British Journal of Nutrition (2001), **85**, 23–31
DOI: 10.1079/BJN2000220

Susceptibility of LDL to oxidative modification in healthy volunteers supplemented with low doses of *n*-3 polyunsaturated fatty acids

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(Received 30 November 1999 – Revised 2 June 2000 – Accepted 18 July 2000)

The objective of the present study was to evaluate the oxidative susceptibility of LDL in human volunteers following supplementation with various low doses (<1 g/d) of *n*-3 polyunsaturated fatty acids (PUFA). Sixty-two healthy volunteers (thirty-seven males and twenty-five females, aged 19–63 years) were recruited to take part in a randomised placebo-controlled trial. Volunteers were required to take 0·9, 0·6 or 0·3 g *n*-3 PUFA as fish oil or placebo capsules daily for 16 weeks. Susceptibility of LDL to oxidative modification was assessed by measuring the production of conjugated dienes and thiobarbituric acid-reactive substances in LDL oxidised by Cu^{2+} (15 μM) or 2,2′-azobis(2-amidinopropane) dihydrochloride (1 mM) for 5 h. Plasma fatty acid and LDL-fatty acid composition, cholesterol levels and antioxidant concentrations were also measured. While post-treatment *n*-3 PUFA compositions of plasma and LDL reflected the capsule contents, no meaningful differences in antioxidant concentrations or cholesterol levels were observed between the groups. Supplementation with low doses of *n*-3 PUFA as fish oil did not influence the oxidative susceptibility of LDL. The results of the present study suggest that moderate dietary intakes of *n*-3 PUFA do not significantly influence the susceptibility of LDL to oxidative modification *in vitro*.

***n*-3 Polyunsaturated fatty acids: LDL: Fish oil supplementation**

Consumption of moderate amounts of fatty fish has been shown to be beneficial in protecting against CHD (Kromhout *et al.* 1985; Burr *et al.* 1989). These favourable effects have been attributed to the high levels of *n*-3 polyunsaturated fatty acids (PUFA), especially eicosapentaenoic acid (20:5*n*-3; EPA) and docosahexaenoic acid (22:6*n*-3; DHA) found in fish. Recently, in a large multicentre randomised placebo-controlled trial carried out in Italy treatment with *n*-3 PUFA supplements (1 g daily) significantly decreased ($P = 0{\cdot}023$) over 3·5 years, the rate of death, non-fatal myocardial infarction, and stroke in a group of patients who had recently recovered from a myocardial infarction (GISSI-Prevenzione Investigators, 1999). Various effects, including a reduction in fasting (Harris, 1996) and postprandial triacylglycerols (TAG; Ågren *et al.* 1996), a reduction in platelet activity (Levine *et al.* 1989; Ågren *et al.* 1997), a reduction in blood pressure (Sanders & Hinds, 1992; Schmidt *et al.* 1992), a reduction in blood viscosity (Green *et al.* 1990) and inflammatory processes (Wallace *et al.* 1995), have been reported following *n*-3 PUFA consumption.

In the countries of the EU, the average *per capita* fish consumption is very low (Scientific Committee for Food, 1993). The most obvious way of increasing the intake of *n*-3 PUFA is to eat more oily fish, and the British Nutrition Foundation (1993) recommended weekly consumption of two portions of fish such as mackerel, herring, salmon, pilchards and sardines, providing approximately 1 g *n*-3 PUFA/d.

Increased consumption of fatty fish gives rise to concern over possible enhancement in LDL oxidation which is thought to play a pivotal role in atherogenesis (Steinberg

Abbreviations: AAPH, 2,2′-azobis(2-amidinopropane) dihydrochloride; DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; PUFA, polyunsaturated fatty acid; TAG, triacylglycerol; TBARS, thiobarbituric acid-reactive substances.

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et al. 1989). The fatty acid content of LDL is reflective of dietary intake and could influence the susceptibility of LDL to oxidative modification (Reaven *et al.* 1993). However, results from studies examining the effect of *n*-3 PUFA on LDL oxidation have been inconsistent. A number of human supplementation studies (Lussier-Cacan *et al.* 1993; Oostenbrug *et al.* 1994; Suzukawa *et al.* 1995; Wander *et al.* 1996, 1998) have shown a deleterious effect of *n*-3 PUFA supplementation on the susceptibility of LDL to oxidative modification, while other studies (Nenseter *et al.* 1992; Frankel *et al.* 1994; Bonanome *et al.* 1996; Brude *et al.* 1997; Hansen *et al.* 1998) showed no effect. Findings from animal studies have also been conflicting (Saito *et al.* 1991; Thomas *et al.* 1994; Whitman *et al.* 1994; Calviello *et al.* 1997; Vaagenes *et al.* 1998).

Oxidation of LDL is a lipid peroxidation process, and the present study has focused on measuring the products of lipid peroxidation such as thiobarbituric acid-reactive substances (TBARS) and conjugated dienes. Two different types of water-soluble initiators, Cu^{2+} and 2,2′-azobis(2-amidinopropane) dihydrochloride (AAPH) were used to initiate LDL oxidation. The main difference between these initiators is that AAPH produces a random attack of free radicals in LDL, whereas Cu^{2+} involves a more site-specific mechanism which may be more relevant *in vivo* (Esterbauer & Jurgens, 1993).

