

Olive oil- and fish oil-enriched diets modify plasma lipids and susceptibility of LDL to oxidative modification in free-living male patients with peripheral vascular disease: the Spanish Nutrition Study

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(Received 10 February 1998 – Revised 14 December 1998 – Accepted 9 February 1999)

The present study describes a clinical trial in which Spanish patients suffering from peripheral vascular disease (Fontaine stage II) were given specific lipid supplements. Designed as a longitudinal intervention study, patients were provided with olive oil for 3 months, followed by a 3 month wash-out period, then supplemented with a combination of fish oil and olive oil for the final 3 months. Changes in plasma and lipoprotein fatty acid composition and susceptibility of LDL to *in vitro* oxidation were examined. Furthermore, lipid-supplement-induced changes in LDL properties were measured as relative electrophoretic mobility and macrophage uptake. In addition, thirteen patients not provided with olive oil and fish oil were included as a control group and twenty healthy age-matched individuals were used as a reference group. A complete clinical study and a nutritional survey concerning food habits and lifestyle were performed every 3 months. Yao indices and claudicometry did not change significantly with dietary intervention although changes in plasma lipid composition suggested an improvement in the condition of the patients. The intake of the fish-oil supplement resulted in significantly increased plasma levels of eicosapentaenoic acid (20:5*n*-3) and docosahexaenoic acid (22:6*n*-3) in comparison with baseline concentrations, olive-oil and control groups. Fish-oil consumption significantly decreased plasma triacylglycerol levels compared with the olive-oil period, control and reference groups. The susceptibility of LDL to Cu-mediated oxidation was lower in the patients consuming olive oil and the fish-oil supplement than in the control group, and the uptake of LDL by macrophages was significantly lower in the group supplemented with fish oil. In conclusion, consumption of olive oil together with a dietary supplement of fish oil may be useful in the nutritional management of patients suffering from peripheral vascular disease in terms of increasing plasma *n*-3 long-chain polyunsaturated fatty acids and decreasing susceptibility of LDL to oxidation.

Fish oil: Olive oil: Peripheral vascular disease: LDL oxidation

Cardiovascular diseases (CVD) are still the main cause of death, disability and economic loss in most developed countries. Peripheral vascular disease (PVD) is characterized by lower extremity ischaemia due to arteriosclerosis obliterans; this disease is highly prevalent and patients classified in the Fontaine stage II exhibit intermittent claudication as a result of stenosis in their leg arteries.

Epidemiological analysis of the relationship between nutrition and CVD shows that societies becoming more affluent are adopting dietary habits similar to those of the industrialized nations, leading to atherosclerotic consequences

(Keys *et al.* 1986). Hypertension, hypercholesterolaemia and smoking are the three major risk factors for the development of atherosclerosis (Gurr, 1992) and improvement of nutritional habits and other lifestyle factors would help to ameliorate this situation.

Dietary fatty acids are among the most important nutrients determining plasma lipid concentration. Increases in dietary saturated fatty acids (SFA) have been proven to elevate total plasma and LDL-cholesterol concentrations (Vega *et al.* 1982), whereas increases in dietary *n*-3 polyunsaturated fatty acids (PUFA) decrease serum triacylglycerols without

Abbreviations: C group, control group; CVD, cardiovascular diseases; DHA, docosahexaenoic acid; DiI, 1,1'-dioctadecyl-3,3,3'-tetramethylindocarbocyanine perchlorate; EPA, eicosapentaenoic acid; MUFA, monounsaturated fatty acids; O group, olive oil group; OF group, olive oil plus fish oil group; PUFA, polyunsaturated fatty acids; PVD, peripheral vascular disease; R group, reference group; SFA, saturated fatty acids.

