Effects of fish oil fatty acids on low density lipoprotein size, oxidizability, and uptake by macrophages

Michio Suzukawa, Mavis Abbey,[1] Peter R. C. Howe, and Paul J. Nestel

CSIRO, Division of Human Nutrition, P.O. Box 10041 Gouger Street, Adelaide, South Australia 5000, Australia

Abstract The effect of fish oil and corn oil supplementation on plasma lipids and lipoproteins and on low density lipoprotein (LDL) oxidation was examined in 20 treated hypertensive subjects. The randomized double-blind crossover study consisted of two 6-week interventions with 4 g/day of a highly purified fish oil or corn oil. Fish oil significantly (−24%, $P < 0.01$) reduced plasma triglyceride, and increased LDL-cholesterol (+6%, $P < 0.01$ compared to corn oil). LDL particles were larger ($P < 0.01$) after fish oil compared to baseline and LDL size was inversely correlated with plasma triglyceride ($P < 0.001$) both before and after fish oil supplementation, and positively correlated with high density lipoprotein cholesterol ($P < 0.01$). Fish oil reduced lag time before onset of copper-induced LDL oxidation (−25%, $P < 0.001$) and significantly increased production of thiobarbituric acid-reactive substances (TBARS) during oxidation, compared with corn oil. Corn oil had no significant effect on lag time and oxidation rate. Fish oil increased macrophage uptake of copper-oxidized LDL and of macrophage-modified LDL. Corn oil was without effect. Additionally, macrophages that were supplemented with fish oil fatty acids in vitro displayed a significantly ($P < 0.001$) higher capacity to oxidize LDL than either control cells or cells supplemented with corn oil fatty acids. We conclude that from the standpoint of atherosclerosis, fish oil fatty acids adversely raise the susceptibility of LDL to copper-induced and macrophage-mediated oxidation but that the increase in plasma LDL cholesterol concentration reflects an increase in size that may be favorable.—**Suzukawa, M., M. Abbey, P. R. C. Howe, and P. J. Nestel.** Effects of fish oil fatty acids on low density lipoprotein size, oxidizability, and uptake by macrophages. *J. Lipid Res.* 1995. **36:** 473–484.

Supplementary key words atherosclerosis • n-3 fatty acids • corn oil • LDL oxidation • triglyceride • human study • macrophage • LDL size

Epidemiological studies in Greenland Eskimos led to the hypothesis that fish oil rich in n-3 polyunsaturated fatty acids (PUFAs), eicosapentaenoic acid (EPA, C20:5), and docosahexaenoic acid (DHA, C22:6), is useful for ameliorating atherosclerosis (1, 2). Animal studies in swine (3) and nonhuman primates (4) have provided convincing evidence that fish oil prevents atherosclerosis. However, Thiery and Seidel (5) have reported that fish oil feeding results in an enhancement of cholesterol-induced atherosclerosis in rabbits. In a clinical study of patients with coronary heart disease, Burr et al. (6) have reported that the number of non-fatal acute myocardial infarctions was higher in the group advised to increase intake of dietary fatty fish and fish oil, although incidence of sudden death decreased. Dietary fish oil is reported to affect many cardiovascular risk factors such as lipid metabolism (7–9), platelet function (10), blood pressure (11), blood viscosity (12), and inflammatory processes (13). Trials using fish oil supplements have shown a hypotriglyceridemic effect, and a reduction in low density lipoprotein cholesterol (LDL-C) levels when saturated fat intake is partially replaced by fish oil (7). However, the atherogenicity of LDL from patients supplemented with fish oil is not yet clear. This could derive from increased vulnerability to oxidation or from the presence of smaller, denser LDL. Recently, it was proposed that oxidation of LDL increases its atherogenicity, and susceptibility of LDL to oxidative modification is one of the most important factors in its atherogenicity (14, 15). The generation of oxidized LDL leads to enhanced uptake by macrophages, stimulation of macrophage acyl-CoA:cholesterol acyltransferase (ACAT) activity and foam cell formation in arterial walls. Diets rich in n-6 PUFAs have been shown to increase susceptibility of LDL to oxidative modification (16–19). However, the effects of dietary n-3 PUFAs are contradictory (20–22).

Abbreviations: LDL, low density lipoprotein; TBARS, thiobarbituric acid-reactive substances; PUFA, polyunsaturated fatty acid; EPA, eicosapentaenoic acid; DHA, docosahexaenoic acid; LDL-C, LDL-cholesterol; ACAT, acyl-CoA:cholesterol acyltransferase; FCS, fetal calf serum; DMEM, Dulbecco's modified Eagle's medium; BSA, bovine serum albumin; HDL, high density lipoprotein; CE, cholesteryl ester; HBSS, Hanks balanced salt solution; LPDS, lipoprotein-deficient serum; LOOH, lipid peroxide; VLDL, very low density lipoprotein; CETP, cholesteryl ester transfer protein; CAD, coronary artery disease.

[1]To whom correspondence should be addressed.

In this randomized double-blind crossover study of hypertensive subjects (fish oil and corn oil), we examined changes in lipids and lipoprotein composition and size and the susceptibility of LDL to copper-induced and macrophage-mediated oxidation. Additionally, we reported that macrophages supplemented with fish oil fatty acids in vitro had enhanced ability to oxidize LDL.

MATERIALS AND METHODS

Subjects

This study was organized as part of a dietary intervention trial that investigated the effect of n-3 fatty acids as an adjunct to drug treatment of hypertension in 42 subjects recruited from general practice. Blood pressure and plasma lipid results for the full study (n = 42) will be reported elsewhere. We report here on a sub-set of 20 subjects (14 women, 6 men) with stable uncomplicated hypertension. The subjects, with a mean age of 60 ± 10 years (mean ± SD) were being managed with either beta-blocker (atenolol) monotherapy (10 women, 5 men) or a combination of beta-blocker (atenolol) and diuretic (4 women, 1 man). Diuretics that were used in this study were indapamide hemihydrate (2 subjects), chlorothiazide (2 subjects), and hydrochlorothiazide (1 subject). Subjects continued their usual medication throughout the study. Baseline characteristics (mean ± SEM) were systolic blood pressure 130 ± 3 mm Hg, diastolic blood pressure 77 ± 2 mm Hg, plasma cholesterol 5.63 ± 0.34 mmol/l, plasma triglyceride 1.73 ± 0.20 mmol/l, and body mass index 26.6 ± 3.8 kg/m². The study was approved by the Human Ethics Committee of CSIRO Division of Human Nutrition, Adelaide, South Australia.

Study design

The study was a randomized double-blind crossover design. Subjects maintained their normal diet and exercise patterns during a 4-week run-in period after which they were randomly assigned to two groups (groups A and B) who took each test supplement for 6 weeks in a different sequence. In each 6-week intervention phase, subjects took four 1-g capsules per day containing either Omacor (Pronova, Oslo, Norway), which provided 3.4 g of n-3 fatty acids, or corn oil. Ten subjects in group A took corn oil capsules first, and the others in group B took fish oil capsules first. The fatty acid composition and α-tocopherol content of the capsules are shown in **Table 1.** Compliance was monitored by interview at fortnightly intervals. Body weight was monitored throughout the study.

Materials

Dulbecco's modified Eagle's medium (DMEM), Ham's F-10 medium, penicillin (50,000 U/ml), streptomycin (50,000 μg/ml), l-glutamine (200 mM) and fetal calf serum (FCS) were obtained from Cytosystems (Sydney, N.S.W. Australia). [1α,2α(n)-³H]cholesterol was purchased from Amersham (Sydney, N.S.W. Australia). Essentially fatty acid-free bovine serum albumin (BSA) was from Sigma Chemical Co. (Sydney, N.S.W., Australia).

TABLE 1. Fatty acid composition and α-tocopherol content of corn oil and fish oil supplements

	Corn Oil	Fish Oil
Fatty acid (%)[a]		
16:0	10.2	0.2
18:0	2.0	0.6
18:1	28.0	0.4
18:2	57.5	0.3
18:3	0.9	
20:4		2.8
20:5		48.3
22:5		3.1
22:6		35.6
α-Tocopherol (mg/ml)	2.2	2.0

[a]The number before the colon specifies the number of carbon atoms, and that after the colon the number of double bonds.

Cell culture

Monolayer cultures of J774 macrophages were grown and maintained in DMEM containing 10% (vol/vol) FCS, penicillin (100 U/ml), streptomycin (100 μg/ml) and l-glutamine (292 μg/ml). For each experiment, the cells were cultured in Multiwell™ Tissue Culture Plates (6-Well, Becton Dickson Labware, Rutherford, NJ) at 37°C in an atmosphere containing 5% CO_2/95% air.