The purpose of the present study was to evaluate the effect of supplementation with low doses of *n*-3 PUFA in fish oil for a relatively long period of time, on the susceptibility of LDL to oxidative modification in a group of healthy volunteers.

Materials and methods

Subjects

Sixty-two volunteers (thirty-seven males and twenty-five females, aged 19–63 years) were recruited to participate in the study. They were healthy, as verified by physical examination, medical history and biochemical and haematological profile. Exclusion criteria for the study were as follows: smoking; those on special diets; taking nutritional supplements; excessive alcohol consumption (>28 units and >21 units alcohol/week for males and females, respectively); BMI <20 or >30 kg/m^2; pregnancy; heavy exerciser (more than three 30 min periods of strenuous exercise per week); diabetes; endocrine disease; liver disease; those consuming an habitual diet rich in *n*-3 PUFA. The present study was part of a study funded by the EU (NUTRIFISH) which was carried out at the Unit of Nutrition and Dietetics, Department of Clinical Medicine, Trinity Health Sciences Centre, St James Hospital, Dublin 8, Republic of Ireland. Approval for the study was obtained from the Ethics Committee of the Federated Dublin Voluntary Hospitals at Trinity College, Dublin, and informed consent was given by all volunteers.

Study design

In this placebo-controlled study, subjects were randomised on the basis of fasting plasma TAG levels into one of four treatment groups, so that mean baseline TAG levels did not differ significantly between the groups. The treatment groups were 0·9, 0·6, or 0·3 g *n*-3 PUFA as fish oil/d, or 0·9 g control oil/d. Subjects were required to take three 1 g capsules daily for 16 weeks. A capsule count showed that subjects consumed 93–95 % of the capsules prescribed. Capsules were prepared by Hoffman-La Roche Ltd., Basel, Switzerland. The 0·9 g *n*-3 PUFA (containing 0·337 g *n*-3 PUFA/g fish oil; 0·172 g EPA and 0·11 g DHA/g fish oil) capsule contained fish oil only. The 0·6 g *n*-3 PUFA (containing 0·227 g *n*-3 PUFA/g fish oil; 0·114 g EPA and 0·072 g DHA/g fish oil) and 0·3 g *n*-3 PUFA (containing 0·118 g *n*-3 PUFA/g fish oil; 0·056 g EPA and 0·036 g DHA/g fish oil) capsules contained a blend of fish oil and olive oil. Placebo capsules (containing 0·007 g *n*-3 PUFA/g fish oil; EPA and DHA not present) contained olive oil only. The fish oil and control oil supplements contained the same quantities (mg/kg) of α-tocopherol (1134), β-tocopherol (8) and γ-tocopherol (20). Compliance was ascertained by interviewing volunteers when supplying capsules on a monthly basis, counting returned capsules and by monitoring platelet phospholipid-EPA and -DHA levels at baseline (0 weeks) and after completion of 4, 8, 12 and 16 weeks of supplementation.

Blood collection

Fasting blood samples were obtained at baseline (0 weeks) and after 4, 8, 12 and 16 weeks of supplementation. However, the sample analysis reported here was carried out on blood collected at baseline and following 16 weeks of supplementation only. Blood samples were collected into evacuated tubes containing 0·39 M-K_3 EDTA. Plasma was immediately separated and stored at −80°C before analysis. Samples were protected from natural light. EDTA-treated plasma stored at −80°C is stable for several weeks (Esterbauer *et al.* 1992; Kleinveld *et al.* 1992).

Isolation of LDL

LDL was prepared immediately after thawing by a rapid isolation technique (Chung *et al.* 1986). Plasma adjusted to density 1·3 g/ml with solid KBr was overlayered with density solution (1·006 g/ml, 2·7 mM-EDTA) in Beckman Optiseal ultracentrifuge tubes (Beckman Instruments, Palo Alto, CA, USA). The tubes were centrifuged at 645 000 ***g*** for 90 min at 7°C, acceleration 6 and deceleration 8 using an NVT 90 near vertical rotor in a Beckman Optima XL-90 ultracentrifuge (Beckman Instruments). Following ultracentrifugation, the tubes were sliced to obtain the LDL fraction. LDL isolates for the estimation of carotenoid, tocopherol and retinol concentrations and for fatty acid composition analysis were stored at −80°C. LDL isolated for oxidation experiments was mixed with density solution (density 1·063 g/ml) and centrifuged a second time under the same conditions to eliminate contamination with albumin. The top layer containing LDL was removed by tube slicing.

Dialysis of LDL

LDL samples were dialysed at 4°C, in the dark against 40 litres PBS containing 10 μM-Na_2 EDTA, pH 7·4, for 42 h while shaking gently in a microdialysis unit (GibcoBRL, Life Technologies Ltd, Paisley, Strathclyde, UK). Dialysis membrane was stored in Chelex® 100 resin-treated (Bio-Rad Laboratories, Hercules, CA, USA) double-distilled deionised water before use.

