ELSEVIER

Atherosclerosis 193 (2007) 159–167

ATHEROSCLEROSIS

www.elsevier.com/locate/atherosclerosis

Age- and dose-dependent effects of an eicosapentaenoic acid-rich oil on cardiovascular risk factors in healthy male subjects

Roberta Cazzola[a,*], Samantha Russo-Volpe[a], Elizabeth A. Miles[b], Dinka Rees[c], Tapati Banerjee[b], Catherine E. Roynette[b], Solenne J. Wells[b], Marie Goua[c], Klaus W.J. Wahle[c], Philip C. Calder[b], Benvenuto Cestaro[a]

[a] *Department of Preclinical Sciences "LITA Vialba", University of Milan, 74 Via G.B. Grassi, Milan 20157, Italy*
[b] *Institute of Human Nutrition, University of Southampton, Southampton, United Kingdom*
[c] *Rowett Research Institute and Robert Gordon's University, Aberdeen, United Kingdom*

Received 15 February 2006; received in revised form 25 May 2006; accepted 2 June 2006
Available online 1 August 2006

Abstract

Supplementation with fish oils, rich in n − 3 polyunsaturated fatty acids, modifies cardiovascular risk factors. However, dose–response relationships are poorly defined and whether similar effects are seen in young and older subjects is not known. This study determined the effect of supplementing the diet of young and older male subjects with different amounts of an eicosapentaenoic acid (EPA)-rich oil. Healthy young (18–42 years) and older (53–70 years) males were randomized to placebo or 1.35, 2.7 or 4.05 g EPA/day for 12 weeks. There was no effect of EPA on blood pressure or on plasma total, LDL or HDL cholesterol. EPA lowered plasma triacylglycerols, with the maximal effect at the lowest dose. Plasma lipoperoxides decreased in all groups. EPA decreased the lag time of copper-induced lipoprotein peroxidation and the ratio of reduced to total glutathione in the older subjects. The highest dose of EPA increased soluble E-selectin in young subjects, while increasing EPA tended to decrease soluble intercellular adhesion molecule 1 in young and older subjects. Young and older males will gain cardiovascular benefit from increased intake of EPA. Young males are unlikely to suffer adverse consequences from high EPA intake, whereas older males may have an increased risk of lipoprotein peroxidation.

Keywords: Fish oil; Polyunsaturated fatty acid; Cardiovascular disease; Blood lipids; Oxidative stress; Adhesion molecules

1. Introduction

The observed low incidence of cardiovascular mortality among Inuit populations [1] and the Japanese [2] has been ascribed to their high intake of long chain n − 3 polyunsaturated fatty acids (PUFAs) [3]. These fatty acids are found in fish and other seafood, especially so-called oily fish, and many epidemiological and case–control studies conducted in Western Europe and North America have reported significant inverse correlations between the intake of fish, oily fish or long chain n − 3 PUFAs or the status of long chain n − 3 PUFAs and cardiovascular outcomes [see 4–6]. The protective effects of long chain n − 3 PUFAs are explained by a reduction in risk factors for atherosclerosis, with much information coming from studies using fish oils, which are typically rich in the two major long chain n − 3 PUFAs, eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA). Supplementation of the diet with fish oils for periods of a few weeks to a few months has been shown to result in a lowering of systolic and diastolic blood pressure [see 7 for a meta-analysis] and of fasting plasma triacylglycerol concentrations [see 8,9]. Endothelial dysfunction and inflammation are now considered to be major risk factors for cardiovascular disease [10,11] and elevated plasma concentrations of soluble adhesion molecules, indicative of inflammatory processes affecting the endothelium, have been observed in subjects at risk of or suffering from cardiovascular disease

* Corresponding author. Tel.: +39 02 50319690; fax: +39 02 700536744.
E-mail address: roberta.cazzola@unimi.it (R. Cazzola).

0021-9150/$ – see front matter © 2006 Elsevier Ireland Ltd. All rights reserved.
doi:10.1016/j.atherosclerosis.2006.06.008

[12–14] and are considered by some to be useful markers of risk [15,16]. Fish oil supplementation has been shown to result in decreased plasma concentrations of some soluble adhesion molecules [17], suggesting decreased endothelial inflammation or decreased release from the endothelium. Paradoxically, fish oil supplementation has been demonstrated to cause a small increase in plasma total and LDL cholesterol concentrations [see 9] and to increase the susceptibility of lipoproteins to ex vivo peroxidation [18–20]. The latter effect is likely due to the high number of double bonds present in long chain n − 3 PUFAs which would provide increased substrate for lipid peroxidation once incorporated into lipoproteins. Indeed, fish oil supplementation of the diet has been shown to increase the circulating concentrations of indicators of oxidative stress such as lipid hydroperoxides [21,22]. Despite these apparently deleterious effects of long chain n − 3 PUFAs, recommendations to increase their intake have been made [5,23–25].

Despite the large number of studies with fish oil investigating cardiovascular risk factors, there are relatively few dose–response studies. Furthermore, there are few attempts to ascribe the effects of fish oil to the individual long chain n − 3 PUFAs present (i.e. EPA or DHA). Additionally, there is very little information on whether long chain n − 3 PUFAs exert similar effects in individuals of different ages. Finally, some of the potentially deleterious effects of fish oil could be avoided if lipid-soluble antioxidant vitamins such as α-tocopherol were co-administered in sufficient amounts. Therefore, this study investigated the dose–response effects of an EPA-rich oil, administered in combination with α-tocopherol, in young and older male subjects. Outcomes investigated were blood pressure, plasma lipid, α-tocopherol and soluble adhesion molecule concentrations, the susceptibility of lipoproteins to copper-induced peroxidation, and indices of oxidative stress. The overall aim of the study was to produce findings that will allow nutritional policy makers to develop scientifically sound recommendations and to develop improved preventative and therapeutic strategies for different age groups regarding cardiovascular health.

2. Subjects, materials and methods

2.1. Subjects and study design

Ethical permission for all procedures involving human volunteers was obtained from the relevant ethical committees. Healthy young and older adult males aged 18–42 and 53–70 years, respectively, were invited to participate in the study. All volunteers completed a health and lifestyle questionnaire prior to entering the study. Volunteers were excluded if they were taking any prescribed medication; were vegetarian; consumed fish oil, evening primrose oil or vitamin supplements; smoked >10 cigarettes/day; drank >10 units of alcohol/week; had a body mass index >32 kg/m^2; or consumed >1 portion of oily fish/week. One hundred young male and 69 older male subjects were recruited to the study. Ninety-three young male and 62 older male subjects completed the study. Drop-outs were distributed across all treatment groups. Data are reported for those subjects who completed the study.