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having a major effect on serum total cholesterol and LDL-cholesterol concentrations in healthy subjects (Schmidt *et al.* 1992). Several studies have shown that both *n*-6 PUFA and monounsaturated fatty acids (MUFA) have similar hypocholesterolaemic effects when replacing SFA in the diet (Mattson & Grundy, 1985), although MUFA do not reduce HDL-cholesterol (Grundy & Denke, 1990). The oxidative modifications of LDL have been involved in the initiation and progression of atherosclerosis (Witztum & Steinberg, 1991). Oxidative modifications of LDL are due mainly to the peroxidation of unsaturated fatty acids. The macrophages take up oxidized LDL rapidly, leading to foam-cell formation, which in turn results in the generation of atheromatous plaques (Steinberg *et al.* 1989).

Recent studies have shown that various types of fatty acids can alter the susceptibility of LDL particles to oxidative modification. It has been reported that replacement of *n*-6 PUFA with MUFA in both rabbit and human plasma LDL particles by dietary manipulation results in the formation of lipoproteins that are less susceptible to the generation of conjugated dienes during incubation with CuSO₄ and to the degradation by macrophages after incubation with endothelial cells, two standard assays used to monitor the oxidative modification of LDL (Reaven *et al.* 1991).

The rationale for a number of studies involving fish oil and atherogenesis is based on the observation that the frequency of CVD in populations consuming large quantities of fish (rich in *n*-3 fatty acids) is significantly lower than that seen in comparable populations consuming a diet low in *n*-3 fatty acids (Hirai *et al.* 1982). Data from epidemiological studies have shown a lower CVD mortality in the Mediterranean countries, where the diet is traditionally rich in olive oil, compared with other industrialized countries (Keys *et al.* 1986; Mancini *et al.* 1995). Spain is a Mediterranean country, so we wondered if it would be possible to decrease further the risk of CVD in the Spanish population by increasing the olive-oil as well as fish-oil intake. On the other hand, it has been shown that high-MUFA diets reduce the susceptibility of LDL to oxidative modification but *n*-3 and *n*-6 PUFA increase it (Visioli *et al.* 1995). LDL particles rich in PUFA, presenting more double bonds, are more vulnerable to oxidative attack and, in principle, should be more atherogenic than LDL particles rich in MUFA. However, would the intake of olive oil help to decrease lipid oxidation induced by the intake of *n*-3 PUFA in patients with PVD?

In addition, there is a paucity of information on the effects of different dietary lipids on lipoprotein metabolism in patients with vascular disease because biochemical research on the effect of fish oils rich in *n*-3 PUFA has concentrated mainly on healthy volunteers (Berry *et al.* 1991) and individuals with abnormal lipid concentrations (Radack *et al.* 1990). We hypothesize that eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) from the *n*-3 PUFA capsules could help to lower the risk of thrombosis, a common problem in patients with PVD.

The present study was designed to evaluate the effect of diets enriched in MUFA (olive oil) or MUFA plus *n*-3 PUFA (olive oil plus fish oil) on the plasma lipid profile and susceptibility of LDL to oxidative modification in free-living Spanish male patients with PVD.

Materials and methods

Subjects

The study was performed on thirty-seven male subjects from Granada (Spain) diagnosed with PVD (Fontaine degree II), aged 59.3 (SE 2.3) years, who were recruited from a group of cardiovascular outpatients followed-up by the Department of Vascular Surgery, Clinic Hospital, University of Granada, Spain. The Fontaine degree II is a peripheral arterial obstruction, characterized by intermittent claudication and pain while walking which disappears in a few minutes after stopping, and which is due to 75% stenosis in the larger leg arteries (Bollinger, 1982). All subjects underwent a complete physical examination and medical history. A total of twenty-four patients underwent dietary intervention with different lipid sources: olive oil (O group) and olive oil plus fish oil (OF group); and thirteen patients with no dietary lipid intervention served as a control group (C group). A reference group (R group) of twenty healthy individuals was selected from within the population served by Clinic Hospital. All these subjects were recruited from a local dairy industry.