Susceptibility of LDL to oxidative modification

Samples of dialysed LDL were removed for analysis of protein concentration (Markwell *et al.* 1981) using bovine serum albumin as the standard. Samples were adjusted to a protein concentration of 50 μg LDL protein/ml and 1 μM-EDTA in quartz cuvettes (Kleinveld *et al.* 1992). Oxidation of LDL was initiated by addition of $CuSO_4$ (15 μM; Kleinveld *et al.* 1992) or AAPH (1 mM; Univar PLC, Basingstoke, Hants, UK; Frei & Gaziano, 1993). In the case of AAPH, the initiator was added to both the samples and PBS reference blanks. Absorbance of the samples was monitored at wavelength 234 nm, every 2 min at 30°C and 37°C respectively using a Cary 1E u.v./visible Varian spectrophotometer with twelve-position automated cell-changer and peltier heater accessories (Varian Chromatography Systems, Walnut Creek, CA, USA). Maximal oxidation rate (nmol/mg LDL per min) and lag phase (min) were estimated using exact coordinates provided by the kinetics software application (Varian Chromatography Systems).

Thiobarbituric acid-reactive substances assay

LDL which was oxidised for measurement of conjugated dienes with either Cu^{2+} (15 μM) or AAPH (1 mM) was subsequently used for the measurement of TBARS (Buege & Aust, 1978). Following 5 h oxidation, the reaction was stopped by addition of 200 μM-EDTA and 40 μM-butylated hydroxytoluene and the samples stored at 4°C for a maximum of 7 d. Absorbance was measured at 535 nm and quantification of TBARS (nmol equivalents malondialdehyde/mg LDL-protein) was performed by comparison with a standard curve of malondialdehyde equivalents generated by acid-catalysed hydrolysis of 1,1,3,3,-tetraethoxypropane (Sigma Chemical Co., St Louis, MO, USA).

Estimation of carotenoids, tocopherols and retinol in plasma and LDL

Carotenoids, tocopherols and retinol were extracted from 0·2 ml plasma and 0·6 ml LDL of concentration 400 μg LDL-protein/ml PBS according to the method of Burton *et al.* (1985). Samples were injected onto a temperature-controlled (25°C) reverse-phase HPLC system (Scott & Hart, 1993) with two on-line u.v./visible detectors (Shimatzu Corporation, Kyoto, Japan). The data were processed using Millennium 2.1 software data processing package (Waters Chromatograph Division, Milford, MA, USA). Retinol, tocopherols (α and γ) and carotenoids (lutein, zeaxanthin, β-cryptoxanthin, all-*trans* lycopene, α-carotene and all-*trans* β-carotene) were quantified by reference to five-point calibration curves. For each subject, duplicate pre- and post-treatment plasma or LDL samples were run on the same day. Plasma antioxidants are expressed as absolute amounts (μmol/l) and cholesterol standardised amounts (μmol/mmol total plasma cholesterol), while LDL antioxidants are expressed in terms of cholesterol (μmol/mmol cholesterol) and also as mol antioxidants/mol LDL. The molar antioxidant content of LDL was calculated using $2{\cdot}5 \times 10^6$ as the mean molecular weight and 34·7 % as the mean cholesterol content (Esterbauer *et al.* 1992).

Total fatty acid composition of plasma and LDL

Lipid was extracted from 0·1 ml plasma and 1 ml LDL of concentration 400 μg LDL-protein/ml for analysis by the one-step extraction and direct trans-esterification method of Lepage & Roy (1986). Fatty acid composition analysis was performed by GC (Varian 3800 equipped with an 8200 standalone autosampler and connected to Star software package; Varian Chromatography Systems). The column was a DB-wax capillary column (30 m × 0·32 mm i.d.) and a splitless mode of injection was used. N_2 was the carrier gas used. The temperatures of the injector port and the detector were 250°C and 300°C, respectively. The column temperature was held at 50°C for 1 min and increased stepwise to a plateau of 250°C. A standard mixture of nineteen fatty acid methyl esters (Sigma Chemical Co., Poole, Dorset, UK) was used to calibrate the GC. Results are expressed as g/100 g total fatty acids present.

Other analytical methods

Plasma total cholesterol and LDL-cholesterol levels (mmol/l) were analysed using an enzymic, colorimetric kit (CHOD-PAP, MRP 2; Boehringer Mannheim, Mannheim, Germany) and using a Cary 1E u.v./visible Varian spectrophotometer (Varian Chromatography Systems). PRECINORM L (Boehringer Mannheim), control serum was included in each assay. Analysis of plasma TAG concentrations was carried out at the Unit of Nutrition and Dietetics, Department of Clinical Medicine, Trinity Health Sciences Centre, St James Hospital, Dublin, Republic of Ireland, and were determined using enzymic colorimetric assays (TAG PAP; BioMerieux SA, Lyon, France) on a Technicon RA-XT analyser (Bayer, Dublin, Republic of Ireland).