Table 1
Characteristics at baseline of the population

	Young subjects	Older subjects
Age (years)	24.4 ± 0.6	60.7 ± 0.8*
Body weight (kg)	76.2 ± 1.07	83.7 ± 1.67*
BMI (kg/m^2)	24.1 ± 0.3	27.6 ± 0.5*
SBP (mm)	134.8 ± 1.4	139.3 ± 2.1
DBP (mm)	80.5 ± 1.2	86.4 ± 1.0*
Total cholesterol (mM)	3.69 ± 0.08	4.68 ± 0.10*
HDL cholesterol (mM)	1.13 ± 0.03	1.03 ± 0.04
LDL cholesterol (mM)	2.18 ± 0.06	3.16 ± 0.07*
Triacylglycerols (mM)	0.83 ± 0.04	1.03 ± 0.06*
Plasma sVCAM-1 (μg)	610.6 ± 30.6	977.4 ± 54.6*
Plasma sICAM-1 (μg/l)	60.5 ± 2.4	56.3 ± 4.1
Plasma sE-selectin (μg/l)	67.3 ± 3.7	122.0 ± 11.0*

Values are mean ± S.E.M. for subjects who completed the study. BMI, body mass index; ICAM-1, intercellular adhesion molecule 1; SBP, systolic blood pressure; DBP, diastolic blood pressure; VCAM-1: vascular cell adhesion molecule 1.

* Significantly different from the young subjects ($P < 0.01$; Student's t-test).

Subjects were randomly allocated in a double-blind fashion to one of four treatment groups; placebo, low EPA, moderate EPA and high EPA. Baseline characteristics of the young and older subjects are shown in Table 1; there were no differences in these characteristics across the treatment groups at baseline within each age group. Older subjects had significantly higher body weight, BMI, diastolic blood pressure, and plasma total and LDL cholesterol, triacylglycerol, sVCAM-1, and sE-selectin concentrations (Table 1). Systolic blood pressure also tended to be higher but not significantly different in older subjects ($P = 0.087$).

Subjects consumed nine 1 g capsules/day for 12 weeks. The capsules were provided by Pronova Biocare AS, Lysaker, Norway. Subjects in the placebo group consumed 9 × 1 g corn oil capsules/day. Subjects in the low EPA group consumed 6 g corn oil plus 3 g EPA-rich oil (EPAX 4510TG) in capsules (9 × 1 g) per day. Subjects in the moderate EPA group consumed 3 g corn oil plus 6 g EPA-rich oil in capsules (9 × 1 g) per day. Subjects in the high EPA group consumed 9 g EPA-rich oil in capsules (9 × 1 g) per day. Capsules were provided to subjects in small plastic pots each containing the daily allocation (i.e. nine capsules) with instructions to take three capsules three times daily. The EPA-rich oil contained 45% of fatty acids as EPA and 9% of fatty acids as docosahexaenoic acid (DHA; 22:6n − 3) (Table 2), mainly (>90%) in triacylglycerol form. Thus, the intakes of EPA from the capsules were 1.35, 2.7 and 4.05 g/day, respectively, in the low, moderate and high EPA groups and the intakes of DHA were approximately 0.27, 0.54 and 0.81 g/day. Intakes of linoleic acid from the capsules were 5.5, 3.7, 1.8 and 0.1 g/day, respectively, in the placebo, low EPA, moderate EPA and high EPA groups. Habitual intakes of linoleic acid

Table 2
Fatty acid composition of the placebo and EPA-rich capsules

Fatty acid	Placebo	EPA-rich
14:0	1.2	0.5
16:0	9.2	4.4
16:1n − 7	0	1.9
18:0	1.9	4.5
18:1n − 7	0	4.5
18:1n − 9	24.8	8.2
18:2n − 6	61.7	0.9
18:3n − 3	0	0.7
18:4n − 3	0	3.6
20:1n − 9	0	3.8
20:4n − 6	0	2.5
20:4n − 3	0	1.6
20:5n − 3 (EPA)	0	44.9
22:5n − 3 (DPA)	0	2.6
22:6n − 3 (DHA)	0	8.7

Values are g/100 g total fatty acid.

and EPA were not measured, but would be expected to be in the range of 8–16 (mean approximately 12.5) [26] and <0.2 g/day [23], respectively. All capsules contained 3.6 mg α-tocopherol. Thus, all subjects consumed an additional 32 mg α-tocopherol/day from the capsules (average habitual intake would be expected to be in the range 5–20 (mean approximately 10) mg/day [27]). Compliance was assessed by returned capsule counting and plasma lipid fatty acid profiles.

2.2. Blood collection

Blood was collected (using heparin as anticoagulant) from subjects in the fasted state (>10 h without food) in the morning at study entry and after 12 weeks of treatment. Plasma was prepared by centrifugation at 1000 × *g* for 15 min at room temperature. Plasma samples were immediately divided into aliquots then stored at −80 °C until further assays. Plasma samples were thawed only once, at the time of the assays, and samples collected at baseline and at the end study from a given subject were analyzed in the same assay to eliminate inter-assay variability.

2.3. Plasma phospholipid fatty acid composition

Lipid was extracted from plasma with chloroform/methanol (2:1, v/v) and phospholipids isolated by thin layer chromatography using a mixture of hexane/diethyl ether/acetic acid (90:30:1, v/v/v) as the elution phase. Fatty acid methyl esters were prepared by incubation with 140 g/l boron trifluoride in methanol at 80 °C for 60 min, and analyzed by gas chromatography as described elsewhere [28]. Peroxidation index was calculated as

$$\sum((\text{concentration of each PUFA(mmol/L)}) \times (\text{the number of double bonds in that PUFA} - 1))$$

2.4. Plasma lipid concentrations

Total cholesterol, HDL cholesterol and triacylglycerol concentrations in plasma were determined by enzymatic methods using Roche Diagnostics standards and kits (Roche Diagnostics S.p.A., Milan, Italy). HDL cholesterol concentrations were quantified after precipitation of LDL and VLDL fractions with phosphotungstic acid and magnesium chloride (Sigma–Aldrich S.r.l., Milan, Italy), and the LDL concentrations were calculated using the Friedewald formula [29].

