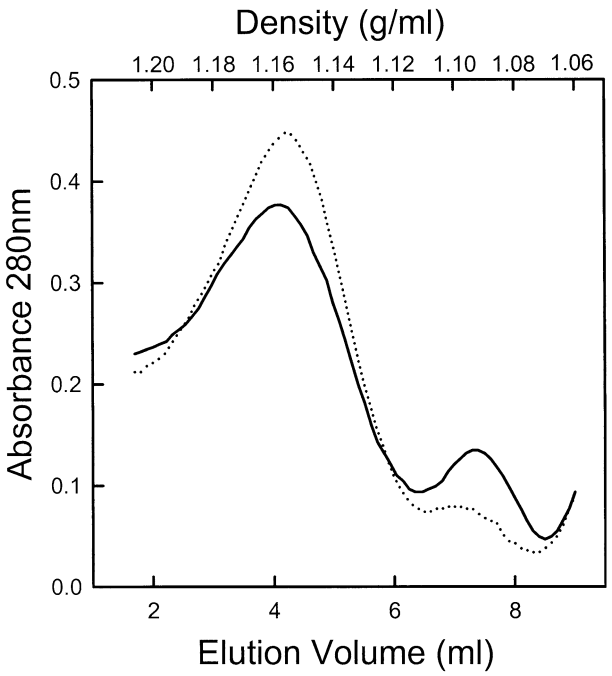

Fig 1. Mean rate zonal ultracentrifugation profiles of HDL subfractions from FCHL patients before (dotted line) and after (continuous line) treatment with Omacor. The left peak represents HDL_3, the right peak represents HDL_2.

0.191 ± 0.042 and 0.141 ± 0.037 (baseline) to 0.283 ± 0.074 and 0.188 ± 0.072 (Omacor), respectively. The structure of HDL subfractions before and after Omacor or placebo was analyzed by evaluating their flotation rate, particle diameter, and chemical composition. The mean elution profiles of HDL after rate zonal ultracentrifugation are reported in Fig 1. After Omacor, the content of slow-floating HDL_3 particles decreased, with a concomitant increase of fast-floating HDL_2. Slight, nonsignificant increases were found in flotation rate of both HDL_3 (elution volume: 4.46 ± 0.12 mL [baseline], 4.53 ± 0.12 mL [Omacor]) and HDL_2 (elution volume: 7.63 ± 0.08 mL [baseline], 7.75 ± 0.07 mL [Omacor]); the mean HDL_3 and HDL_2 particle diameter did not change. Minor variations were also observed in the composition of HDL subfractions (Table 2): both HDL_2 and HDL_3 became CE-enriched and triglycerides-depleted after Omacor, but only the increase in HDL_2 CE content achieved statistical significance.

We also evaluated the plasma concentration of 2 factors critically involved in the determination of the plasma concentration and structure of the HDL, and of their subfractions, ie, LCAT and CETP. No significant changes were observed after either placebo or Omacor (Table 3). By contrast, the plasma concentration of paraoxonase, an antioxidant enzyme that circulates in plasma bound to HDL,[23] increased by 10% after Omacor compared to baseline and placebo (Table 3).

Finally, no significant changes were observed in the capacity of patients' plasma to promote cell cholesterol efflux, expressed as percent cholesterol efflux from Fu5AH cells during a 4-hour incubation with plasma samples collected before (20.0% ± 0.9%) and after placebo (20.3% ± 0.9%) or Omacor (20.3% ± 0.8%).

Table 2. HDL Subfractions Composition at Baseline, and During Placebo and Omacor

	Baseline	Placebo	Omacor
HDL_2			
Free cholesterol	3.43 ± 0.22	3.56 ± 0.24	3.34 ± 0.21
Cholesteryl esters	20.26 ± 1.15	19.99 ± 1.13	22.89 ± 1.28*
Triglycerides	9.59 ± 0.98	8.69 ± 0.77	7.30 ± 1.00
Phospholipids	22.00 ± 1.69	22.04 ± 1.70	22.34 ± 1.40
Proteins	44.73 ± 1.05	45.71 ± 0.8	44.12 ± 0.79
HDL_3			
Free cholesterol	1.99 ± 0.15	1.80 ± 0.08	1.80 ± 0.13
Cholesteryl esters	15.02 ± 0.91	15.08 ± 0.87	15.67 ± 0.64
Triglycerides	8.24 ± 0.84	8.34 ± 0.94	6.74 ± 0.77
Phospholipids	20.84 ± 0.81	20.98 ± 0.79	20.69 ± 0.66
Proteins	53.91 ± 0.78	53.86 ± 0.76	55.11 ± 0.70

NOTE. Results are expressed as percentage of weight, means ± SEM, N = 14.
*Significantly different from baseline and placebo.

DISCUSSION

The dyslipidemia in FCHL patients is characterized by elevations of plasma cholesterol and/or triglycerides, predominance of small and dense LDL, and reduced plasma HDL_2 levels.[2-4] We have previously reported that a concentrate of ω-3 FAs lowers plasma triglycerides, and shifts LDL subclass distribution towards more buoyant particles, without affecting LDL size, in FCHL patients.[11] We show now that the same ω-3 FA concentrate remarkably increases plasma HDL_2 cholesterol and mass levels, without affecting the concentration of the denser HDL_3 subfraction. Moreover, the ω-3 FA concentrate raises the plasma concentration of the HDL-bound, antioxidant enzyme paraoxonase.