Statistical analysis

Results are expressed as means and standard deviations. Power calculations were based on the reduction in fasting plasma TAG observed in a previous *n*-3 PUFA supplementation study (Roche & Gibney, 1996). Following screening sixty-two eligible volunteers were recruited, and sixty-one were included in the statistical analysis as one volunteer was excluded for poor compliance. Chi-square test was used to detect differences in gender profile between the groups. Pearsons's correlation coefficient was used to examine relationships between variables. Analysis of covariance was used to test for differences between the

Table 1. Initial characteristics of volunteers given 0·9, 0·6 or 0·3 g *n*-3 polyunsaturated fatty acids (PUFA)/d or a placebo for 16 weeks
(Values (apart from gender) are mean and standard deviations)

Treatment group	0·9 g *n*-3 PUFA/d (*n* 14)		0·6 g *n*-3 PUFA/d (*n* 16)		0·3 g *n*-3 PUFA/d (*n* 17)		Placebo (*n* 14)	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD
Age (years)	39·8	13·2	34·1	11·0	32·8	9·1	40·3	12·8
Gender (M/F)	8/6		11/5		10/7		8/6	
Height (m)	1·71	0·10	1·77	0·10	1·74	0·12	1·74	0·11
Weight (kg)	71·2	13·9	77·8	15·0	75·6	12·7	78·6	12·6
BMI (kg/m^2)	24·2	2·9	24·7	3·1	25·0	2·0	25·8	2·3

M, male; F, female.

treatment groups at the end of the treatment period, with post-treatment as the dependent variable, treatment group as the fixed factor and the pretreatment assigned as covariate. Dunnett's tests were performed on post-treatment variables which were found to be significantly different. Due to the large number of statistical observations made, the $P \leq 0{\cdot}01$ was used to assess statistical significance. Statistical analysis was performed using SPSS (SPSS Inc., Chicago, IL, USA) statistical software package.

Results

The baseline characteristics of the subjects are shown in Table 1. There were no differences among the treatment groups with respect to age, gender profile, height, weight or BMI, either at baseline or following the supplementation period.

Plasma fatty acid compositions before and after supplementation are shown in Table 2. A clear relationship between the level of the *n*-3 PUFA supplement and the subsequent increase in plasma levels of EPA and DHA was evident. Plasma EPA was significantly ($P < 0{\cdot}01$) greater in the 0·9 g *n*-3 PUFA/d group compared with the placebo group.

A dose–response relationship between the level of the *n*-3 PUFA supplement and the subsequent increases in LDL-*n*-3 PUFA compositions was also apparent (Table 3). LDL-EPA was significantly ($P < 0{\cdot}01$) greater in the 0·9 g *n*-3 PUFA group compared with the placebo group.

Plasma total cholesterol and LDL-cholesterol levels were not significantly altered by *n*-3 PUFA supplementation (Table 4). Using analysis of covariance (baseline values as covariates), there was no significant difference between groups in plasma total cholesterol ($P = 0{\cdot}255$) or LDL-cholesterol ($P = 0{\cdot}253$) concentrations following supplementation. Fasting plasma TAG concentrations were significantly reduced ($P = 0{\cdot}0001$) in the group who received the highest dose of *n*-3 PUFA only (Table 4).

Antioxidant concentrations in plasma and LDL are shown in Tables 5 and 6. Treatment did not affect antioxidant levels.

There were no significant differences in maximum diene production ($P = 0{\cdot}0149$), rate of conjugated diene production ($P = 0{\cdot}045$), lag phase ($P = 0{\cdot}534$) or TBARS ($P = 0{\cdot}208$) in Cu^{2+}-oxidised LDL between the groups following supplementation (Table 7). Treatment had no significant effect on maximum diene production ($P = 0{\cdot}784$), maximal rate of conjugated diene production ($P = 0{\cdot}869$), lag phase

Table 2. Fatty acid composition† (g/100 g total fatty acids) of plasma for volunteers given 0·9, 0·6 or 0·3 g *n*-3 polyunsaturated fatty acid (PUFA)/d or a placebo for 16 weeks‡
(Values are means and standard deviations)

Treatment group	0·9 g *n*-3 PUFA/d (*n* 14)				0·6 g *n*-3 PUFA/d (*n* 16)				0·3 g *n*-3 PUFA/d (*n* 17)				Placebo (*n* 14)			
	Before treatment		After 16 weeks		Before treatment		After 16 weeks		Before treatment		After 16 weeks		Before treatment		After 16 weeks	
Fatty acid	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD
14 : 0	1·38	0·63	1·44	0·74	1·27	0·46	1·32	0·39	0·97	0·37	1·22	0·32	1·34	0·46	1·09	0·32
16 : 0	23·24	2·46	23·03	1·93	22·95	2·19	23·81	1·60	22·95	1·34	23·37	1·12	23·11	2·10	22·49	1·13
16 : 1	2·38	0·54	1·94	0·75	2·39	0·90	2·35	0·62	2·39	0·55	2·42	0·59	2·15	0·84	2·30	0·71
18 : 0	7·10	0·64	7·51	0·31	7·11	0·86	7·23	0·90	7·47	1·02	7·46	0·57	6·92	2·13	7·00	0·53
18 : 1	20·87	3·64	19·14	1·94	20·40	2·48	19·51	2·68	21·29	2·68	20·60	2·41	20·38	1·75	20·55	1·67
18 : 2*n*-6	32·21	4·69	32·68	3·70	33·09	4·07	32·75	3·17	32·07	3·69	32·35	2·51	33·04	3·35	33·27	4·02
18 : 3*n*-3	0·92	0·27	0·84	0·28	0·76	0·20	0·67	0·17	0·87	0·66	0·70	0·22	0·81	0·33	0·95	0·35
20 : 0	0·33	0·09	0·33	0·07	0·32	0·05	0·32	0·06	0·36	0·05	0·35	0·06	0·34	0·05	0·33	0·04
20 : 3*n*-6	1·36	0·24	1·17	0·14	1·45	0·37	1·35	0·34	1·51	0·24	1·34	0·28	1·32	0·20	1·32	0·24
20 : 4*n*-6	5·81	1·40	5·22	0·58	5·85	0·76	5·62	0·90	6·18	1·41	5·71	1·28	5·93	1·41	5·94	0·99
20 : 5*n*-3	1·22	0·71	2·65**	0·73	1·18	0·43	1·70	0·43	0·99	0·37	1·35	0·24	1·47	1·26	1·38	0·72
22 : 0	0·75	0·25	0·74	0·15	0·67	0·36	0·47	0·39	0·59	0·40	0·57	0·39	0·72	0·12	0·72	0·09
22 : 6*n*-3	1·83	0·84	2·76	0·69	2·00	0·58	2·26	0·49	1·74	0·42	2·00	0·31	1·95	0·94	2·11	0·88