Methods

At the end of the run-in period (visit 1, baseline) and after each 6-week intervention period (visit 2 or 3) blood was collected in EDTA (1 mg/ml) after an overnight fast of at least 12 h. Plasma was separated by low speed centrifugation at 600 *g* for 10 min at 4°C.

Plasma lipid measurements

Aliquots of plasma were stored at −80°C for analysis at the end of the study. Plasma cholesterol and triglyceride were measured by enzymatic colorimetric techniques in a Cobas-Bio automated centrifugal analyzer (F. Hoffmann-La Roche Ltd. Basle, Switzerland) using test kits (Roche Diagnostic Systems, Basle, Switzerland). Total high density lipoprotein (HDL) and HDL_3 were measured as for plasma cholesterol after selective precipitation with Dextralip (Sochibo, France) (23). HDL_2 was calculated by difference. LDL cholesterol was calculated using a modification of the Friedewald equation (24).

Lipoprotein preparation

LDL was prepared from plasma that was stored at −80°C by a rapid isolation technique using a Beckman

Optima TLX benchtop ultracentrifuge (Beckman Instruments, Palo Alto, CA) as reported by Chung et al. (25). LDL for oxidation experiments was dialyzed at 4°C against phosphate-buffered saline (PBS, pH 7.4) which had been purged with N_2, and sterilized by filtration (0.2 μm). Radiolabeled LDL was prepared from plasma that was incubated for 24 h with [^{3}H]cholesterol and washed with red blood cells to remove unincorporated label according to the technique reported by Barter and Jones (26), [^{3}H]CE(cholesteryl ester)-LDL was isolated by ultracentrifugation, dialyzed, and sterilized as described above. The final preparation of [^{3}H]CE-labeled LDL contained 83.6 ± 3.3% (n = 3) of the radioactivity in CE and had a specific activity of 6040 ± 1086 cpm/μg protein.

Copper oxidation of LDL

Oxidation of LDL was determined as the production of conjugated dienes by continuously monitoring the change in absorbance at 234 nm according to the method of Esterbauer et al. (27). Freshly prepared LDL (50 μg protein/ml) was incubated with 5 μM $CuSO_4$ at 37°C in a Beckman DU65 Spectrophotometer fitted with a peltier heater (Beckman Instruments). Absorbance at 234 nm was automatically recorded at 2-min intervals for 120 min. Lag time and propagation rate were determined as previously described (27).

Uptake of copper-oxidized [^{3}H]CE-LDL in J774 macrophages

Because of the possibility of between-experiment variability, plasma was stored at −80°C and three LDL samples from the same subject (LDL separated from plasma at the end of baseline, corn oil, and fish oil periods) were measured at the same time. The same number of J774 cells (6×10^5 cells/dish) were seeded and cultured for 3 days before adding the LDL. Values in the experiments using cultured cells are presented as percent of baseline for each subject. Kleinveld et al. (28) have reported that EDTA-treated plasma stored at −80°C is stable for several weeks. We have shown that freezing plasma at −80°C prior to isolation of LDL has no significant effect on lag time or oxidation rate during incubation of LDL with copper. Lag time and oxidation rate were 53 ± 1.4 min and 18.5 ± 0.3 nmol diene/min per mg protein (means ± SD, n = 4), respectively, on LDL prepared from fresh plasma and 52 ± 1.5 min and 17.1 ± 1.4 nmol diene/min per mg protein, respectively, on LDL prepared from the same plasma that had been stored at −80°C for 2 months. Additionally, in a preliminary study, we tested the effect of freezing the plasma sample on uptake of oxidized LDL by macrophages. Fresh plasma samples from nine healthy volunteers were stored at 4°C or −80°C for 24 h. There were no significant differences between LDL separated from fresh plasma and LDL separated from frozen plasma either in uptake of copper-oxidized LDL (2.51 ± 0.62, 2.99 ± 0.87 μg LDL/mg protein, fresh and frozen, respectively) or in uptake of macrophage-oxidized LDL (3.22 ± 0.78, 3.05 ± 0.48 μg LDL/mg protein, fresh and frozen, respectively). These results confirm that there is no significant effect of freezing on uptake of copper-oxidized LDL and macrophage-oxidized LDL.

[^{3}H]CE-LDL (50 μg/ml protein) was oxidized by incubation with 5 μM $CuSO_4$ in PBS (incubation volume 1 ml) at 37°C for 90 min. The reaction was stopped by adding EDTA (100 μM final concentration). A 250-μl aliquot of the incubation mix was added to a monolayer of J774 macrophages that were cultured in 1 ml DMEM with 10% FCS (final concentration of copper-oxidized [^{3}H]CE-LDL was 10 μg/ml). In this medium, macrophages do not oxidize LDL (29), and Zhang, Harkamal, and Steinbrecher (30) have reported that [^{3}H]CE-LDL does not lose cholesteryl ester radioactivity by oxidation. After incubation for 24 h with copper-oxidized [^{3}H]CE-LDL, each monolayer was washed twice with buffer A (150 mM NaCl, 50 mM Tris-HCl at pH 7.4) containing 2 mg/ml BSA, followed by three washes with buffer A containing no BSA. One ml of hexane–isopropanol (3:2) was added to each dish and incubated for 30 min at room temperature. The organic solvent was collected and each monolayer was rinsed with 0.5 ml of the same solvent. The recovery of [^{3}H]cholesteryl ester in this collection was 98 ± 2% (n = 6). Radioactivity in the organic solvent extracts was measured by scintillation counting. After extraction of lipids in situ, the cells in each monolayer were dissolved in 2 ml of 0.1 M NaOH and aliquots were taken for protein determination.

As exposure to copper for 90 min leads only to partial oxidation of LDL, further experiments were carried out on pooled samples of plasmas from each experimental period (baseline, corn oil, and fish oil) using the plasmas of the 20 subjects in this study. LDL were labeled and isolated as before but oxidation was carried out for 180 min as well as for 90 min.

Macrophage-mediated oxidation

To measure susceptibility of [^{3}H]CE-LDL to macrophage-mediated oxidation, 10 μg/ml [^{3}H]CE-LDL was incubated for 24 h with J774 macrophages in Ham's F10 medium and uptake of [^{3}H]CE-LDL in this 24 h was determined as described above for copper-oxidized [^{3}H]CE-LDL.

As this design measures uptake of LDL from medium in which the LDL were oxidized by the macrophages, additional two-step studies were carried out in which oxidized LDL were incubated with fresh macrophages. This was performed with pooled samples from each of the three periods (baseline, corn oil, and fish oil) using the plasmas of the 20 subjects in the study. [^{3}H]CE-LDL (20 μg/ml) was oxidized by 24 h incubation with a monolayer of J774

macrophages in Hanks Balanced Salt Solution (HBSS) supplemented with iron (5 μM) and copper (1 μM). This conditioned [^{3}H]CE-LDL was diluted twice with DMEM containing 10% lipoprotein-deficient serum (LPDS) to protect against further oxidation, and incubated for 6 h with a fresh monolayer of J774 macrophages to measure uptake. TBARS in the conditioned [^{3}H]CE-LDL was also measured.

Supplementation of macrophages with fish oil fatty acids or corn oil fatty acids to measure macrophages potential to oxidize LDL

A fish oil fatty acids- or a corn oil fatty acids-albumin mixture was made by adding fish oil fatty acids (0.8 ml from a Omacor capsule) or corn oil fatty acids (0.8 ml from a corn oil capsule) to 5.2 ml of 0.15 M sodium chloride at pH 7.4 containing 12% (w/v) fatty acid-free BSA. The solution was stirred gently with a magnetic stirrer for 4 h at 4°C, sterilized by 0.45 μm filter and kept frozen at −80°C until use. J774 macrophages, which had been cultured in Multiwell™ for 2 days, were supplemented with fish oil fatty acids or corn oil fatty acids by culturing in 2 ml of DMEM with 10% FCS containing 25 μl of fish oil fatty acids- or corn oil fatty acids-albumin mixture. The same amount of 0.15 M sodium chloride at pH 7.4 containing 12% (w/v) fatty acid-free BSA was added to the medium of control cells. After 24 h incubation, cells were washed carefully once with PBS containing 2 mg/ml BSA, followed by three washes with PBS. Twenty μg of [^{3}H]CE-LDL was added to the washed cells incubated in 1 ml of HBSS containing 5 μM iron and 1 μM copper. After 24 h incubation, conditioned [^{3}H]CE-LDL was diluted twice with DMEM containing 10% LPDS and added to fresh macrophages. Uptake of [^{3}H]CE-LDL was measured as described above. TBARS in the conditioned [^{3}H]CE-LDL was also measured.