2.5. Lipid-soluble vitamin concentrations and markers of oxidative stress

The concentrations of lipid-soluble vitamins were measured by reverse-phase high performance liquid chromatography (HPLC) as previously described [30].

Plasma lipid hydroperoxide concentrations were measured using a colorimetric kit (D-ROM test Diacron, Grosseto, Italy); results are expressed as micromoles per liter of H_2O_2 equivalents, according to the manufacturer's instructions.

Lipoprotein susceptibility to peroxidation was measured in plasma by determining the kinetics of copper-induced peroxidation resulting in the formation of fluorescent adducts originating from the reaction of aldehydes derived from lipid peroxidation with amino groups of apolipoproteins and/or phospholipids [31]. Fluorescence emission was monitored at 430 nm, setting the excitation at 360 nm, every 30 min for 8 h. The lag time before the propagation phase occurred was determined and is expressed in minutes. Lag time is an index of lipoprotein resistance to oxidation and is influenced by both lipid- and water-soluble plasma components, since the oxidation was carried out in whole plasma. The rate of the propagation phase was also determined.

Reduced and oxidized glutathione concentrations were measured according to Hissin and Hilf [32].

2.6. Plasma soluble adhesion molecule concentrations

The concentrations of sVCAM-1, sICAM-1 and sE-selectin in plasma were measured by specific ELISAs obtained from BioSource (Nivelles, Belgium). Limits of detection for these assays were <0.5 μg/l (sVCAM-1), <0.04 μg/l (sICAM-1), and <0.5 μg/l (sE-selectin) (data supplied by the manufacturer's of the kits).

2.7. Statistical analysis

Results are reported as mean ± standard error of mean (S.E.M.) for each treatment group. Data were checked for normality, and skewed parameters were log transformed before statistical analysis. The differences between baseline values of young and older subjects were analyzed using Student's *t*-test. The statistical significances of age and treatment were analyzed by using two-factor repeated measures analysis of

variance (ANOVA) with the baseline value as the covariate. When a significant interaction between factors was found, post hoc tests with Bonferroni's correction were used. In all analyses a value of $P < 0.05$ was considered statistically significant. All statistical analyses were performed by using StatistiXL software (Version 1.5; StatistiXL, Western Australia).

3. Results

3.1. BMI and blood pressure

There were no significant effects of treatment on body weight, BMI or blood pressure in either young or older subjects (data not shown).

3.2. Plasma phospholipid fatty acid composition

The total concentration of phospholipid fatty acids was significantly higher in the older than in the young subjects at baseline (approximately 4.2 mM versus 1.7 mM) and this difference was maintained after treatment in all groups (data not shown). The fatty acid composition of plasma phospholipids from subjects in this study has been reported recently [33] and will be summarised here. The proportions of total saturated fatty acids (approximately 48%), monounsaturated fatty acids (approximately 14%) and PUFAs (approximately 38%) in plasma phospholipids were not different between the young and older subjects at baseline and were not affected by treatment. At baseline, young subjects had lower proportions of di-homo-γ-linolenic acid ($2.6 \pm 0.1\%$ versus $3.2 \pm 0.1\%$; $P < 0.001$), EPA ($1.1 \pm 0.1\%$ versus $1.4 \pm 0.1\%$; $P = 0.014$), docosapentaenoic acid (DPA; $0.98 \pm 0.03\%$ versus $1.10 \pm 0.04\%$; $P = 0.009$) and DHA ($3.3 \pm 0.1\%$ versus $4.7 \pm 0.2\%$; $P < 0.001$) than older subjects. Fatty acid composition of plasma phospholipids did not change in the placebo group [33]. However, plasma phospholipid EPA and DPA increased from baseline in the groups receiving the EPA-rich oil [33] (Fig. 1), and in these groups EPA and DPA were significantly higher after treatment compared with the placebo group in both old and young subjects [33]. The increase in the EPA content of plasma phospholipids was dose-dependently related to the amount of EPA provided by the capsules ($\rho = 0.74$ for young and $\rho = 0.882$ for older subjects; both $P < 0.001$) and was greater in the older subjects when compared with the young subjects at each EPA dose [33]. The changes in DPA were also greater in the older than the young subjects at each treatment dose [33]. DHA increased in plasma phospholipids in young and older subjects receiving the moderate and high doses of EPA [33] (Fig. 1). The increase in the n − 3 PUFAs in plasma phospholipids after supplementing the diet with an EPA-rich oil was mirrored by a dose-dependent decrease in the proportion of the n − 6 PUFAs, linoleic, di-homo-γ-linolenic and arachidonic [33] (Fig. 1).

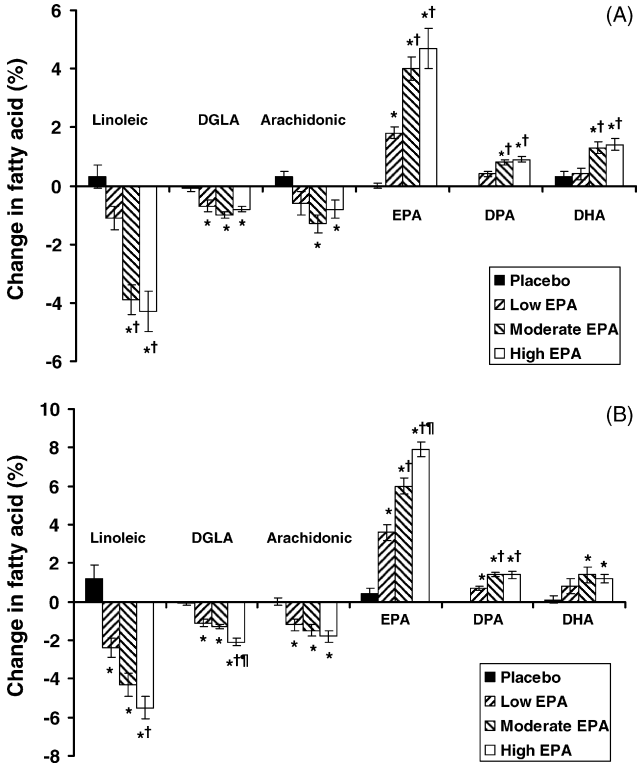

Fig. 1. Changes in proportions of n − 6 and n − 3 PUFAs in plasma phospholipids of young (A) and older (B) male subjects supplementing their diet with placebo or with one of three doses of an eicosapentaenoic acid-rich oil. Data are mean ± S.E.M. change after 12 weeks of supplementation. DGLA, di-homo-γ-linolenic acid; DHA, docosahexaenoic acid; DPA, docosapentaenoic acid; EPA, eicosapentaenoic acid. *Significantly different from placebo group; †significantly different from low EPA group; ¶significantly different from moderate EPA group.