PVD was identified by measuring blood flow using a Doppler probe and by Yao indices. The Yao index is defined as the ratio of systolic blood pressure in the inferior member to that in the superior one, usually measured at the ankle and arm respectively. In healthy subjects both pressures are markedly similar; however, when leg arteries are partly obliterated due to arteriosclerosis and thrombosis the pressure behind the stenosis site drops. Thus, the ankle pressure is lower than the arm pressure. A value <1 is considered pathological. Resting patients classified as Fontaine stage II have a Yao index of about 0.5–0.7. Yao indices can also be determined by measuring arterial pressures in the forearm and thigh (Bollinger, 1982). Yao indices for the present study were calculated from maximum arterial pressures determined in right and left arm and ankle as well as forearm and thigh. The presence and distribution of atheromatous plaques in both leg and arm were assessed for each subject using scanning duplex, and treadmill running was determined as the longest distance run on a treadmill in 5 min.

Patients with endocrine or metabolic disturbances such as diabetes mellitus, hypothyroidism and obesity, or affected by cardiac episodes such as angina pectoris, previous myocardial infarction and/or electrocardiographic signs of myocardial infarct or some other chronic disease were not included in the study. All participants gave their written informed consent to participate in the study which was approved by the Ethics Committee of Clinic Hospital.

Study design

The study spanned a total of 9 months. A complete clinical study was performed during the first 15 d of the study and in the last 15 d of each 3-month period of the study, by the Department of Vascular Surgery at Clinic Hospital, and a nutritional survey was conducted to assess food habits and lifestyle. C and R groups were examined once half way through the study.

A clinical trial was designed to study the effects of

providing specific lipid supplements to patients suffering from PVD. Designed as a longitudinal intervention study, twenty-four patients were provided with olive oil for 3 months to cook all meals (initial values at 0 months are considered to be baseline values for the O group and values after intake of olive oil for 3 months are reported as 3 month values for the O group), followed by a 3 month wash-out period (baseline levels for the OF group), then supplemented with a fish oil–olive oil combination (3 month values for the OF group) for the final 3 months. The fish oil was administered in powder form as maltodextrin–starch microcapsules. The supplement was supplied in sachets containing 8 g fish oil and a total of 20 g solids. The sachets were reconstituted in approximately 150 ml water or fruit juice for consumption. Each patient had two sachets, equivalent to 16 g fish oil, daily, 8 g after lunch and 8 g after dinner. The sachets were prepared in the pilot plant of the Research and Development Department of Abbott Laboratories, Granada, Spain, following good manufacturing practices. The fatty acid compositions of the olive and fish oils are shown in Table 1.

All subjects and their wives attended a session of dietary counselling for 1 h before starting the study. This nutritional education session was designed to teach the patients basic food composition and characteristics, appropriate serving sizes, the effects of alcohol consumption and recommended cooking techniques. A dietary survey for each of the periods and patients was taken by the research dietitians to estimate the current dietary energy, protein, fat and carbohydrate intakes (Aguilera *et al.* 1996). Patients were recommended to walk 1 km or more daily and to stop smoking. Body weight was recorded every 3 months. On these occasions, compliance with all recommendations (diet, reducing the number of cigarettes per day and exercising) was assessed and problems with the diet were discussed in meetings with the subjects. Compliance with dietary recommendations was determined at the end of each period by a dietitian using food records of measured and weighed food intake

Table 1. Selected fatty acid composition (g/100g total fatty acids) of olive and fish oils used as lipid sources for dietary treatment of free-living male patients with peripheral vascular disease

Fatty acid	Olive oil	Sachets of fish oil*
16:0	6.61	19.9
18:0	2.78	3.4
18:1 <i>n</i> -9	83.10	12.3
18:2 <i>n</i> -6	5.11	1.8
18:3 <i>n</i> -3	0.37	0.1
20:2 <i>n</i> -6	0.63	0.6
20:5 <i>n</i> -3	ND	18.0
22:5 <i>n</i> -3	ND	2.3
22:6 <i>n</i> -3	ND	10.4
Saturated	9.63	26.0
Monounsaturated	83.67	28.0
<i>n</i> -6 PUFA > C ₁₈	6.7	11.0
<i>n</i> -3 PUFA > C ₁₈	ND	35.0

ND, not detected.