Other analyses

Protein concentrations of LDL and cells were determined on the Cobas-Bio automated centrifugal analyzer. (Roche Diagnostica, Nutley, NJ) by the method of Clifton, Chang, and Mackinnon (31). Malondialdehyde generated in oxidized LDL and in medium was measured by the TBARS method as described by Buege and Aust (32) except that the sample volume was 0.1 ml, the reagent volume was 0.2 ml, and the sample absorbance was measured at 535 nm in a Cobas-Bio automated centrifugal analyzer. The concentration of MDA was calculated using the extinction coefficient for MDA ($1.56 \times 10^5 M^{-1}cm^{-1}$) as previously described (32). LDL α-tocopherol was measured by high performance liquid chromatography using the method of Yang and Lee (33). Total cholesterol content of isolated LDL was measured as described above for plasma cholesterol. Fatty acid methyl esters in LDL and macrophages were determined by gas chromatography as previously described (34). LDL particle size was determined by the method of McNamara et al. (35). Plasma apolipoprotein B (apoB) was measured by immunonephelometry using anti-human-apoB antiserum (Boehringer-Mannheim, Germany) (36) on a Cobas-Bio automated centrifugal analyzer. Plasma lipid peroxide (LOOH) was measured colorimetrically (37) on the Cobas-Bio automated centrifugal analyzer using a test kit (Kamiya Biochemical Co., Thousand Oaks, CA).

Data analysis

Statistical analysis of oxidation measures was conducted using StatWorks™ computer software (Version 1.2, Cricket Software Inc., Philadelphia, PA). All other statistical analyses were conducted using SPSS-PC computer software (SPSS, Chicago, IL). Comparisons of data between periods were performed by *t*-test.

RESULTS

Plasma measurements (Table 2)

Plasma triglyceride was significantly lower after supplementation with fish oil (−24% and −20% compared with baseline and corn oil, respectively, $P < 0.01$). As

TABLE 2. Plasma lipids and lipoproteins, lipid peroxide (LOOH), apoB, and LDL size after the baseline, corn oil, and fish oil periods

	Baseline	Corn Oil	Fish Oil
Lipid fraction (mmol/l)			
Cholesterol	5.63 ± 1.51	5.52 ± 1.36	5.68 ± 1.39
Triglyceride	1.73 ± 0.89	1.63 ± 0.77	1.31 ± 0.52[a]
LDL-C	3.95 ± 1.34	3.81 ± 1.25	4.10 ± 1.26[b]
HDL-C	0.99 ± 0.24	0.97 ± 0.25	0.99 ± 0.30
HDL_3-C	0.74 ± 0.16	0.75 ± 0.21	0.75 ± 0.21
HDL_2-C	0.25 ± 0.13	0.22 ± 0.12	0.24 ± 0.15
LDL size (nm radius)	12.42 ± 0.35	12.49 ± 0.34	12.58 ± 0.34[c]
Plasma LOOH (nmol/ml)	1.79 ± 1.42	1.40 ± 1.10	1.90 ± 1.44
Plasma apoB (g/l)	0.84 ± 0.25	0.81 ± 0.24	0.85 ± 0.24

Values are means ± SD, n = 20.
[a]Significantly different from baseline and corn oil, $P < 0.01$.
[b]Significantly different from corn oil, $P < 0.01$.
[c]Significantly different from baseline, $P < 0.01$.

shown in **Fig. 1A,** there was no apparent carryover effect of triglyceride lowering from fish oil to the end of the corn oil period in group B. There were no significant differences in total plasma cholesterol among the three periods although there was a 7.6% increase in LDL-cholesterol ($P < 0.01$) with fish oil compared to corn oil. There were no significant differences among the three groups for total HDL-cholesterol, HDL_2-cholesterol, or HDL_3-cholesterol. The LDL:HDL cholesterol ratio did not change during the study (4.2 ± 1.7, 4.1 ± 1.5, 4.4 ± 1.7, means ± SD for the baseline, corn oil, and fish oil periods, respectively). After fish oil fatty acid supplementation, the major species of LDL particles were significantly larger ($P < 0.01$) compared with LDL after the baseline period. Supplementation had no effect on plasma apoB or plasma lipid peroxide levels.

In each dietary period LDL particle size was inversely correlated with plasma triglyceride ($r = -0.71$, $r = -0.76$ and $r = -0.75$ for baseline, corn oil, and fish oil, respectively, $P < 0.001$) and positively correlated with HDL cholesterol ($r = 0.62$ ($P < 0.01$), 0.73 and 0.76 ($P < 0.001$) for baseline, corn oil, and fish oil, respectively). The change in plasma triglyceride from baseline to the fish oil period was also negatively correlated with the change in LDL particle size ($r = -0.55$, $P < 0.02$).

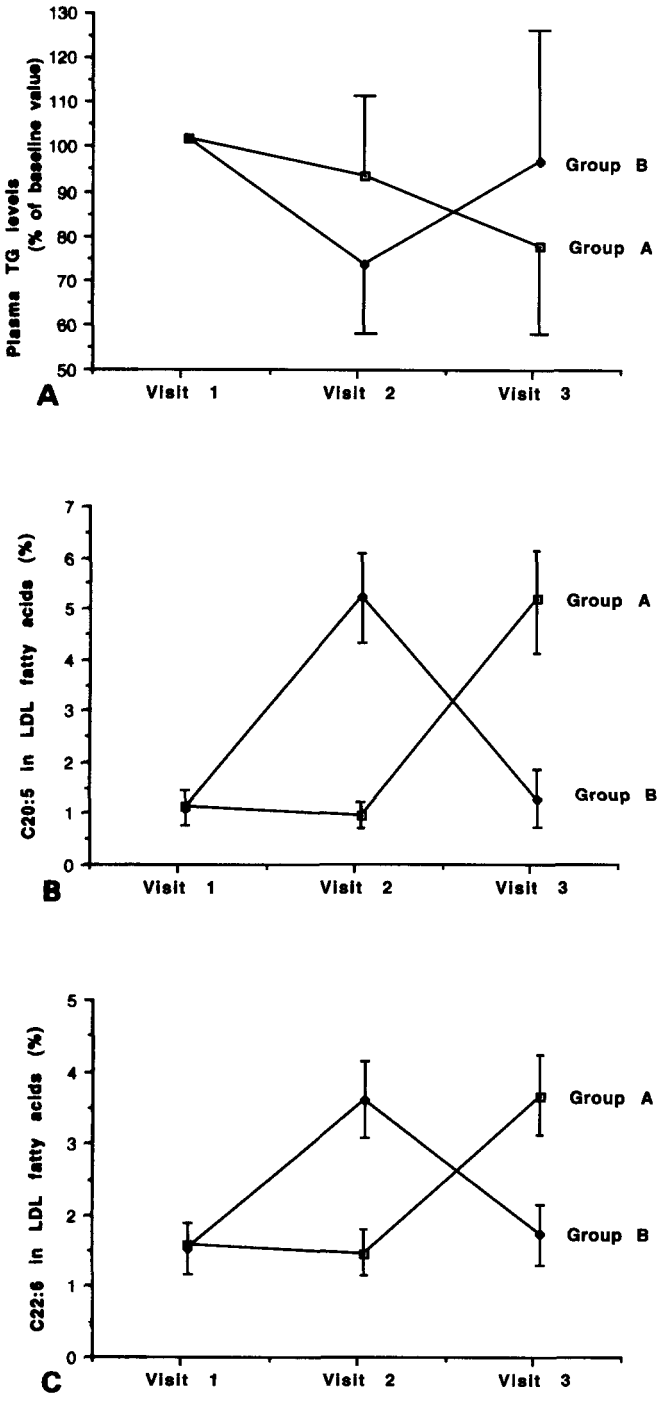

Fig. 1. Plasma triglyceride (TG) levels (panel A), C20:5 (panel B), and C22:6 (panel C) in LDL fatty acids at visit 1, 2, and 3 in group A or B. Values of plasma TG, C20:5, or C22:6 in LDL fatty acids represent means ± SD (n = 10 in group A, 10 in group B).

Characteristics of LDL used in oxidation experiments

The fatty acid composition of LDL used in oxidation experiments is shown in **Table 3.** After 6 weeks of fish oil supplementation, EPA (20:5) and DHA (22:6) increased significantly (5-fold, $P < 0.001$ and 2.4-fold, $P < 0.001$, respectively) while oleic acid (18:1), linoleic acid (18:2), dihomogammalinolenic acid (20:3), and arachidonic acid (20:4) decreased significantly ($P < 0.001$). Corn oil supplementation significantly decreased oleic acid ($P < 0.05$), and significantly increased linoleic acid ($P < 0.05$). As shown in Figs. 1B and 1C, there was no carryover effect on EPA and DHA from the fish oil period to the end of the corn oil period in group B where measurements were made.