The peroxidation index calculated for plasma phospholipids did not change in the subjects receiving placebo but increased significantly in young subjects in the high EPA group and in older subjects in the moderate and high EPA groups (Table 3). The increases in the subjects in the high EPA groups were approximately 25% and these changes were significantly greater than those seen in the corresponding placebo groups (approximately 2% increase).

3.3. Plasma lipid concentrations

No significant effects of treatment on the plasma concentrations of total, LDL and HDL cholesterol were observed (Table 4). However, plasma triacylglycerol concentrations were significantly decreased in both young and older subjects receiving EPA (Table 4). The extent of the decrease was approximately 25% in the young subjects in all three groups receiving EPA and this change was significantly different from that seen in the placebo group. The extent of the decrease in older subjects receiving EPA was similar to that seen in the young subjects (25–30%) but a similar decrease was also seen in the placebo group.

Table 3
Peroxidation index of plasma phospholipids, ratio of plasma phospholipid PUFA and lipids (cholesterol + triacylglycerol) to plasma α-tocopherol at baseline and after 12 weeks of supplementation

Treatment group	Time (week)	Peroxidation index		PUFA/α-tocopherol (mmol/mmol)		Plasma lipids/α-tocopherol (mmol/mmol)	
		Young	Older	Young	Older	Young	Older
Placebo	0	1.06 ± 0.04	2.67 ± 0.18	35.2 ± 1.8	60.8 ± 2.8	241.4 ± 7.6	239.7 ± 19.3
	12	1.07 ± 0.06	2.67 ± 0.19	32.1 ± 2.0	52.4 ± 2.6	214.2 ± 10.2*	167.6 ± 8.3*
Low EPA	0	1.06 ± 0.05	2.84 ± 0.18	33.9 ± 1.8	59.7 ± 2.6	241.3 ± 10.6	211.5 ± 8.3
	12	1.12 ± 0.07	2.97 ± 0.19	25.6 ± 1.5*	52.2 ± 2.7*	176.6 ± 7.6*	164.5 ± 5.4*
Moderate EPA	0	1.08 ± 0.04	2.71 ± 0.18	35.5 ± 2.6	63.1 ± 4.1	209.9 ± 12.0	247.5 ± 20.3
	12	1.15 ± 0.07	3.34 ± 0.24*	27.2 ± 1.5*	54.1 ± 3.1	189.8 ± 6.5*	185.5 ± 9.2*
High EPA	0	1.01 ± 0.06	2.86 ± 0.18	31.6 ± 1.7	65.0 ± 3.3	213.8 ± 7.5	240.6 ± 11.9
	12	1.27 ± 0.08*	3.55 ± 0.25*	26.0 ± 1.0*	53.7 ± 3.8	177.3 ± 6.9*	181.7 ± 9.5*

Values are mean ± S.E.M. for subjects who completed the study.
* Significantly different from baseline ($P < 0.05$; paired Student's t-test).

3.4. Plasma lipid-soluble vitamin concentrations and markers of oxidative stress

At baseline older subjects had significantly higher concentrations of α-tocopherol and β-carotene than young subjects (α-tocopherol: young subjects 20.5 ± 0.5 μM, older subjects 25.3 ± 0.7 μM; β-carotene: young subjects 0.33 ± 0.01 μM, older subjects 0.39 ± 0.02 μM; both $P < 0.01$; Student's t-test). Plasma concentrations of α-tocopherol, but not of β-carotene, increased similarly in all treatment groups, including the placebo group (data not shown). The increase in plasma α-tocopherol was approximately 20% (data not shown). The ratio of plasma phospholipid PUFA to α-tocopherol decreased significantly in young subjects receiving the EPA-rich oil (Table 3). Older subjects receiving EPA showed a similar decrease in this ratio, but this was significant only in the low EPA group (Table 3). The ratio of plasma lipids (total cholesterol + triacylglycerols) to α-tocopherol decreased significantly in all treatment groups, including the placebo group (Table 3).

Plasma lipid hydroperoxide concentrations decreased by approximately 10% in both young and older subjects irrespective of treatment group (Table 5). Lag time of copper-induced lipoprotein peroxidation and the ratio of reduced to total glutathione were not altered in young subjects (Table 5). However, in the older subjects treated with EPA, the lag time of lipoprotein peroxidation and the ratio of reduced to total glutathione were significantly decreased (Table 5). In the moderate and high EPA groups the changes in these parameters were significantly different from those seen in the placebo group (data not shown). It is notable that the lag time of lipoprotein peroxidation in response to copper in the older subjects in moderate and high EPA groups was decreased by 50%. There was no effect of treatment on the rate of the propagation phase of lipoprotein peroxidation (data not shown).

3.5. Plasma soluble adhesion molecule concentrations

Plasma sICAM-1 concentrations were not significantly affected by treatment with EPA in either young or older subjects (Table 6). In contrast, the plasma concentration of sE-selectin was significantly increased in young subjects in the high EPA group (Table 6). Plasma sVCAM-1 concentrations tended to decrease with increasing intake of EPA, although the observed changes were not significant (Table 6). Consistent with this trend, the relationship between the change in the proportion of EPA in plasma phospholipids and the change in sVCAM-1 concentration was significant for the

Table 4
Plasma lipid concentrations at baseline and after 12 weeks of supplementation