* The fish oil was administered as maltodextrin–starch microcapsules. The supplement was supplied in sachets containing 8 g fish oil. Every patient had two sachets (16 g fish oil daily), one after lunch and the other after dinner. Fish-oil intake represented about 2% of the total daily energy.

and evaluation of recipes of all home-made dishes during 1 week.

Once all foods were quantified and coded to introduce different items into a computer program, nutrient values were computed using the program *Alimentacion y Salud (Food and Health)* developed in the Institute of Nutrition of the University of Granada (Mataix *et al.* 1994a). The database used was *Spanish Food Composition Tables* (Mataix *et al.* 1994b).

Blood sampling and biochemical determinations

Blood samples for plasma lipid and lipoprotein-related determinations were taken at the beginning of the study and every 3 months for O and OF groups and once for the R and C groups. Blood (25 ml) was collected by venepuncture into EDTA-containing vacutainer tubes after a 12 h overnight fast. The samples were kept on ice before centrifugation at 1700 g for 15 min at 4° to obtain plasma.

Total plasma cholesterol and triacylglycerol concentrations were measured by enzymic colorimetric methods using commercial kits (CHOD-PAP for cholesterol; Boehringer Mannheim, Mannheim, Germany). Serum HDL-cholesterol was determined in the supernatant fraction after precipitation of the lower-density lipoproteins with phosphotungstic acid and MgCl₂. LDL-cholesterol was calculated according to Friedewald's equation (Friedewald *et al.* 1972).

Lipoprotein isolation

VLDL, LDL and HDL were isolated by a single discontinuous density gradient ultracentrifugation in a vertical rotor (Chung *et al.* 1981). A discontinuous NaCl–KBr density gradient was formed by adjusting the density of the plasma to 1.30 kg/l with KBr and layering normal saline solution (d = 1.006 kg/l) over the adjusted plasma. The isolated LDL was exhaustively dialysed against 150 mM-NaCl, 1 mM-EDTA, pH 7.4 at 4° for 24 h.

LDL protein was measured by Bradford's (1979) method using bovine serum albumin as a standard. Plasma and LDL fatty-acid patterns were determined by GLC as previously described (Lepage & Roy, 1986).

Determination of LDL oxidation susceptibility

Determination of thiobarbituric acid-reactive substances. A total of 200 mg LDL protein/l was oxidized in the presence of Cu²⁺ at levels of 1.25, 2.5, 5, 10 and 20 μmol/l in phosphate-buffered saline for 24 h at 37°. LDL (200 mg LDL protein/l) was also oxidized in the presence of Cu²⁺ 5 μmol/l in PBS for 0, 1, 3, 5, 8 and 24 h at 37°. After the incubation oxidation was stopped by cooling samples to 4° and adding 200 mmol EDTA/l and 40 mmol butylated hydroxytoluene/l (Jialal & Grundy, 1991).

The lipid peroxide content of the oxidized LDL was determined as thiobarbituric acid-reactive substances. Oxidized LDL was combined with 1 ml TCA–thiobarbituric acid–HCl mixture (150 ml/l TCA; 3.7 g/l thiobarbituric acid, 2.5 ml/l HCl) and vortex-mixed, as described by Buege & Aust (1978). The solution was heated for 20 min

in a water bath at 100° to precipitate protein. After cooling, samples were centrifuged at 1700 *g* for 20 min. The supernatant fraction was carefully withdrawn and absorbance was determined at 532 nm against a blank containing all the reagents except LDL. The absorbance was expressed in malondialdehyde equivalents/mg LDL protein, using a standard curve for 1,1,3,3-tetramethoxypropane. Absorbances obtained were plotted against Cu concentrations and lag phase and slope of the corresponding curves were calculated using the Slidewrite Plus software (Sunnyvale, CA, USA).