Cholesterol and α-tocopherol contents of LDL used in the oxidation experiments are shown in **Table 4.** After corn oil supplementation, α-tocopherol in LDL was sig-

TABLE 3. Effect of corn oil and fish oil supplementation on LDL fatty acids

Fatty Acids[a]	Baseline	Corn Oil	Fish Oil
		%	
16:0	19.7 ± 1.9	19.5 ± 1.5	19.9 ± 2.1
16:1	3.5 ± 1.3	3.3 ± 0.9	3.1 ± 1.5
18:0	6.1 ± 0.7	6.0 ± 0.7	6.2 ± 0.6
18:1	21.1 ± 2.6	20.2 ± 2.4[b]	19.2 ± 2.0[c]
18:2	36.3 ± 6.1	38.0 ± 4.9[b]	33.9 ± 4.8[c]
20:3 (n-6)	1.9 ± 0.4	1.9 ± 0.4	1.2 ± 0.3[c]
20:4 (n-6)	6.6 ± 1.6	6.5 ± 1.5	5.6 ± 1.0[c]
20:5	1.0 ± 0.3	1.0 ± 0.5	5.1 ± 1.0[c]
22:6	1.5 ± 0.5	1.5 ± 0.4	3.5 ± 0.6[c]

Values are means ± SD, n = 20.
[a]The number before the colon specifies the number of carbon atoms, and that after the colon the number of double bonds.
[b]Significantly different from baseline, $P < 0.05$.
[c]Significantly different from baseline and corn oil, $P < 0.001$.

TABLE 4. Effect of corn oil and fish oil supplementation on α-tocopherol and cholesterol content of LDL

	Baseline	Corn Oil	Fish Oil
α-Tocopherol,			
μg/mg protein	5.99 ± 1.42	6.89 ± 1.96[a]	6.46 ± 1.61
mmol/mol cholesterol	4.42 ± 0.74	4.74 ± 1.04	4.54 ± 0.83
Cholesterol, μmol/mg protein	3.13 ± 0.49	3.32 ± 0.56	3.26 ± 0.65

Values are means ± SD, n = 20.
[a]Significantly different from baseline, $P < 0.01$.

nificantly higher (15%, $P < 0.01$) than at baseline when expressed relative to protein concentration, but not when expressed as a molar ratio to cholesterol. Supplementation with fish oil had no significant effect on LDL α-tocopherol. Total cholesterol content of LDL did not differ between dietary periods.

Copper oxidation of LDL

Lag time before initiation of LDL oxidation and propagation rate of oxidation are shown in **Table 5.** After fish oil supplementation lag time and propagation rate were significantly reduced (−25%, $P < 0.001$ and −10%, $P < 0.05$, respectively, compared to baseline). Corn oil supplementation had no effect on lag time or propagation rate. Oxidizability studies of LDL isolated from five subjects treated with both diuretic and beta-blocker showed a shorter lag time compared to that of the others (48.4 ± 2.5 min, 60.6 ± 11.0 min, respectively). The vitamin E content of these LDL was lower than that of the others (4.87 ± 1.26 μg/mg, 6.39 ± 1.29 μg/mg, respectively). In the subjects taking both diuretic and beta-blocker, lag time was significantly ($P < 0.01$) reduced by fish oil supplementation (48.4 ± 2.5 min, 35.3 ± 2.8 min, for baseline and fish oil, respectively).

Supplementation with fish oil significantly increased TBARS in pooled LDL samples after incubation with copper for 90 min or 180 min ($P < 0.001$, compared to baseline and corn oil, **Fig. 2A**). TBARS in [^{3}H]CE-LDL isolated from individual subjects were also significantly ($P < 0.001$ compared to baseline and corn oil) increased after incubation with copper for 90 min (116 ± 213, 492 ± 427% increase from baseline, corn oil, and fish oil, respectively). There was no significant change in TBARS in [^{3}H]CE-LDL (pooled samples and individual samples) oxidized for 90 min after corn oil supplementation. TBARS (pooled samples) produced after 180 min oxidation with corn oil supplementation was significantly ($P < 0.001$) lower than that at baseline. Uptake of [^{3}H]CE-LDL (pooled samples) that was oxidized for 90 min or 180 min increased significantly ($P < 0.001$, compared to baseline and corn oil, Fig. 2B) after fish oil supplementation. Uptake of individual samples of [^{3}H]CE-LDL oxidized for 90 min was also increased significantly after fish oil supplementation compared to baseline ($P < 0.001$) and corn oil ($P < 0.01$) (81 ± 169, 229 ± 180% increase from baseline, corn oil, and fish oil, respectively). There was no significant change after corn oil supplementation in uptake of copper-oxidized [^{3}H]CE-LDL (pooled samples and individual samples).

Macrophage-mediated LDL oxidation

As shown in **Fig. 3,** uptake of macrophage-oxidized [^{3}H]CE-LDL separated from pooled plasma after fish oil supplementation increased significantly compared to that at baseline ($P < 0.001$) and after corn oil ($P < 0.001$). There was no significant difference in the uptake after corn oil supplementation compared to that at baseline. Uptake of individual samples of [^{3}H]CE-LDL incubated with macrophages for 24 h was also increased significantly ($P < 0.01$) after fish oil supplementation compared to baseline and corn oil (9 ± 26, 26 ± 34% increase from baseline, corn oil, and fish oil, respectively). Corn oil supplementation did not significantly change the amount of TBARS in the medium; however, fish oil significantly increased TBARS compared to baseline ($P < 0.001$) and to corn oil ($P < 0.001$) (Fig. 3). TBARS in [^{3}H]CE-LDL isolated from individual subjects were also increased after incubation with macrophages for 24 h (5.1 ± 60.8, 55.4 ± 92% increase from baseline, corn oil, and fish oil, respectively).

Effect of supplementing macrophages with fish oil fatty acids on fatty acid composition of macrophages and capacity to oxidize LDL

Fish oil fatty acids were incorporated and metabolized by macrophages. C20:5, C22:5, and C22:6 in macrophage

TABLE 5. Effect of corn oil and fish oil supplementation on susceptibility of LDL to copper-induced oxidation

	Baseline	Corn Oil	Fish Oil
Lag time (min)	57.9 ± 9.4	57.9 ± 10.8	43.1 ± 7.5[a]
Propagation rate (nmole diene/min/mg protein)	11.9 ± 2.6	12.1 ± 2.0	10.6 ± 2.0[b]

Values are means ± SD, n ' 20.
[a]Significantly different from baseline and corn oil, $P < 0.001$.
[b]Significantly different from baseline and corn oil, $P < 0.05$.

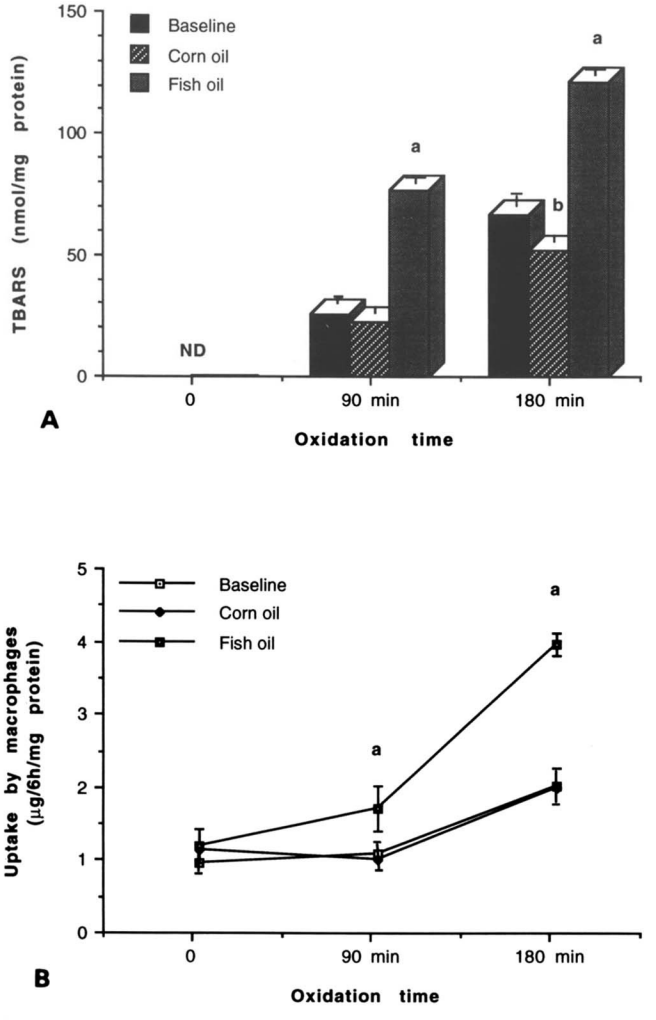

Fig. 2. Effect of corn oil and fish oil supplementation on TBARS in copper-oxidized LDL separated from pooled plasma (panel A), and on uptake of copper-oxidized LDL from pooled plasma (panel B). [^{3}H]CE-LDL separated from pooled plasma were oxidized by incubation with 5 μM $CuSO_4$ at 37°C in PBS for 90 min or 180 min. The reaction was stopped by adding EDTA (100 μM), and TBARS was measured. Oxidized [^{3}H]CE-LDL (final concentration, 10 μg/ml) was added to a monolayer of J774 macrophages cultured in DMEM with 10% FCS. After incubation for 6 h, uptake of copper-oxidized [^{3}H]CE-LDL was measured. Values in panel A or B represent means ± SD (n = 4); a, significant ($P < 0.001$) compared to baseline and corn oil; b, significant ($P < 0.001$) compared to baseline.