Treatment group	Time (week)	Total cholesterol (mM)		LDL cholesterol (mM)		HDL cholesterol (mM)		Triacylglycerols (mM)	
		Young	Older	Young	Older	Young	Older	Young	Older
Placebo	0	3.8 ± 0.1	4.8 ± 0.1	2.3 ± 0.1	3.2 ± 0.1	1.2 ± 0.1	1.1 ± 0.1	0.7 ± 0.1	1.1 ± 0.1
	12	3.7 ± 0.1	4.4 ± 0.1	2.0 ± 0.1	3.0 ± 0.1	1.3 ± 0.1	1.0 ± 0.1	0.8 ± 0.1	0.9 ± 0.1*
Low EPA	0	3.6 ± 0.2	4.5 ± 0.2	2.2 ± 0.1	3.1 ± 0.2	1.0 ± 0.1	1.0 ± 0.1	0.9 ± 0.1	0.9 ± 0.1
	12	3.5 ± 0.2	4.1 ± 0.1	2.2 ± 0.2	2.8 ± 0.1	1.0 ± 0.1	0.9 ± 0.1	0.6 ± 0.1*	0.7 ± 0.1*
Moderate EPA	0	3.8 ± 0.1	4.7 ± 0.2	2.2 ± 0.1	3.2 ± 0.2	1.2 ± 0.1	0.9 ± 0.1	0.9 ± 0.1	1.2 ± 0.1
	12	3.6 ± 0.2	4.4 ± 0.1	2.0 ± 0.1	2.9 ± 0.1	1.2 ± 0.1	1.0 ± 0.1	0.6 ± 0.1*	0.9 ± 0.1*
High EPA	0	3.5 ± 0.1	4.7 ± 0.2	2.0 ± 0.1	3.1 ± 0.1	1.1 ± 0.1	1.2 ± 0.1	0.8 ± 0.1	1.0 ± 0.1
	12	3.4 ± 0.2	4.5 ± 0.1	1.9 ± 0.2	3.0 ± 0.1	1.2 ± 0.2	1.1 ± 0.1	0.6 ± 0.1*	0.7 ± 0.1*

Values are mean ± S.E.M. for subjects who completed the study.
* Significantly different from baseline ($P < 0.05$; paired Student's t-test).

Table 5
Indices of plasma oxidative stress at baseline and after 12 weeks of supplementation

Treatment group	Time (week)	Lipid hydroperoxides (μM)		Lag time of lipoprotein peroxidation (min)		GSH/glutathione	
		Young	Older	Young	Older	Young	*Older*
Placebo	0	6.2 ± 0.1	6.8 ± 0.1	183.2 ± 6.2	116.7 ± 8.0	0.88 ± 0.01	0.77 ± 0.01
	12	5.3 ± 0.1*	5.8 ± 0.1*	188.8 ± 5.9	118.5 ± 8.0	0.90 ± 0.01	0.78 ± 0.01
Low EPA	0	6.2 ± 0.1	6.8 ± 0.2	182.1 ± 7.6	122.2 ± 9.0	0.86 ± 0.02	0.79 ± 0.01
	12	5.7 ± 0.1*	6.2 ± 0.1*	188.4 ± 7.3	97.5 ± 8.7*	0.87 ± 0.01	0.71 ± 0.01*
Moderate EPA	0	6.3 ± 0.2	7.0 ± 0.2	188.5 ± 6.4	130.1 ± 5.7	0.89 ± 0.01	0.81 ± 0.01
	12	5.6 ± 0.1*	6.3 ± 0.1*	204.4 ± 5.4	61.6 ± 8.4*	0.90 ± 0.01	0.64 ± 0.01*
High EPA	0	6.2 ± 0.1	6.7 ± 0.1	189.8 ± 7.3	133.0 ± 5.8	0.87 ± 0.01	0.81 ± 0.01
	12	5.7 ± 0.09*	6.1 ± 0.1*	184.7 ± 7.1	63.9 ± 5.1*	0.87 ± 0.01	0.62 ± 0.01*

Values are mean ± S.E.M. for subjects who completed the study.
* Significantly different from baseline ($P < 0.05$; paired Student's *t*-test).

Table 6
Plasma soluble adhesion molecule concentrations at baseline and after 12 weeks of supplementation

Treatment group	Time (week)	sVCAM-1 (μg/l)		sICAM-1 (μg/l)		sE-selectin (μg/l)	
		Young	Older	Young	Older	Young	*Older*
Placebo	0	567.4 ± 49.1	1188.0 ± 141.9	54.5 ± 4.8	57.1 ± 6.9	59.8 ± 6.5	96.0 ± 5.8
	12	655.7 ± 60.9	1016.4 ± 103.2	51.0 ± 4.7	48.8 ± 6.0	55.5 ± 4.1	114.1 ± 10.0
Low EPA	0	668.7 ± 80.3	993.9 ± 64.5	60.1 ± 3.9	52.0 ± 7.6	64.9 ± 5.7	144.5 ± 31.4
	12	578.6 ± 78.2	893.0 ± 67.6	55.6 ± 3.8	49.6 ± 7.1	62.9 ± 6.9	135.8 ± 21.8
Moderate EPA	0	599.5 ± 53.1	905.0 ± 103.5	65.8 ± 4.1	68.3 ± 9.4	72.4 ± 9.9	143.5 ± 27.6
	12	571.7 ± 54.2	738.5 ± 50.1	65.9 ± 5.6	51.0 ± 8.0	75.5 ± 9.4	119.1 ± 9.1
High EPA	0	608.9 ± 61.1	977.0 ± 152.1	61.9 ± 6.0	48.2 ± 7.2	71.8 ± 6.9	127.3 ± 25.6
	12	530.5 ± 37.6	770.9 ± 62.4	59.2 ± 3.7	48.2 ± 7.4	88.0 ± 9.2*,†	133.3 ± 36.6

Values are mean ± S.E.M. for subjects who completed the study. ICAM-1: intercellular adhesion molecule 1; VCAM-1: vascular cell adhesion molecule 1.
* Significantly different from baseline ($P < 0.05$; paired Student's *t*-test).
† Significantly different from placebo ($P < 0.02$; one-factor ANOVA).

young subjects ($\rho = -0.255$; $P = 0.015$) and tended towards significance for all subjects ($\rho = -0.153$ for young subjects, $P = 0.065$).