Oxidized LDL relative electrophoretic mobility. LDL electrophoresis was performed at pH 8.6 in 0.01 M-barbital buffer on 10 g/l agarose gel based on the method of Noble (1968). LDL (6 mg) was applied in each well of a prepared agarose gel. The electrophoretic separation was performed for 145 min at 300 V. Gel was fixed in a solution of ethanol–glacial acetic acid–water (60:10:30, by vol.), and dried. Dried gel was stained for 10–15 min in 5 ml/l Blue Coomassie R-250 in ethanol–glacial acetic acid–water (9:2:9, by vol.). After destaining in a solution of methanol–glacial acetic acid–water (35:25:40, by vol.), the gel was rinsed with water and dried. The change in LDL electrophoretic mobility was expressed relative to the mobility of non-oxidized LDL.

Labelling and macrophage uptake of oxidized LDL. LDL was labelled with 1,1'-dioctadecyl-3,3,3'-tetramethylindocarbocyanine perchlorate (DiI) by the method described by Zouhair & Edna (1993). A stock solution of the fluorescent probe DiI (Molecular Probes, Inc., Eugene, OR, USA) was prepared by dissolving 30 mg DiI in 1 ml dimethyl sulfoxide, and this solution was added to the LDL to yield a final proportion of 300 mg DiI/mg LDL protein. After incubating this mixture for 18 h at 37°, the labelled LDL was isolated by ultracentrifugation (189 000 *g* for 24 h at 4°) dialysed against normal saline and filter-sterilized (0.45 µm). A standard solution of DiI-LDL was prepared in saline to give a concentration range of 100–1600 mg/l. Fluorescence was determined in a Perkin Elmer Model LS-50 (Perkin Elmer, Norwalk, CT, USA) with excitation and emission wavelengths set at 520 and 578 nm respectively.

For the assay of macrophage oxidized-LDL uptake, we used the U-937 macrophage human cell line obtained from the American Tissue Culture Collection (ATCC, Rockville, MD, USA). U-937 cells were maintained in RPMI-1640 containing 100 g/l heat inactivated (56° for 30 min) fetal bovine serum. Cells were plated at 2×10^6 macrophages per well. DiI-LDL (100 g protein/l) was incubated with the U-937 macrophage cell line for 24 h at 37° in CO₂–air (5:95, v/v). After the incubation period, cells and medium were centrifuged at 1700 *g* for 15 min and fluorescence was determined in the supernatant fraction. Cells were added to 1 ml isopropanol and were shaken gently for 2 min. The isopropanol-extracted DiI was then centrifuged at 1700 *g* for 15 min and fluorescence was determined in the supernatant fraction. The percentage of fluorescence relative to the initial value was considered as the macrophage LDL uptake.

LDL tocopherol, retinol and β-carotene determination. A total of 200 mg LDL protein/l was mixed with 250 ml cold methanol containing 100 ml α-DL-tocopherol

acetate solution as internal standard and extracted with hexane (2.5 ml). The hexane phase was dried under N₂ and dissolved in ethanol (50:1, v/v) as described by Frei & Gaziano (1993). The ethanol extract was analysed by reverse-phase-HPLC using methanol–water (99:1, v/v) as the mobile phase and a flow rate of 1 ml/min on a Lichrospher 60 RP-select B column (Merck, Darmstadt, Germany). Under these conditions retinol, β-carotene and α-tocopherol were determined simultaneously.