fatty acids increased significantly ($P < 0.01$) and C18:1 and C18:2 decreased significantly ($P < 0.05$) through in vitro supplementation (**Table 6**). When the fish oil fatty acid-enriched macrophages were preincubated with native [^{3}H]CE-LDL, it became apparent that some oxidation of the [^{3}H]CE-LDL had taken place because TBARS in the medium was significantly ($P < 0.01$) increased compared to that of fish oil fatty acid-enriched macrophages without LDL (4.27 ± 0.26, 2.55 ± 0.16 μM, mean ± SD n = 4, respectively). Incubation of these oxidized [^{3}H]CE-LDL led to enhanced uptake by fresh macrophages ($P < 0.001$ compared to LDL pre-incubated with control cells, Table 6). Uptake of the [^{3}H]CE-LDL conditioned by corn oil fatty acid-enriched macrophages (1.88 ± 0.39 μg/6 h per mg protein, mean ± SD n = 4) was significantly ($P < 0.001$) higher than that of control cells, but significantly ($P < 0.01$) lower than that of fish oil fatty acid-enriched cells. TBARS in the medium after pre-incubation of LDL with corn oil fatty acid-enriched cells (2.16 ± 0.17 μM, mean ± SD n = 4) was significantly ($P < 0.01$) increased compared to that with corn oil fatty acid-enriched cells in the absence of LDL (1.34 ± 0.18 μM, mean ± SD n = 4), but significantly ($P < 0.001$) lower than that of fish oil fatty acid-enriched cells (4.27 ± 0.26 μM).

DISCUSSION

The purpose of the study was to explore the effects of n–3 fatty acids in fish oil on LDL and in particular on the concentration, size, and oxidizability, as each of these characteristics influences atherosclerosis. The main outcomes were a small but significant rise in LDL cholesterol concentration such as has been reported by others (8), a small but significant increase in LDL size, and, most importantly, clear evidence for increased LDL oxidizability. This evidence comprised increased in vitro copper-oxidized and macrophage-modified changes in LDL that led to their increased uptake by macrophages. These findings define a potential atherogenic property of dietary fish oil, although it must be emphasized that these are in vitro observations and that the sum of the metabolic outcomes of marine n–3 fatty acids appears to be antiatherogenic in life. Nevertheless, our findings indicate a need for more antioxidant capacity, such as α-tocopherol, if large amounts of fish oil are to be taken.

Effect of fish oil on LDL composition and size

The LDL cholesterol concentration rose with fish oil (Table 2) but without a concomitant increase in plasma apoB. This is consistent with the observed increase in size without an increase in particle number, although any rise in apoB due to LDL particles would have been masked by the fall in very low density lipoprotein (VLDL) concentration. The reasons for the common increase in LDL cholesterol concentration after dietary fish oil have been reviewed by others (7, 8) and include the high palmitate content of many fish oils but certainly not of the present preparation. Metabolic explanations include a greater conversion of the predominantly smaller species of VLDL and LDL, and a down-regulation of LDL receptor activity seen in some but not all studies.

The reduction in plasma triglyceride with fish oil supplementation and the concomitant increase in LDL particle size observed in our study have also been reported by Contacas, Barter, and Sullivan (38) after daily supple-

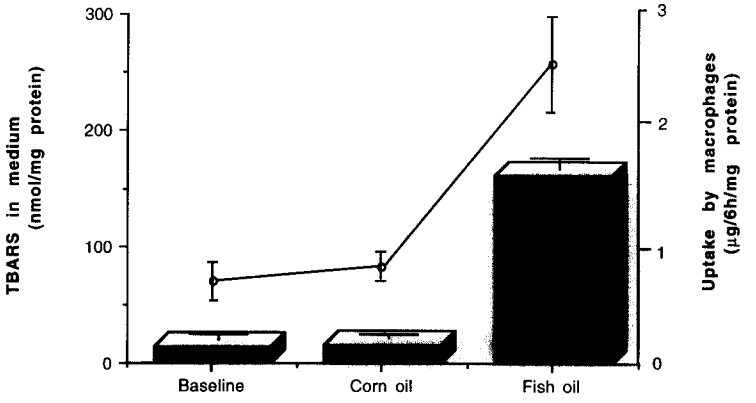

Fig. 3. Effect of corn oil and fish oil supplementation of macrophages on TBARS in medium (bar graph), and uptake of macrophage-oxidized LDL (line graph). [^{3}H]CE-LDL separated from pooled plasmas were first co-cultured with macrophages for 24 h and then reincubated with fresh macrophages for 6 h. TBARS in medium and uptake of macrophage-oxidized LDL were measured as described in methods. Values represent means ± SD (n = 4).

mentation with 3 g n-3 fatty acids. Small dense LDL are known to be associated with coronary artery disease (CAD) (39-41), and results from the Framingham Offspring Study indicate that small dense LDL are associated with an increase in plasma triglyceride (42). Using a two-variable model, the major factors determining LDL size have been shown to be plasma triglyceride and HDL cholesterol (35, 43), factors that in our study were significantly correlated with LDL size. The reason for these associations can be explained in part by the exchange of triglyceride from VLDL for cholesteryl ester in LDL which is mediated by cholesteryl ester transfer protein (CETP) (44). The enrichment of LDL with triglyceride promotes hydrolysis by lipase which results in the formation of smaller LDL particles. Thus, the reduction in plasma triglyceride, as a result of n-3 fatty acid supplementation, would be expected to reduce the triglyceride content of LDL leading to an increase in LDL size. In addition, we have shown previously that n-3 fatty acid supplementation reduces the activity of CETP (34) which may also contribute to the increase in LDL size. Further, as shown recently by Watson et al. (45), the associations between large LDL (LDL_1), VLDL triglyceride, and HDL cholesterol are also a function of hepatic triglyceride lipase activity. Small dense LDL particles are more susceptible than larger LDL to oxidative damage (46) and the increase in LDL size after n-3 fatty acid supplementation might be expected to contribute to a reduction in atherogenic risk.

LDL oxidizability

Several studies suggest that replacement of dietary saturated fats with n-6 PUFAs results in higher susceptibility of LDL to oxidative modification (16-19). The role of oxidized LDL in the formation of atherosclerosis is thought to be important. In animal studies, antioxidants, vitamin E (47) and probucol (48), have been reported to prevent atherosclerosis. In humans, susceptibility of LDL

TABLE 6. Effect of supplementation of macrophages with fish oil fatty acids on fatty acid composition of macrophages and capacity to oxidize LDL

	Control Cells	n-3-Enriched Cells
Fatty acid (%)[a]		
C16:0	19.0 ± 4.6	16.0 ± 3.0
C16:1	6.2 ± 1.7	6.4 ± 0.7
C18:0	6.7 ± 1.1	5.3 ± 2.6
C18:1	41.4 ± 13.4	18.5 ± 2.9[b]
C18:2	8.8 ± 3.4	2.7 ± 1.2[b]
C18:3	2.0 ± 0.6	1.6 ± 1.0
C20:3 (n-6)	1.6 ± 1.1	0.8 ± 0.1
C20:4 (n-6)	5.1 ± 2.6	3.9 ± 1.1
C20:5	1.8 ± 0.4	21.6 ± 6.2[c]
C22:5	2.0 ± 0.5	10.2 ± 4.0[c]
C22:6	4.9 ± 3.0	13.1 ± 0.8[c]
Uptake of pre-incubated [^{3}H]CE-LDL by macrophages (μg/6 h/mg cell protein)	0.54 ± 0.14	2.60 ± 0.24[d]

Values are means ± SD, n = 3 in fatty acids, n = 4 in uptake.
[a]The number before the colon specifies the number of carbon atoms, and that after the colon the number of double bonds.
[b]Significantly different from control, $P < 0.05$.
[c]Significantly different from control, $P < 0.01$.
[d]Significantly different from control, $P < 0.001$.

to oxidation has been reported to correlate significantly with the stage of development of coronary stenosis (49). Epidemiological studies show an inverse relationship between plasma vitamin E levels and mortality from ischemic heart disease (50) and also between dietary vitamin E intake and the incidence of coronary heart disease (51, 52). Our data show that there are adverse effects of n-3 PUFAs on LDL oxidizability and raise the paradoxical question of why then does fish oil protect against atherosclerosis, at least under experimental conditions. Demonstration of enhanced in vitro susceptibility does not, however, necessarily reflect the in vivo situation.