Mean value was significantly different from that for the placebo group: **$P < 0{\cdot}01$.
† Lipid was extracted from plasma (0·1 ml) and fatty acid methyl esters were analysed by GC. For details, see p. 25.
‡ For details of subjects and treatments, see Table 1 and pp. 25–26.

Table 3. Fatty acid composition† (g/100 g total fatty acids) of LDL for volunteers given 0·9, 0·6 or 0·3 g *n*-3 polyunsaturated fatty acid (PUFA)/d or a placebo for 16 weeks‡

(Values are means and standard deviations)

Treatment group	0·9 g *n*-3 PUFA/d (*n* 14)				0·6 g *n*-3 PUFA/d (*n* 16)				0·3 g *n*-3 PUFA/d (*n* 17)				Placebo (*n* 14)			
	Before treatment		After 16 weeks		Before treatment		After 16 weeks		Before treatment		After 16 weeks		Before treatment		After 16 weeks	
Fatty acids	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD
14:0	0·82	0·15	0·76	0·18	0·77	0·24	0·80	0·15	0·65	0·14	0·75	0·21	0·77	0·13	0·69	0·20
16:0	33·08	2·02	33·49	1·46	32·51	2·04	33·43	1·86	32·73	1·92	33·19	2·36	33·09	2·68	32·59	2·30
16:1	0·91	0·29	0·77	0·16	1·03	0·31	0·97	0·27	1·02	0·28	1·05	0·30	0·98	0·41	0·97	0·36
18:0	13·33	1·06	13·55	0·91	12·54	1·34	12·76	0·92	12·92	1·75	12·74	1·11	12·32	1·18	12·34	0·97
18:1	10·61	1·41	9·47	0·89	11·22	2·18	10·43	1·65	11·37	2·46	10·96	1·75	10·46	1·61	10·74	1·79
18:2*n*-6	25·98	2·72	25·08	2·41	25·20	2·57	24·28	1·70	25·28	2·05	24·86	1·74	26·81	3·43	26·87	3·16
18:3*n*-3	0·39	0·18	0·33	0·07	0·36	0·12	0·34	0·17	0·31	0·15	0·53	0·87	0·54	0·75	0·39	0·16
20:0	0·57	0·18	0·60	0·15	0·71	0·17	0·73	0·16	0·70	0·28	0·75	0·26	0·65	0·17	0·66	0·12
20:3*n*-6	2·35	0·26	2·06	0·48	2·49	0·65	2·41	0·71	2·57	0·66	2·28	0·42	2·19	0·26	2·19	0·49
20:4*n*-6	6·96	1·13	6·42	1·06	7·04	1·01	6·93	0·93	7·04	1·06	6·93	1·26	6·92	1·24	7·06	1·31
20:5*n*-3	1·39	0·92	2·61**	0·74	1·20	0·43	1·79	0·42	1·04	0·38	1·20	0·37	1·37	1·08	1·28	0·63
22:0	1·01	0·54	1·02	0·38	1·50	0·51	1·33	0·66	1·51	0·48	1·60	0·50	1·15	0·51	1·27	0·27
22:6*n*-3	2·52	0·84	3·77	0·84	2·95	0·78	3·54	0·81	2·51	0·73	2·88	0·77	2·62	1·20	2·87	1·10

Mean value was significantly different from that of the placebo group: $^{**}P < 0{\cdot}01$.
† Lipid was extracted from LDL (1 ml of concentration 400 μg LDL protein/ml) and fatty acid methyl esters were analysed by GC. For details, see. p. 25.
‡ For details of subjects and treatments, see Table 1 and pp. 25–26.