4. Discussion

This study sought to identify the extent to which an EPA-rich oil could mimic the documented effects of fish oil on selected cardiovascular risk factors, to identify the dose–response effects of EPA, and to see whether EPA has the same effects in young and older male subjects. EPA was used at three doses, with the highest being 4.05 g/day. Although dietary information was not gathered from these subjects, data available for comparable groups of the UK population suggest that typical intake of EPA would be <0.2 g/day [23]. The study was placebo controlled and double-blind. EPA was incorporated in a dose–response fashion into plasma phospholipids, with greater incorporation occurring in the older subjects. These data are indicative of different handling of EPA between young and older subjects. Other data in the literature is also suggestive of this [34,35]. Likely reasons for the different handling of EPA between young and older are discussed elsewhere [33] and may relate to differences in body composition resulting in differences in fatty acid oxidation, storage and incorporation into plasma lipid pools. Incorporation of DPA and DHA in plasma phospholipids also increased with increasing EPA intake and was higher in the older subjects. The capsules contained DHA and some DPA, which likely accounts for the increased appearance of these fatty acids in plasma phospholipids, although there may have been some synthesis, especially of DPA, from EPA.

Fish oil has been demonstrated to decrease plasma triacylglycerol concentrations [8,9]. Harris [9] reported an approximately 25% lowering in normotriacylglycerolaemic subjects when data from a number of placebo controlled studies in humans were pooled, while Roche [8] estimated the dose–response relationship between long chain n − 3 PUFA intake and reduction in plasma triacylglycerol concentrations. In the current study, EPA lowered plasma triacylglycerol concentrations, with the maximal effect occurring at the lowest EPA dose and a similar effect (approximately 25–30% lowering) occurring in both young and older subjects. Clearly these data are in accordance with Harris' estimate of the extent of triacylglycerol-lowering with fish oil [9]. Roche's estimated dose–response relationship [8] suggests expected

lowering of about 15, 20 and 25% at the three doses. The data obtained in the current study indicate that EPA can mimic the triacylglycerol-lowering effect of fish oil and suggest that EPA may be responsible for the latter effect. The reason why the maximum effect is seen at the lowest dose of EPA used (1.35 g/day), which is clearly not in accordance with Roche's estimated dose–response relationship [8], could be that EPA is more potent than DHA; Roche's estimate is based upon the combination of these two fatty acids. However, recent studies that have compared the triacylglycerol-lowering effects of pure EPA and pure DHA (each at about 4 g/day for 4 or 6 weeks) in healthy subjects aged 20–70 years [36], in mildly hyperlipidemic males [37], in hyperlipidemic, overweight males [38] and in type-2 diabetics [39] found that both EPA and DHA caused lowering of plasma triacylglycerol concentrations of about 20%.

Fish oil has been demonstrated to decrease blood pressure [7] and to slightly elevate plasma total and LDL cholesterol concentrations [9]. However, in the current study there was no significant effect of EPA on blood pressure or on plasma total, LDL or HDL cholesterol concentrations. Thus, the beneficial effect of fish oil on blood pressure and the potentially detrimental effect on plasma cholesterol concentration appear not to be due to EPA, and may be due to DHA or DPA. Woodman et al. [39] found no effect of 4 g EPA/day for 6 weeks on 24 h systolic and diastolic blood pressures in type-2 diabetics, while Buckley et al. [36] found no effect of 4.9 g/day EPA for 4 weeks on plasma total, LDL or HDL cholesterol concentrations in healthy adults. Likewise, there was no effect of 4 g/day EPA for 6 weeks on total, LDL or HDL cholesterol concentrations in mildly hyperlipidemic males [37], in hyperlipidemic, overweight males [38] and in type-2 diabetics [39]. In contrast, some recent studies using pure DHA have shown that low doses (0.7 or 1.5 g/day) can raise total and LDL cholesterol concentrations [40,41], confirming that the slight cholesterol-raising effect of fish oil is most likely due to this fatty acid rather than to EPA.

Fish oil has been associated with an increase in markers of oxidative stress [18–22], most likely due to the increased presence of highly unsaturated n − 3 fatty acids in plasma lipids and cells. In the current study, the lag time of copper-induced lipoprotein peroxidation and the ratio of reduced to total glutathione were decreased in the older subjects treated with EPA, although not in the young subjects. In apparent contrast to these effects, the plasma concentration of oxidized lipids was decreased after all the dietary treatments, including placebo, in both young and older groups. Although lipid hydroperoxides decreased by approximately 10%, plasma triacylglycerols decreased by approximately 25–30%. Thus, the ratio between the oxidized and native lipids, indicative of a higher risk of in vivo susceptibility to oxidation, is increased after EPA supplementation in both young and older subjects. At study entry, the young subjects appear to have better antioxidant defences and lower susceptibility to lipid peroxidation than the older subjects, as evidenced by a higher ratio of reduced to total glutathione, lower plasma lipid hydroperoxide concentrations, a longer lag time of lipoprotein oxidation, a lower peroxidation index of phospholipid PUFA and a lower phospholipid PUFA/α-tocopherol ratio. The treatment with EPA promoted an increase in the peroxidation index of PUFA and a negligible amelioration of the PUFA to α-tocopherol ratio in the older subjects and a moderate increase in the peroxidation index of PUFA and a significant amelioration of the PUFA to α-tocopherol ratio in the young subjects. Thus, the latter may have been able to cope better with the added "EPA load", especially with the additional α-tocopherol, than the older subjects. It appears that the older subjects still experienced an additional oxidative burden while consuming EPA, since the ratio of reduced to total glutathione was decreased. Since EPA decreased lag time of copper-induced lipoprotein peroxidation, effects of fish oil on such outcomes [18–20] could be due to EPA. However, Mesa et al. [42] reported that 4.9 g/day of EPA or DHA had similar effects (a 12% reduction) on the lag time of copper-induced oxidation of LDL isolated from plasma. In that study the LDL were from a mixed age group (23–65 years of age) of males and females and it is not apparent if different effects occurred in young and older subjects. Nevertheless, the current study indicates that an EPA-rich oil results in increased lipoprotein susceptibility to oxidation in older but not young subjects, even when the oil is co-administered with α-tocopherol to provide an intake of 32 mg/day. This amount of α-tocopherol seems to be enough to counteract deleterious peroxidation of PUFA in young but not in older subjects. At study entry, the ratio between plasma lipids (triacylglycerols plus cholesterol) and α-tocopherol was similar in the young and older subjects. After the administration of the different oils and α-tocopherol, this ratio was significantly decreased in all the groups, including placebo. This reflects the decrease in plasma triacylglycerol and the increase in plasma α-tocopherol concentrations, which occurred in all groups. This was interesting in that corn oil, which is mainly 18:2n − 6, also reduced triacylglycerol in the placebo group.