Plasma lipid peroxide relative to TG tended to be higher after fish oil supplementation compared to baseline and corn oil supplementation although it did not reach significance (Table 2). Plasma MDA:TG ratio increases significantly after fish oil supplementation despite lower plasma TG (20, 53), and suggests that increased plasma n-3 PUFAs provide excess substrate for oxidation. Several papers have reported on the effect of dietary fish oil on LDL oxidation with controversial results. LDL from rabbits on diets supplemented with EPA-ethyl ester (300 mg/kg) was less susceptible to oxidation (54), whereas fatty acid hydroperoxide levels in plasma from Watanabe heritable hyperlipidemic rabbits treated with 2.5 ml of MaxEPA daily were similar to those in unsupplemented rabbits (55). Nenseter et al. (21) reported that LDL from subjects supplemented with fish oil did not show increased susceptibility to copper-induced lipid peroxidation. However, in another study, fish oil (10 g MaxEPA/day) supplementation for 4 weeks raised TBARS in plasma and LDL, raised TBARS in conditioned LDL (by incubation with copper or smooth muscle cells), and stimulated uptake of the conditioned LDL by macrophages (22). In hypertriglyceridemic patients, dietary supplementation with fish oil (12 g/day Promega) for 6 weeks was reported to lead to higher susceptibility of LDL to copper-induced oxidation as evaluated by TBARS formation, free amino group levels, and changes in LDL electrophoretic mobility (20). In this current paper, we have demonstrated clearly that n-3 fatty acid supplementation increased the susceptibility of LDL to copper-induced and macrophage-mediated oxidation. The inconsistency among the various reports is hard to identify, but more favour heightened oxidizability.

Lag time, evaluated by monitoring diene formation, indicates the total antioxidative status of LDL (27). Many reports (56-58), including work from our own laboratory (59), have shown that vitamin E supplementation increases the lag time before the onset of LDL oxidation. Several groups have reported the effect of fish oil supplementation on vitamin E levels in plasma and LDL. Nair et al. (60) reported that fish oil supplementation decreased vitamin E levels in plasma, platelets, and red blood cells, whereas others (22, 53) have reported that there were no changes in vitamin E levels in plasma and LDL after fish oil supplementation. In our present study, vitamin E content of LDL did not change after fish oil supplementation (Table 4); the fish oil contained 2 mg vitamin E/ml, a total dose of 8 mg/day. Cosgrove, Church, and Pryor (61) reported that the oxidizability of PUFAs in homogenous solutions (in units of $M^{-½}sec^{-½}$) were: 2.03×10^{-1} (18:2), 4.07×10^{-2} (18:3), 5.75×10^{-2} (20:4), and 10.15×10^{-2} (22:6). Thus, the oxidizability of DHA (22:6) is five times greater than that of linoleic acid (18:2). We propose that more vitamin E is required to protect against oxidative stress when EPA and DHA intake is increased. Indeed, vitamin E supplementation has been shown to counteract the fish oil-induced increase in LDL oxidizability (22, 62). We have shown recently that increasing the α-tocopherol content of either LDL or of macrophages reduces the oxidizability of LDL by copper or by macrophages, respectively (63).

A potential confounding effect of the beta-blocking drug, atenolol, is that other drugs of that category have been shown to influence the oxidation of LDL (64). However, the effect has been to reduce both TBARS formation in the presence of macrophages or copper, and to reduce the degradation of LDL by macrophages. These results were obtained with pindolol, propranolol, and metoprolol added in vitro to the incubation media. Indapamide, taken by two subjects in this study, has been reported to inhibit LDL oxidation induced by copper or by cultured endothelial cells (65). A hydrochlorothiazide (also taken by one of our subjects) has been found not to affect oxidation of LDL (65). Although we cannot assess whether in vivo exposure of LDL to atenolol or indapamide would lead to partial protection as observed in other studies (64, 65), similar drug treatments were experienced during the taking of fish oil or of corn oil and so the comparison between oil treatments remains valid. However, the thiazides appeared to reduce the α-tocopherol content of LDL, which in turn led to a reduced lag time during in vitro oxidation. The reason for this is unknown.

Both lag time and propagation rate were significantly decreased with fish oil. A reduction in lag time, an indicator of enhanced oxidizability, and a reduction in oxidation rate, an indicator of decreased oxidizability, were observed after fish oil supplementation (Table 5). Recently Whitman et al. (66) reported similar results. We cannot offer an explanation for this apparent paradox. We would place greater credence on the increased uptake of EPA/DHA-enriched LDL by macrophages as clear evidence for enhanced oxidizability (Fig. 2).

Effect of fish oil on macrophage-mediated LDL oxidation

The three major cell types in the arterial wall (endothelial cells, smooth muscle cells, and macrophages) have been reported to be able to modify LDL to a form recog-

nized by scavenger receptors in macrophages (67–69). It is suggested that LDL is oxidized by these cells in the arterial wall, and that the susceptibility of LDL to cell-induced oxidation is important in atherogenesis. The predominant cell type in the early atherosclerotic lesion fatty streak is the foam cell derived from monocyte-macrophage loaded with cholesteryl ester (70). The cholesterol in foam cells originates primarily from plasma lipoproteins, including LDL. The mechanism for the formation of foam cells is not yet clear, but it is speculated that macrophages take up cell-oxidized LDL via scavenger receptors, without feedback regulation. As shown in our results, LDL after fish oil supplementation was highly oxidized by macrophages. As shown in Table 6, macrophages enriched in n-3 fatty acids displayed a higher capacity to oxidize LDL. The amount of n-3 fatty acids that the LDL might have acquired during the incubation would have been too small to be responsible for the increased uptake.

In conclusion, these results indicate that dietary supplementation with n-3 fatty acids increases the oxidizability of LDL which may counteract some of the beneficial effects of fish oil which relate to the risk of developing atherosclerosis. Consumption of adequate amounts of antioxidants such as vitamin E should be considered to minimize these adverse effects.

We would like to thank Y. Lungershausen for her excellent work in co-ordinating the study and G. B. Belling and C. Doran for their technical assistance. We gratefully acknowledge Pronova Biocare a.s. for providing the Omacor and corn oil capsules.

Manuscript received 4 April 1994, in revised form 31 August 1994, and in re-revised form 17 October 1994.