Table 4. Plasma total cholesterol concentrations, LDL-cholesterol concentrations and fasting plasma triacylglycerol (TAG) concentrations (mmol/l)† for volunteers given 0·9, 0·6 or 0·3 g *n*-3 polyunsaturated fatty acids (PUFA)/d or a placebo for 16 weeks‡

(Values are means and standard deviations)

Treatment groups	0·9 g *n*-3 PUFA/d (*n* 14)				0·6 g *n*-3 PUFA/d (*n* 16)				0·3 g *n*-3 PUFA/d (*n* 17)				Placebo (*n* 14)			
	Before treatment		After 16 weeks		Before treatment		After 16 weeks		Before treatment		After 16 weeks		Before treatment		After 16 weeks	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD
Plasma total cholesterol	4·01	0·96	4·27	1·00	4·34	1·33	4·16	1·15	4·07	0·74	3·96	0·74	4·21	0·94	4·20	1·18
LDL-cholesterol	2·47	0·73	2·61	0·67	2·72	1·02	2·58	0·97	2·57	0·71	2·70	0·67	2·89	0·97	2·71	0·84
Fasting TAG	1·38	0·24	1·14***	0·18	0·95	0·08	1·00	0·12	0·89	0·10	0·97	0·08	1·26	0·15	1·06	0·10

Mean value was significantly different from that of the placebo group: $^{***}P < 0{\cdot}0001$.
† Cholesterol and TAG were determined using enzymic procedures. For details, see. p. 25.
‡ For details of subjects and treatments, see Table 1 and pp. 25–26.

Table 5. Plasma tocopherols, retinal and carotenoids† (μmol/l) for volunteers given 0·9, 0·6 or 0·3 g *n*-3 polyunsaturated fatty acids (PUFA)/d or a placebo for 16 weeks‡

(Values are means and standard deviations)

Treatment group	0·9 g *n*-3 PUFA/d (*n* 14)				0·6 g *n*-3 PUFA/d (*n* 16)				0·3 g *n*-3 PUFA/d (*n* 17)				Placebo (*n* 14)			
	Before treatment		After 16 weeks		Before treatment		After 16 weeks		Before treatment		After 16 weeks		Before treatment		After 16 weeks	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD
α-Tocopherol	28·66	12·91	27·67	6·90	23·74	4·90	23·05	5·99	22·39	5·50	22·51	4·23	27·62	6·03	28·21	7·97
γ-Tocopherol	1·38	0·88	1·27	0·44	1·64	0·76	1·29	0·81	1·53	0·75	1·34	0·49	1·54	0·60	1·45	0·66
Retinol	1·92	0·61	1·93	0·45	1·85	0·38	1·77	0·35	1·79	0·50	1·64	0·38	1·86	0·38	1·89	0·31
β-Carotene	0·31	0·13	0·35	0·13	0·44	0·24	0·35**	0·18	0·34	0·29	0·29	0·27	0·36	0·20	0·39	0·25
α-Carotene	0·10	0·05	0·11	0·04	0·10	0·05	0·08	0·06	0·11	0·14	0·08	0·09	0·11	0·06	0·10	0·07
Lycopene	0·21	0·11	0·22	0·09	0·23	0·09	0·23	0·08	0·22	0·12	0·21	0·11	0·23	0·10	0·24	0·11
β-Cryptoxanthin	0·26	0·14	0·23	0·12	0·16	0·09	0·21	0·14	0·13	0·07	0·17	0·11	0·20	0·13	0·19	0·13
Lutein	0·25	0·10	0·28	0·09	0·24	0·07	0·22	0·08	0·23	0·09	0·20	0·08	0·31	0·12	0·31	0·15
Zeaxanthin	0·06	0·03	0·08	0·04	0·09	0·06	0·09	0·05	0·08	0·03	0·07	0·03	0·08	0·04	0·09	0·05

Mean value was significantly different from baseline value (passed *t* test): $^{**}P \leq 0{\cdot}01$.
† Tocopherols, retinol and carotenoids were extracted from plasma (0·2 ml) and analysed by HPLC. For details, see. p. 24.
‡ For details of subjects and treatments, see Table 1 and pp. 25–26.

Table 6. Tocopherols and carotenoids* (μmol/mmol cholesterol) in LDL from volunteers given 0·9, 0·6 or 0·3 g *n*-3 polyunsaturated fatty acids (PUFA)/d or a placebo for 16 weeks†

(Values are means and standard deviations)

Treatment group	0·9 g *n*-3 PUFA/d (*n* 14)				0·6 g *n*-3 PUFA/d (*n* 16)				0·3 g *n*-3 PUFA/d (*n* 17)				Placebo (*n* 14)			
	Before treatment		After 16 weeks		Before treatment		After 16 weeks		Before treatment		After 16 weeks		Before treatment		After 16 weeks	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD
α-Tocopherol	5·29	3·00	5·26	2·93	3·55	1·11	3·91	1·04	3·33	0·92	3·38	0·89	5·26	3·31	5·39	3·00
γ-Tocopherol	0·31	0·19	0·30	0·14	0·28	0·14	0·23	0·11	0·27	0·11	0·24	0·13	0·30	0·14	0·34	0·14
β-Carotene	0·14	0·08	0·14	0·08	0·17	0·12	0·15	0·11	0·14	0·13	0·10	0·07	0·13	0·10	0·15	0·13
α-Carotene	0·04	0·03	0·04	0·03	0·03	0·01	0·02	0·01	0·04	0·05	0·02	0·02	0·04	0·04	0·04	0·04
Lycopene	0·09	0·08	0·10	0·09	0·09	0·07	0·11	0·09	0·08	0·05	0·08	0·05	0·09	0·08	0·09	0·08
β-Cryptoxanthin	0·06	0·04	0·05	0·05	0·03	0·02	0·04	0·03	0·02	0·02	0·03	0·02	0·04	0·04	0·04	0·03
Lutein	0·03	0·02	0·03	0·02	0·02	0·01	0·02	0·01	0·02	0·01	0·02	0·01	0·04	0·02	0·04	0·02
Zeaxanthin	0·01	0·01	0·01	0·01	0·01	0·01	0·01	0·01	0·01	0·01	0·01	0·00	0·01	0·01	0·01	0·01