Drug treatment of FCHL has essentially focused on the lowering of the total amount of LDL, and the reduction in the atherogenicity of LDL, ie, reduction in the amount of small and dense LDL, with little attention paid to the correction of the defective HDL profile. Statins have little effect[16,24,25] while fibrates increase[24-27] plasma HDL cholesterol in FCHL patients. In the present study, a ω-3 FA concentrate slightly increased plasma HDL cholesterol and did not affect the concentration of major HDL apolipoproteins and lipoprotein particles, but it did enhance the plasma content of the HDL_2 subfraction. The increase of plasma HDL_2 levels may be consequent to the remarkable decrease of plasma triglyceride-rich lipoproteins, leading to a reduced CE transfer out of HDL,[18]

Table 3. Plasma LCAT, CETP, and Paraoxonase Concentrations at Baseline, and During Placebo and Omacor Treatment

	Baseline	Placebo	Omacor
CAT (mg/mL)	4.90 ± 0.44	4.96 ± 0.61	5.03 ± 0.60
CETP (mg/mL)	1.19 ± 0.20	1.20 ± 0.17	1.32 ± 0.27
Paraoxonase (μg/mL)	117.0 ± 7.5	117.2 ± 7.8	129.7 ± 9.3*

NOTE. Results are expressed as means ± SEM, N = 14.
*Significantly different from baseline and placebo.

and accumulation in plasma of CE-enriched and slowly catabolized HDL_2 particles.[28] The observation that ω-3 FAs decrease the net mass transfer of CE from HDL to lower density lipoproteins,[29] without affecting plasma CETP concentration, as shown in the present study, is consistent with this mechanism. However, no significant correlation was found between the reciprocal changes in plasma triglyceride and HDL_2 levels ($r = 0.127$, $P = .67$). Another possibility is that HDL phospholipids enriched with ω-3 FAs are poor substrates for hepatic lipase, as shown for the phospholipase activity of LCAT,[30] preventing hepatic lipase–mediated HDL_2 to HDL_3 conversion, thus again leading to a selective accumulation of HDL_2 in plasma.

Oxidation of LDL is recognized to be a critical early step in atherogenesis, and various investigators have found a correlation between LDL oxidizability and atherosclerosis progression.[31] The small LDL of FCHL patients are more prone to oxidation than normal, large LDL particles,[32] and both LDL size and in vitro oxidizability are correlated with the extent of preclinical atherosclerosis, as measured by B-mode ultrasonography in FCHL patients.[33] Therefore, the accumulation of small, oxidation-prone LDL in the plasma of FCHL patients may contribute to the high CHD risk. The HDL-bound paraoxonase prevents LDL oxidation in vitro[34] and in the arterial wall,[35] a mechanisms that provides a link between HDL, lipoprotein oxidation, and atherosclerosis. Paraoxonase activity and concentration are under genetic and environmental regulation. Environmental factors that alter paraoxonase activity/concentration include cigarette smoking,[36] and alcohol, fat, and vitamin intake.[37-39] There is obvious interest in pharmacological interventions able to increase paraoxonase activity/concentration, especially in high-risk individuals, but very little information has been made available so far. Hormone-replacement therapy was reported to increase paraoxonase activity in postmenopausal women.[40] Among lipid-affecting drugs, simvastatin increased paraoxonase activity in patients with familial hypercholesterolemia,[41] while fibrates had no effect in patients with mixed hyperlipidemia.[42] The present double-blind, placebo-controlled study shows that the administration of an ω-3 FA concentrate to high-risk FCHL patients causes a modest but significant increase of plasma paraoxonase concentration. Some studies have reported significant correlations between paraoxonase activity/concentration and plasma HDL and apoA-I levels[36,43,44]; however, the ω-3 FA concentrate increased plasma paraoxonase concentration without affecting the plasma levels of major HDL apolipoproteins. Paraoxonase has been described as preferentially associated with apoA-I–containing HDL particles,[45] but the increase of paraoxonase induced here by the ω-3 FA concentrate is clearly independent of changes in plasma LpA-I levels. It is noteworthy that a similar rise of paraoxonase activity without changes in plasma HDL cholesterol, apoA-I and LpA-I levels was previously reported in simvastatin-treated hypercholesterolemic patients.[41] Both the ω-3 FA concentrate and simvastatin, however, cause a change in the distribution of HDL subfractions, selectively increasing HDL_2 levels.[46] It thus seems that changes in HDL size/density distribution, rather than variations in total HDL concentrations, are important in mediating the effects of ω-3 FAs and simvastatin on plasma paraoxonase content. Indeed, in vitro studies have shown that large HDL are more efficient than small HDL in promoting the release of paraoxonase from cells and stabilizing the enzyme.[47]

The relevance of the present findings for the objective of reducing the high CHD risk in FCHL patients remains to be defined. FCHL patients present with elevated plasma cholesterol and/or triglycerides, predominance of small and dense LDL particles, and low concentrations of HDL_2 cholesterol,[2-4] and each of these abnormalities may contribute to the high CHD risk.[5-7] The administration of an ω-3 FA concentrate to FCHL patients does not change plasma cholesterol and lowers plasma triglycerides. The potentially harmful increase of LDL cholesterol may be balanced with a shift of LDL distribution towards more buoyant particles.[11] The ω-3 FA concentrate selectively raises the plasma concentrations of the "protective" HDL_2 subfraction,[7,48-51] and of the antioxidant enzyme paraoxonase. All together these changes may imply a beneficial effect of the ω-3 FA concentrate on CHD risk in FCHL patients, which needs to be demonstrated in large prospective studies.

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