REFERENCES

1. Bang, H. O., J. Dyeberg, and N. Hjorne. 1976. The composition of food consumed by Greenland Eskimos. *Acta Med. Scand.* **200:** 69–73.
2. Dyerberg, J., H. D. Bang, and N. Hjorne. 1975. Fatty acid composition of the plasma lipid in Greenland Eskimos. *Am. J. Clin. Nutr.* **28:** 958–966.
3. Kim, D. N., J. Schmee, C. S. Lee, A. Eastman, J. S. Ross, and W. A. Thomas. 1991. Comparison of effects of fish oil and corn oil supplements on hyperlipidemic diet-induced atherogenesis in swine. *Atherosclerosis.* **89:** 191–201.
4. Davis, H. R., R. T. Bridenstine, D. Vesselinovitch, and R. W. Wissler. 1987. Fish oil inhibits development of atherosclerosis in rhesus monkeys. *Arteriosclerosis.* **7:** 441–449.
5. Thiery, J., and D. Seidel. 1987. Fish oil feeding results in an enhancement of cholesterol-induced atherosclerosis in rabbits. *Atherosclerosis.* **63:** 53–56.
6. Burr, M. L., A. M. Fehily, J. F. Gilbert, S. Rogers, P. M. Holliday, P. M. Sweetnam, P. C. Elwood, and N. M. Deadman. 1989. Effects of changes in fat, fish, and fibre intakes on death and myocardial reinfarction in Israel: diet and reinfarction trial (DART). *Lancet.* **ii:** 757–761.
7. Harris, W. 1989. Fish oil and plasma lipid and lipoprotein metabolism in humans: a critical review. *J. Lipid Res.* **30:** 785–807.
8. Nestel, P. J. 1990. Effects of n-3 fatty acid on lipid metabolism. *Annu. Rev. Nutr.* **10:** 149–167.
9. Schmidt, E. B., S. D. Kristensen, R. De Caterina, and D. R. Illingworth. 1993. The effects of n-3 fatty acids on plasma lipids and lipoproteins and the other cardiovascular risk factors in patients with hyperlipidemia. *Atherosclerosis.* **103:** 107–121.
10. Levine, P. H., N. Fisher, P. B. Schneider, R. H. Whitten, B. H. Weiner, I. S. Ockene, B. F. Johnson, M. H. Johnson, E. M. Doyle, P. A. Riendeau, and J. J. Hoogasian. 1989. Dietary supplementation with omega-3 fatty acids prolongs platelet survival in hyperlipidemic patients with atherosclerosis. *Arch. Intern. Med.* **149:** 1113–1116.
11. Singer, P., M. Wirth, I. Berger, S. Voigt, U. Gerike, W. Gdicke, U. Kberle, and H. Heine. 1985. Influence of serum lipids, lipoproteins and blood pressure of mackerel and herring diet in patients with type IV and V hyperlipoproteinemia. *Atherosclerosis.* **56:** 111–118.
12. Green, P., J. Fuchs, and N. Schoenfeld. 1990. Effects of fish oil ingestion on cardiovascular risk factors in hyperlipidemic subjects in Israel: a randomized, double-blind crossover study. *Am. J. Clin. Nutr.* **52:** 1118–1124.
13. Kinsella, J. E., B. Lokesh, and R. A. Stone. 1990. Dietary n-3 polyunsaturated fatty acids and amelioration of cardiovascular disease: possible mechanisms. *Am. J. Clin. Nutr.* **52:** 1–28.
14. Steinberg, D., S. Parthasarathy, T. E. Carew, J. C. Khoo, and J. L. Witztum. 1989. Beyond cholesterol: modifications of low-density lipoprotein that increase its atherogenicity. *N. Engl. J. Med.* **320:** 915–924.
15. Witztum, J. L., and D. Steinberg. 1991. Role of oxidized low density lipoprotein in atherogenesis. *J. Clin. Invest.* **88:** 1785–1792.
16. Bonanome, A., A. Pagnon, S. Biffanti, A. Opportuno, F. Sorgato, M. Dorella, M. Maiorino, and F. Ursini. 1992. Effect of dietary monounsaturated and polyunsaturated fatty acids on the susceptibility of plasma low density lipoprotein to oxidative modification. *Arterioscler. Thromb.* **12:** 529–533.
17. Reaven, P. D., S. Parthasarathy, B. J. Grasse, E. Miler, F. Almazan, F. H. Mattson, J. C. Khoo, D. Steinberg, and J. Witztum. 1991. Feasibility of using an oleate-rich diet to reduce the susceptibility of low density lipoprotein to oxidative modification in humans. *Am. J. Clin. Nutr.* **54:** 701–706.
18. Abbey, M., G. B. Belling, M. Noakes, F. Hirata, and P. J. Nestel. 1993. Oxidation of low-density lipoproteins: intra-individual variability and the effect of dietary linoleate supplementation. *Am. J. Clin. Nutr.* **57:** 391–398.
19. Reaven, P. D., S. Parthasarathy, B. J. Grasse, E. Miller, D. Steinberg, and J. Witztum. 1993. Effects of oleate-rich and linoleate-rich diets on the susceptibility of low density lipoprotein to oxidative modification in mildly hypercholesterolemic subjects. *J. Clin. Invest.* **91:** 668–676.
20. Lussier-Cacan, S., S. Dubreuil-Quidoz, G. Roederer, N. Leboeuf, L. Boulet, G. C. de Langavant, J. Davignon, and M. Naruszewicz. 1993. Influence of probucol on enhanced LDL oxidation after fish oil treatment of hypertriglyceridemic patients. *Arterioscler. Thromb.* **13:** 1790–1797.
21. Nenseter, M. S., A. C. Rustan, S. Lund-Katz, E. Syland, G. Maelandsmo, M. C. Phillips, and C. A. Drevon. 1992. Effect of dietary supplementation with n-3 polyunsaturated fatty acids on physical properties and metabolism of low

density lipoprotein in humans. *Arterioscler. Thromb.* **12:** 369–379.
22. Harats, D., Y. Dabach, G. Hollander, M. Ben-Naim, R. Schwartz, E. M. Berry, O. Stein, and Y. Stein. 1991. Fish oil ingestion in smokers and nonsmokers enhances peroxidation of plasma lipoproteins. *Atherosclerosis.* **90:** 127–139.
23. Warnick, G. R., J. M. Benderson, and J. J. Albers. 1982. Dextran sulphate-Mg^{2+} precipitation procedure for quantitation of high density-lipoprotein cholesterol. *Clin. Chem.* **28:** 1379–1388.
24. DeLong, D. M., E. R. DeLong, P. D. Wood, K. Lippel, and B. M. Rifkind. 1986. A comparison of methods for the estimation of plasma low and very-low density lipoprotein cholesterol. The Lipid Research Clinics Prevalence Study. *J. Am. Med. Assoc.* **256:** 2372–2377.
25. Chung, B. H., J. P. Segrest, M. J. Ray, J. D. Brunzell, J. E. Hokanson, R. M. Krauss, K. Beaudrie, and J. T. Cone. 1986. Single vertical spin gradient ultracentrifugation. *Methods Enzymol.* **128:** 181–209.
26. Barter, P. J., and M. E. Jones. 1979. Rate of exchange of esterified cholesterol between human plasma and high density lipoproteins. *Atherosclerosis.* **34:** 67–74.
27. Esterbauer, H., G. Striegl, H. Puhl, and M. Rothender. 1989. Continuous monitoring of in vitro oxidation of human low density lipoprotein. *Free Radic. Res. Commun.* **6:** 67–75.
28. Kleinveld, H. A., H. L. M. Hak-Lemmers, A. F. H. Stalenhoef, and P. N. M. Demacker. 1992. Improved measurement of low-density-lipoprotein susceptibility to copper-induced oxidation: application of a short procedure for isolating low-density lipoprotein. *Clin. Chem.* **38:** 2066–2072.
29. Leake, D. S., and S. M. Rankin. 1990. The oxidative modification of low-density lipoproteins by macrophages. *Biochem. J.* **270:** 741–748.
30. Zhang, H., J. K. B. Harkamal, and U. S. Steinbrecher. 1990. Effects of oxidatively modified LDL on cholesterol esterification in cultured macrophages. *J. Lipid Res.* **31:** 1361–1369.
31. Clifton, P. M., L. Chang, and A. M. Mackinnon. 1988. Development of an automated Lowry protein assay for the Cobas-Bio centrifugal analyzer. *Anal. Biochem.* **172:** 165–168.
32. Buege, J. A., and S. D. Aust. 1978. Microsomal lipid peroxidation. *Methods Enzymol.* **52:** 302–310.
33. Yang, C. S., and M-J. Lee. 1987. Methodology of plasma retinol, tocopherol, and carotenoid assays in cancer prevention studies. *J. Nutr. Growth Cancer.* **4:** 19–27.
34. Abbey, M., P. Clifton, M. Kestin, B. Belling, and P. Nestel. 1990. Effect of fish oil on lipoproteins, lecithin:cholesterol acyltransferase, and lipid transfer protein activity in humans. *Arteriosclerosis.* **10:** 85–94.
35. McNamara, J. R., H. Campos, J. M. Ordovas, J. Peterson, P. W. F. Wilson, and E. J. Schaefer. 1987. Effect of gender, age, and lipid status on low density lipoprotein subfraction distribution. Results from Framingham Offspring study. *Arteriosclerosis.* **7:** 483–490.
36. Rosseneu, M., R. Vercaemst, K. K. Steinberg, and G. R. Cooper. 1983. Some considerations of methodology and standardization of apolipoprotein B immunoassays. *Clin. Chem.* **29:** 427–433.
37. Tateishi, T., N. Yoshimine, and F. Kuzuya. 1987. Serum lipid peroxide assayed by a new colorometric method. *Exp. Gerantol.* **22:** 103–111.
38. Contacos, C., P. J. Barter, and D. R. Sullivan. 1993. Effect of pravastatin and n-3 fatty acids on plasma lipids and lipoproteins in patients with combined hyperlipidemia. *Arterioscler. Thromb.* **13:** 1755–1762.
39. Crouse, J. R., J. S. Parks, H. M. Schey, and F. R. Kahl. 1985. Studies of low density lipoprotein molecular weight in human beings with coronary artery disease. *J. Lipid Res.* **26:** 566–574.
40. Austin, M. A., J. L. Breslow, C. H. Hennekens, J. E. Buring, W. C. Willett, and R. M. Krauss. 1988. Low-density lipoprotein subclass patterns and risk of myocardial infarction. *J. Am. Med. Assoc.* **260:** 1917–1921.
41. Campos, H., J. J. Genest, Jr., E. Blijlevens, J. R. McNamara, J. L. Jenner, J. M. Ordovas, P. W. F. Wilson, and E. J. Schaefer. 1992. Low density lipoprotein particle size and coronary artery disease. *Arterioscler. Thromb.* **12:** 187–195.
42. Campos, H., E. Blijlevens, J. R. McNamara, J. M. Ordovas, B. M. Posner, P. W. F. Wilson, W. P. Castelli, and E. J. Schaefer. 1992. LDL particle distribution: results from the Framingham Offspring Study. *Arterioscler. Thromb.* **12:** 1410–1419.
43. Swinkels, D. W., P. N. M. Demacker, J. C. M. Hendriks, and A. van't Laar. 1989. Low density lipoprotein subfractions and relationship to other risk factors for coronary artery disease in healthy individuals. *Arteriosclerosis.* **9:** 604–613.
44. Deckelbaum, R. J., E. Granot, Y. Oschry, and S. Eisenberg. 1984. Plasma triglyceride determines structure-composition in low and high density lipoproteins. *Arteriosclerosis.* **4:** 225–231.
45. Watson, T. D. G., M. J. Caslake, D. J. Freeman, B. A. Griffin, J. Hinnie, C. J. Packard, and J. Shepherd. 1994. Determinants of LDL subfraction distribution and concentrations in young normolipidemic subjects. *Arterioscler. Thromb.* **11:** 298–306.
46. de Graaf, J., H. L. M. Hak-Lemmers, M. P. C. Hectors, P. N. M. Demacker, J. C. M. Hendriks, and A. F. H. Stalenhoef. 1991. Enhanced susceptibility to in vitro oxidation of the dense low density lipoprotein subfraction in healthy subjects. *Arterioscler. Thromb.* **11:** 298–306.
47. Anthony, J. V., and M. J. Bush. 1992. Effects of d-α-tocopherol supplementation on experimentally induced primate atherosclerosis. *J. Am. Coll. Nutr.* **11:** 131–138.
48. Kita, T., Y. Nagano, M. Yokode, K. Ishii, N. Kume, A. Ooshima, H. Yoshida, and C. Kawai. 1987. Probucol prevents the progression of atherosclerosis in Watanabe heritable hyperlipidemic rabbit, an animal model for familial hypercholesterolemia. *Proc. Natl. Acad. Sci. USA.* **84:** 5928–5931.
49. Regnstrom, J., J. Nilsson, P. Tornvall, C. Landou, and A. Hamsten. 1992. Susceptibility to low-density lipoprotein oxidation and coronary atherosclerosis in man. *Lancet.* **339:** 1183–1186.
50. Gey, K. F., P. Puska, P. Jordan, and U. K. Moser. 1991. Inverse correlation between plasma vitamin E and mortality from ischemic heart disease in cross-cultural epidemiology. *Am. J. Clin. Nutr.* **53:** 326S–334S.
51. Eric, B. R., M. J. Stampfer, A. Ascherio, E. Giovannucci, G. A. Colditz, and W. C. Willett. 1993. Vitamin E consumption and the risk of coronary heart disease in men. *N. Engl. J. Med.* **328:** 1450–1456.
52. Stampfer, M. J., H. H. Charles, J. E. Manson, G. A. Colditz, B. Rosner, and W. C. Willett. 1993. Vitamin E consumption and the risk of coronary disease in women. *N. Engl. J. Med.* **328:** 1444–1449.
53. Meydani, M., F. Natiello, B. Goldin, N. Free, M. Woods, E. Schaefer, J. B. Blumberg, and S. L. Gorbach. 1991.