* Tocopherols and carotenoids were extracted from LDL (0·6 ml of concentration 400 μg LDL protein/ml) and analysed by HPLC. Retinol was not detected in LDL samples. For details, see. p. 24.
† For details of subjects and treatments, see Table 1 and pp. 25–26.

Table 7. Maximum diene concentration, maximal oxidation rate, lag phase before rapid onset of conjugated diene production and thiobarbituric acid-reactive substances (TBARS) in copper-oxidised LDL* for volunteers given 0·9, 0·6 or 0·3 g *n*-3 polyunsaturated fatty acids (PUFA)/d or a placebo for 16 weeks†

(Values are means and standard deviations)

Treatment group	0·9 g *n*-3 PUFA/d (*n* 14)				0·6 g *n*-3 PUFA/d (*n* 16)				0·3 g *n*-3 PUFA/d (*n* 17)				Placebo (*n* 14)			
	Before treatment		After 16 weeks		Before treatment		After 16 weeks		Before treatment		After 16 weeks		Before treatment		After 16 weeks	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD
Maximum diene concentration (nmol/mg LDL protein)	539·97	93·98	589·05	46·40	530·81	86·53	540·50	62·51	551·62	77·19	535·64	57·12	591·21	63·39	579·15	53·28
Maximal oxidation rate (nmol/mg LDL protein per min)	10·76	2·22	10·88	1·01	8·74	1·54	9·07	1·05	9·18	2·02	9·13	1·63	11·69	1·84	11·18	1·70
Lag phase (min)	66	11	64	8	69	9	68	9	63	7	63	9	66	8	67	7
TBARS (nmol/mg LDL protein)	104	23	110	17	94	13	94	11	88	15	91	17	98	29	99	25

* LDL (50 μg LDL protein/ml) was oxidized by incubation with Cu (15 mM) at 37°C. For TBARS assessment, oxidation was stopped at 5 h. For details, see. p. 26.
† For details of subjects and treatments, see Table 1 and pp. 25–26.

Table 8. Maximum diene concentration, maximal oxidation rate, lag phase before onset of rapid conjugated diene production and thiobarbituric acid-reactive substances (TBARS) in 2,2′-azobis(2-amidinopropane) dihydrochloride (AAPH)-oxidised LDL* from a randomly-selected subset of volunteers given 0·9, 0·6 or 0·3 g *n*-3 polyunsaturated fatty acids (PUFA)/d or a placebo for 16 weeks†

(Values are means and standard deviations)

Treatment group	0·9 g *n*-3 PUFA/d (*n* 7)				0·6 g *n*-3 PUFA/d (*n* 8)				0·3 g *n*-3 PUFA/d (*n* 8)				Placebo (*n* 8)			
	Before treatment		After 16 weeks		Before treatment		After 16 weeks		Before treatment		After 16 weeks		Before treatment		After 16 weeks	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD
Maximum diene concentration (nmol/mg LDL protein)	543·92	43·62	549·24	70·02	523·08	36·05	534·59	36·11	499·37	58·55	507·51	48·30	563·51	47·24	538·02	57·45
Maximal oxidation rate (nmol/mg LDL protein per min)	6·94	0·79	6·50	1·35	6·19	1·67	6·22	1·03	6·30	1·62	6·55	1·74	7·29	1·66	6·40	1·71
Lag phase (min)	65	10	61	14	61	10	60	14	55	12	54	12	56	10	67	8
TBARS (nmol/mg LDL protein)	50	10	55	9	50	5	52	11	50	14	49	9	52	13	52	13

* LDL (50 μg LDL protein/ml) was oxidized by incubation with AAPH (1 mM) at 30°C. For TBARS assessment, oxidation was stopped at 5 h. For details, see p. 26.
† For details of subjects and treatments, see Table 1 and pp. 25–26.

($P = 0{\cdot}198$) or TBARS ($P = 0{\cdot}573$) in AAPH-oxidised LDL (Table 8).

Discussion

The present randomised placebo-controlled study investigated the effects of relatively low doses of *n*-3 PUFA, similar to levels achieved by a weekly intake of about two portions of fatty fish, on the susceptibility of LDL to oxidative modification in human volunteers.

Clear dose–response relationships were found between the dose of *n*-3 PUFA supplemented and post-treatment EPA and DHA compositions of plasma (Table 2) and LDL (Table 3). Overall, the increases in *n*-3 PUFA compositions in plasma and LDL appear to have occurred at the expense of 18:1, 20:3*n*-6 and 20:4*n*-6 fatty acid compositions.