Fish oil supplementation has been shown to increase sE-selectin concentrations in young subjects and to decrease sVCAM-1 concentrations in older subjects [17]. In the current study, the highest dose of EPA increased soluble E-selectin concentrations in young subjects, confirming the earlier finding with fish oil, while increasing intake of EPA was associated with decreasing sVCAM-1 concentrations in both young and older subjects, although the effect was smaller than that seen in older subjects in the earlier study. It is assumed that sVCAM-1 concentration reflects shedding from activated endothelial cells, which in turn is related to the level of expression at the gene and cell surface protein levels. The smaller effect on sVCAM-1 concentrations seen in the current study compared with an earlier study using fish oil, may indicate that EPA is less potent than DHA at inhibiting VCAM-1 expression. This conclusion is consistent with findings of de Caterina et al. [43] who showed that that DHA, but not EPA, decreased VCAM-1 expression (both mRNA and cell surface protein) in cultured human umbil-

ical vein endothelial cells. Collie-Duguid and Wahle [44] showed that both EPA and DHA could attenuate the expression of VCAM-1 on HUVEC, although DHA was more effective.

In summary, EPA, even at a supplemental dose of 4.05 g/day, does not affect blood pressure in normotensive males and does not affect plasma cholesterol, LDL or HDL concentrations. EPA does lower plasma triacylglycerol concentrations in both young and older subjects, with the maximal effect at the lowest EPA dose. However, older subjects, who may have diminished antioxidant defences, may suffer some deleterious effects from high doses of EPA as evidenced by a much shorter lag time of copper-induced lipoprotein peroxidation and a decreased ratio of reduced to total glutathione. EPA can decrease sVCAM-1 concentrations in young and older subjects, indicative of decreased endothelial inflammation, although this effect requires a high EPA intake.

In conclusion, EPA mimics some of the effects of fish oil (e.g. decreasing triacylglycerol and sVCAM-1 concentrations; decreasing lag time of lipoprotein peroxidation, at least in older subjects) but not others (e.g. increasing total and LDL cholesterol and lipid hydroperoxide concentrations; lowering blood pressure). Young and older males will gain cardiovascular benefit from increased intake of EPA (e.g. decreased plasma triacylglycerol and sVCAM-1 concentrations). Young males are unlikely to suffer adverse consequences from increased EPA intake (e.g. elevated cholesterol concentrations and lipid peroxidation), but increased intakes of EPA in older males may increase the risk of lipoprotein peroxidation.

Acknowledgements

We acknowledge Dr. Giovanna Cervato (University of Milan) for the analysis of lipid-soluble vitamin concentrations. This research was funded by the grant “Nutraceuticals for a healthier life”, known as NUHEAL (QLRT-1999-00888) to KWJW, PCC and BC from the European Commission under the Fifth Framework Programme “Quality of life and management of living resources”.

References

[1] Bjerregaard P, Dyerberg J. Mortality from ischemic heart disease and cerebrovascular disease in Greenland. Int J Epidemiol 1988;17:514–9.
[2] Yano K, MacLean CJ, Reed DM, et al. A comparison of the 12-year mortality and predictive factors of coronary heart disease among Japanese men in Japan and Hawaii. Am J Epidemiol 1988;127:476–87.
[3] Bang HO, Dyerberg J, Hjorne N. The composition of foods consumed by Greenland Eskimos. Acta Med Scand 1976;200:69–73.
[4] Bucher HC, Hengstler P, Schindler C, Meier G. N − 3 polyunsaturated fatty acids in coronary heart disease: a meta-analysis of randomized controlled trials. Am J Med 2002;112:298–304.
[5] Kris-Etherton PM, Harris WS, Appel LJ, American Heart Association Nutrition Committee. Fish consumption, fish oil, omega-3 fatty acids, and cardiovascular disease. Circulation 2002;106:2747–57.
[6] Calder PC. N − 3 fatty acids and cardiovascular disease: evidence explained and mechanisms explored. Clin Sci 2004;107:1–11.
[7] Geleijnse JM, Giltay EJ, Grobbee DE, Donders ART, Kok FJ. Blood pressure response to fish oil supplementation: meta-regression analysis of randomized trials. J Hypertens 2002;20:1493–9.
[8] Roche HM. Unsaturated fatty acids. Proc Nutr Soc 1999;58:397–401.
[9] Harris WS. N − 3 fatty acids and lipoproteins: comparison of results from human and animal studies. Lipids 1996;31:243–52.
[10] Sattar N, Ferns G. Endothelial dysfunction. In: Stanner S, editor. Cardiovascular disease: diet, nutrition and emerging risk factors. Oxford: Blackwells; 2005. p. 63–77.
[11] Yaqoob P, Ferns G. Inflammation-related factors. In: Stanner S, editor. Cardiovascular disease: diet, nutrition and emerging risk factors. Oxford: Blackwells; 2005. p. 128–46.
[12] Morisaki N, Saito I, Tamura K, et al. New indices of ischemic heart disease and aging: studies on the serum levels of soluble intercellular adhesion molecule-1 (ICAM-1) and soluble vascular cell adhesion molecule-1 (VCAM-1) in patients with hypercholesterolemia and ischemic heart disease. Atherosclerosis 1997;131:43–8.
[13] Rohde LE, Lee RT, Rivero J, et al. Circulating cell adhesion molecules are correlated with ultrasound-based assessment of carotid atherosclerosis. Arterioscler Thromb Vasc Biol 1998;18:1765–70.
[14] Peter K, Nawroth P, Conradt C, et al. Circulating vascular cell adhesion molecule-1 correlates with the extent of human atherosclerosis in contrast to circulating intercellular adhesion molecule-1, E-selectin, P-selectin, and thrombomodulin. Arterioscler Thromb Vasc Biol 1997;17:505–12.
[15] Ridker PM, Hennekens CH, Roitman-Johnson B, Stampfer MJ, Allen J. Plasma concentration of soluble intercellular adhesion molecule 1 and risks of future myocardial infarction in apparently healthy men. Lancet 1998;351:88–92.
[16] Hwang SJ, Ballantyne CM, Sharrett AR, et al. Circulating adhesion molecules VCAM-1, ICAM-1, and E-selectin in carotid atherosclerosis and incident coronary heart disease cases: the Atherosclerosis Risk In Communities (ARIC) study. Circulation 1997;96:4219–25.
[17] Miles EA, Thies F, Wallace FA, et al. Influence of age and dietary fish oil on plasma soluble adhesion molecule concentrations. Clin Sci 2001;100:91–100.
[18] Leigh-Firbank EC, Minihane AM, Leake DS, et al. Eicosapentaenoic acid and docosahexaenoic acid from fish oils: differential associations with lipid responses. Br J Nutr 2002;87:435–45.
[19] Turini ME, Crozier GL, Donnet-Hughes A, Richelle MA. Short-term fish oil supplementation improved innate immunity, but increased ex vivo oxidation of LDL in man—a pilot study. Eur J Nutr 2001;40:56–65.
[20] Wander RC, Du SH, Ketchum SO, Rowe KE. Effects of interaction of RRR-alpha-tocopheryl acetate and fish oil on low-density-lipoprotein oxidation in postmenopausal women with and without hormone-replacement therapy. Am J Clin Nutr 1996;63:184–93.
[21] Vericel E, Polette A, Bacot S, Calzada C, Lagarde M. Pro- and antioxidant activities of docosahexaenoic acid on human blood platelets. J Thromb Haemost 2003;1:566–72.
[22] Mori TA, Puddey IB, Burke V, et al. Effect of omega 3 fatty acids on oxidative stress in humans: GC–MS measurement of urinary F2-isoprostane excretion. Redox Rep 2000;5:45–6.
[23] British Nutrition Foundation. N − 3 fatty acids and health. London: British Nutrition Foundation; 1999.
[24] Scientific Advisory Committee on Nutrition/Committee on Toxicity. Advice on fish consumption: benefits and risks. London: TSO; 2004.
[25] Van de werf F, Ardissino D, Betriu A, et al. Management of acute myocardial infarction in patients presenting with ST-segment elevation. Eur Heart J 2003;24:26–66.
[26] Henderson L, Gregory J, Irving K, Swan G. The National Diet and Nutrition Survey: adults aged 19–64 years. London: TSO; 2004.
[27] Report of the Panel on Dietary Reference Values of the Committee on Medical Aspects of Food, Policy, Dietary reference values