Effect of long-term fish oil supplementation on vitamin E status and lipid peroxidation in women. *J. Nutr.* **121:** 484–491.
54. Saito, H., K-J. Chang, Y. Tamura, and S. Yoshida. 1991. Ingestion of eicosapentaenoic acid-ethyl ester renders rabbit LDL less susceptible to Cu^{2+}-catalyzed-oxidative modification. *Biochem. Biophys. Res. Commun.* **175:** 61–67.
55. Rich, S., J. F. Miller, Jr., S. Charous, H. R. Davis, P. Shanks, S. Glagov, and W. E. M. Lands. 1989. Development of atherosclerosis in genetically hyperlipidemic rabbits during chronic fish-oil ingestion. *Arteriosclerosis.* **9:** 189–194.
56. Esterbauer, H., M. Dieber-Rotheneder, G. Striegl, and G. Waeg. 1991. Role of vitamin E in preventing the oxidation of low-density lipoprotein. *Am. J. Clin. Nutr.* **53:** 314S–321S.
57. Princen, H. M. G., G. van Poppel, C. Vogelezang, R. Buytenhek, and F. J. Kok. 1992. Supplementation with vitamin E but not β-carotene in vivo protects low density lipoprotein from lipid peroxidation in vitro. Effect of cigarette smoking. *Arterioscler. Thromb.* **12:** 554–562.
58. Reaven, D., and J. L. Witztum. 1993. Comparison of supplementation of RRR-α-tocopherol and racemic α-tocopherol in humans. Effects on lipid levels and lipoprotein susceptibility to oxidation. *Arterioscler. Thromb.* **13:** 601–608.
59. Abbey, M., P. J. Nestel, and P. A. Baghurst. 1993. Antioxidant vitamins and low density lipoprotein oxidation. *Am. J. Clin. Nutr.* **58:** 525–532.
60. Nair, P. P., J. T. Judd, E. Berline, P. R. Taylor, S. Shami, E. Sainz, and H. N. Bhagavan. 1993. Dietary fish oil-induced changes in the distribution of α-tocopherol, retinol, and β-carotene in plasma, red blood cells, and platelets: modulation by vitamin E. *Am. J. Clin. Nutr.* **58:** 98–102.
61. Cosgrove, J. P., D. F. Church, and W. A. Pryor. 1987. The kinetics of the autoxidation of polyunsaturated fatty acids. *Lipids.* **22:** 299–304.
62. Oostenbrug, G. S., R. P. Mensink, and G. Hornstra. 1993. A moderate in vivo vitamin E supplement counteracts the fish-oil-induced increase in in vitro oxidation of human low-density lipoproteins. *Am. J. Clin. Nutr.* **57 (suppl):** 827S.
63. Suzukawa, M., M. Abbey, P. Clifton, and P. J. Nestel. 1994. Effects of supplementing with vitamin E on the uptake of low density lipoprotein and the stimulation of cholesteryl ester formation in macrophages. *Atherosclerosis.* In press.
64. Mazière, C., M. Auclair, and J. C. Mazière. 1992. Lipophilic β-blockers inhibit monocyte and endothelial cell-induced modification of low density lipoproteins. *Biochim. Biophys. Acta.* **1126:** 314–318.
65. Breugnot, C., J. P. Iliou, S. Privat, F. Robin, J. P. Vilaine, and A. Lenaers. 1992. In vitro and ex vivo inhibition of the modification of low-density lipoprotein by indapamide. *J. Cardiovasc. Pharmacol.* **20:** 340–347.
66. Whitman, S. C., J. R. Fish, M. L. Rand, and K. A. Rogers. 1994. N-3 fatty acid incorporation into LDL particles renders them more susceptible to oxidation in vitro but not necessarily more atherogenic in vivo. *Arterioscler. Thromb.* **14:** 1170–1176.
67. Parthasarathy, S., E. Wieland, and D. Steinberg. 1989. A role for endothelial cell lipoxygenase in the oxidative modification of low density lipoprotein. *Proc. Natl. Acad. Sci. USA.* **86:** 1046–1050.
68. Heinecke, J. W., L. Baker, H. Rosen, and A. Chait. 1986. Superoxide-mediated modification of low density lipoprotein by arterial smooth muscle cells. *J. Clin. Invest.* **77:** 757–761.
69. Rankin, S. M., S. Parthasarathy, and D. Steinberg. 1991. Evidence for a dominant role of lipoxygenase(s) in the oxidation of LDL by mouse peritoneal macrophages. *J. Lipid Res.* **32:** 449–456.
70. Aqel, N. M., R. Y. Ball, H. Waldmann, and M. J. Mitchinson. 1984. Monocytic origin of foam cells in human atherosclerotic plaques. *Atherosclerosis.* **53:** 265–271.