Plasma total cholesterol and LDL-cholesterol levels (Table 4) were not affected by treatment. This finding is consistent with fish oil studies reviewed by Harris (1989), which showed that normolipidaemic subjects responded to *n*-3 fatty acid supplementation with essentially little or no change in plasma total cholesterol or LDL-cholesterol levels. Fasting plasma TAG concentrations were significantly reduced in the 0·9 g *n*-3 PUFA treatment group only (Table 4). According to the meta-analysis completed by Hokanson & Austin (1996), which showed that every 1·0 mmol/l increase in plasma TAG concentration was associated with an increase in cardiovascular risk of 32 % in men and 76 % in women, the 0·24 mmol/l reduction in fasting plasma TAG concentrations shown in the present study would be equivalent to a reduction in CHD risk of 8–18 %.

Overall, antioxidant concentrations in plasma (Table 5) and LDL (Table 6) were not altered by fish oil supplementation.

Susceptibility of LDL to oxidative modification, regardless of whether Cu^{2+} or AAPH was used to initiate oxidation (Tables 7 and 8), was not significantly altered by supplementation with low doses of *n*-3 PUFA (<1 g/d), despite incorporation of *n*-3 PUFA into LDL. The fatty acid content of LDL is certainly one of the factors that may influence its susceptibility to oxidation (Reaven *et al.* 1993), and the extent of oxidation has been shown to increase as the number of doubly-allylic positions increases (Liu *et al.* 1997). One would expect, therefore, that LDL enriched with *n*-3 PUFA would be highly susceptible to oxidation. However, studies evaluating this issue have proved contradictory.

Brude *et al.* (1997) found no increase in susceptibility of LDL to oxidative modification, using either Cu^{2+} or AAPH to initiate oxidation, in a group of male smokers with combined hyperlipidaemia following supplementation with 5 g *n*-3 PUFA/d for 6 weeks. Similar findings were observed in other human (Nenseter *et al.* 1992; Frankel *et al.* 1994; Bonanome *et al.* 1996; Hansen *et al.* 1998) and animal (Saito *et al.* 1991; Calviello *et al.* 1997) *n*-3 PUFA supplementation studies. On the other hand, several studies have reported less favourable results. Wander *et al.* (1996, 1998) found that supplementation of post-menopausal women with 15 g fish oil/d shortened lag phase, but slowed oxidation rate with no change in the maximum production of conjugated dienes. Similar results were reported in treated hypertensive subjects (Suzukawa *et al.* 1995), in hypertriacylglycerolaemic patients (Hau *et al.* 1996) in female Yucatan miniature swine (Whitman *et al.* 1994) and in monkeys (Thomas *et al.* 1994). This frequently reported finding of shortened lag phase indicating increased LDL oxidation, but slowed oxidation rate indicating reduced LDL oxidation appears paradoxical, and supports the argument that highly-unsaturated fatty acids are not necessarily oxidised more rapidly than those with fewer double bonds in biological systems (Wander *et al.* 1998). Brude *et al.* (1997) speculated that the reduced oxidation rate following *n*-3 PUFA supplementation may be a result of the tight packing of EPA and DHA in complex membrane lipids making the double bonds less available for free-radical interaction.

It has also been proposed that *n*-3 PUFA could modulate endogenous antioxidant systems (Brude *et al.* 1997). In support of this suggestion, *n*-3 PUFA supplementation has been shown to raise plasma and platelet β-carotene levels (Nair *et al.* 1993; Turley *et al.* 1998). Such an adaptive effect could provide an explanation as to why enhanced oxidisability should be seen more often after short-term supplementation than after longer-term supplementation periods. In agreement with this hypothesis, studies with supplementation periods of 3, 5 or 6 weeks showed increased susceptibility of oxidative modification of LDL (Hornstra *et al.* 1994; Oostenbrug *et al.* 1994; Suzukawa *et al.* 1995; Wander *et al.* 1996, 1998) whereas studies with longer supplementation periods of 5, 6, 8 or 16 weeks showed no effect (Nenseter *et al.* 1992; Frankel *et al.* 1994; Bonanome *et al.* 1996; Brude *et al.* 1997; Hansen *et al.* 1998). The results of the present study of 16 weeks duration also support this hypothesis.

In addition to differences in duration of supplementation, the dose of *n*-3 PUFA may also partly explain why some *n*-3 PUFA supplementation studies demonstrate a deleterious effect on LDL oxidation and other studies fail to do so. Studies which showed no effect on LDL oxidation generally involved supplementation with relatively low doses (≤2·5 g/d) of *n*-3 PUFA (Nenseter *et al.* 1992; Bonanome *et al.* 1996; Calviello *et al.* 1997), while those showing an effect used higher doses (Nair *et al.* 1993; Suzukawa *et al.* 1995; Wander *et al.* 1996, 1998).

In conclusion, moderate dietary intakes *n*-3 PUFA as part of a healthy and varied diet could help improve lipid profile and heart health without causing unwanted enhancement of LDL oxidation.

Acknowledgements

This study was funded by the EU FAIR programs (contract no. FAIR-CT-95-0085, NUTRIFISH). Fish oil capsules and carotenoid standards (β-cryptoxanthin and zeaxanthin) were kindly provided as a gift by Hoffman-La Roche Ltd, Basel, Switzerland.

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