for food energy nutrients in the United Kingdom. London: HMSO; 1991.
[28] Wallace FA, Miles EA, Calder PC. Comparison of the effects of linseed oil and different doses of fish oil on mononuclear cell function in healthy human subjects. Br J Nutr 2003;89:679–89.
[29] Friedewald WT, Levy RI, Fredrickson DS. Estimation of the concentration of low-density lipoprotein cholesterol in plasma, without use of the preparative ultracentrifuge. Clin Chem 1972;18:499–502.
[30] Higdon JV, Liu J, Du S, Morrow JD, Ames BN, Wander RC. Supplementation of postmenopausal women with fish oil rich in eicosapentaenoic acid and docosahexaenoic acid is not associated with greater in vivo lipid peroxidation compared with oils rich in oleate and linoleate as assessed by plasma malondialdehyde and F2-isoprostanes. Am J Clin Nutr 2000;72:714–22.
[31] Cervato G, Viani P, Cazzola R, Cestaro B. A fluorescent method for determination of plasma susceptibility to peroxidation. Clin Biochem 1999;32:171–7.
[32] Hissin PJ, Hilf R. A fluorimetric method for the determination of oxidized and reduced glutathione in tissues. Anal Biochem 1976;74:214–26.
[33] Rees D, Miles EA, Banerjee T, et al. Dose-related effects of eicosapentaenoic acid on innate immune function in healthy humans: a comparison of young and older male subjects. Am J Clin Nutr 2006; 83:331–42.
[34] Bolton-Smith C, Woodward M, Tavendale R. Evidence for age-related differences in the fatty acid composition of human adipose tissue, independent of diet. Eur J Clin Nutr 1997;51:619–24.
[35] Sands SA, Reid KJ, Windsor SL, Harris WS. The impact of age, body mass index, and fish intake on the EPA and DHA content of human erythrocytes. Lipids 2005;40:343–7.
[36] Buckley R, Shewring B, Turner R, Yaqoob P, Minihane AM. Circulating triacylglycerol and apoE levels in response to EPA and docosahexaenoic acid supplementation in adult human subjects. Br J Nutr 2004;92:477–83.
[37] Mori TA, Burke V, Puddey IB, et al. Purified eicosapentaenoic and docosahexaenoic acids have differential effects on serum lipids and lipoproteins, LDL particle size, glucose, and insulin in mildly hyperlipidemic men. Am J Clin Nutr 2000;71:1085–94.
[38] Mori TA, Watts GF, Burke V, Hilme E, Puddey IB, Beilin LJ. Differential effects of eicosapentaenoic acid and docosahexaenoic acid on vascular reactivity of the forearm microcirculation in hyperlipidemic, overweight men. Circulation 2000;102:1264–9.
[39] Woodman RJ, Mori TA, Burke V, Puddey IB, Watts GF, Beilin LJ. Effects of purified eicosapentaenoic and docosahexaenoic acids on glycemic control, blood pressure, and serum lipids in type 2 diabetic patients with treated hypertension. Am J Clin Nutr 2002;76:1007–15.
[40] Theobald HE, Chowienczyk PJ, Whittall R, Humphries SE, Sanders TA. LDL cholesterol-raising effect of low-dose docosahexaenoic acid in middle-aged men and women. Am J Clin Nutr 2004;79:558–63.
[41] Maki KC, Van Elswyk ME, McCarthy D, et al. Lipid responses to a dietary docosahexaenoic acid supplement in men and women with below average levels of high density lipoprotein cholesterol. J Am Coll Nutr 2005;24:189–99.
[42] Mesa MD, Buckley R, Minihane AM, Yaqoob P. Effects of oils rich in eicosapentaenoic and docosahexaenoic acids on the oxidizability and thrombogenicity of low-density lipoprotein. Atherosclerosis 2004;175:333–43.
[43] De Caterina R, Cybulsky MI, Clinton SK, Gimbrone Jr MA, Libby P. The omega-3 fatty acid docosahexaenoate reduces cytokine-induced expression of proatherogenic and proinflammatory proteins in human endothelial cells. Arterioscler Thromb 1994;14:1829–36.
[44] Collie-Duguid ES, Wahle KW. Inhibitory effect of fish oil $n-3$ polyunsaturated fatty acids on the expression of endothelial cell adhesion molecules. Biochem Biophys Res Commun 1996;220: 969–74.