Plasma α-tocopherol, retinol, and β-carotene were determined by HPLC as described by Thurnham *et al.* (1988). A total of 500 µl plasma was mixed with 250 µl tocopherol acetate solution as internal standard and 100 µl pyrogallol solution as antioxidant. The mixture was extracted with *n*-heptane (700 µl). The *n*-heptane phase was dried under N₂ and reconstituted in methanol (700 µl). The mobile phase, acetonitrile–methanol–chloroform (40:40:20, by vol.) with a flow rate of 1 ml/min, was run on a 250 mm Supersher C18 column (Merck, Darmstadt, Germany).

Statistical analyses

Statistical analyses were performed using the SPSS statistical software package (Statistical Package for the Social Sciences, Salem, OR, USA). Data are presented as means with their standard errors. Normality and homogeneity of the data were confirmed before ANOVA; differences among R, C and dietary groups were assessed by one-way ANOVA followed by *a posteriori* *t*-adjusted Bonferroni tests to analyse specific differences between means.

Results

Body weights and BMI, respectively, for the patients did not differ between the O period (72.5 (SE 4.0) kg and 26.9 (SE 1.1) kg/m²) and the OF period (72.2 (SE 3.9) kg and 26.8 (SE 1.1) kg/m²); values for the C group were also similar (77.4 (SE 2.0) kg and 27.7 (SE 0.8) kg/m²). Moreover, no changes were found over time for patients during the O and OF periods.

Clinical functional measures (Yao and claudicometry) allowed us to separate the healthy group (R group) from the patient groups (C, O and OF). For the R group the Yao index was 1.03 (SE 0.02), which was significantly different from the values for the patient groups ($P < 0.05$) but no major changes in this index were detected among the groups of patients; the mean overall values for the patients was 0.59 (SE 0.06). In the present study, claudicometry was defined as the inability of patients to run at least 300 m on the treadmill in 5 min, so patients from groups C, O and OF ran less than 300 m and healthy subjects from R group ran more than 300 m in 5 min. Thus, significant differences were found between patients and healthy individuals ($P < 0.005$). However, there were no significant differences among the groups of patients: C group 250 (SE 18) m/min; O group 244 (SE 14) m/min; OF group 264 (SE 18) m/min.

Table 2 shows the plasma lipid composition in the study groups. Consumption of olive plus fish oils led to a significantly decreased plasma triacylglycerol level compared with the O, C and R groups; no differences in total cholesterol, LDL-cholesterol or HDL-cholesterol concentrations were

Table 2. Plasma triacylglycerol, total cholesterol, LDL-cholesterol and HDL-cholesterol levels, and plasma fatty acid composition in free-living male patients with peripheral vascular disease after dietary intervention with olive oil or olive oil plus fish oil as the main dietary lipid source for 3 months*

Experimental group† . . .	(Mean values with their standard errors)															
	R (n 20)				C (n 13)				O (n 24)				OF (n 24)			
	Baseline		3 months		Baseline		3 months		Baseline		3 months		Baseline		3 months	
	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM
Plasma																
Triacylglycerols (mmol/l)	1.82 ^b	0.22	1.92 ^b	0.30	1.87 ^b	0.14	1.81 ^b	0.17	1.78 ^b	0.12	1.30 ^a	0.09	1.30 ^a	0.09	1.30 ^a	0.09
Total cholesterol (mmol/l)	5.66 ^a	0.23	5.86 ^{ab}	0.21	6.36 ^b	0.18	6.18 ^{ab}	0.22	6.08 ^{ab}	0.18	6.40 ^b	0.22	6.40 ^b	0.22	6.40 ^b	0.22
LDL-cholesterol (mmol/l)	4.34 ^a	0.21	4.55 ^{ab}	0.23	4.86 ^{ab}	0.16	4.82 ^{ab}	0.19	4.82 ^{ab}	0.20	5.03 ^b	0.21	5.03 ^b	0.21	5.03 ^b	0.21
HDL-cholesterol (mmol/l)	1.30	0.06	1.34	0.08	1.4	0.05	1.38	0.06	1.32	0.05	1.35	0.06	1.35	0.06	1.35	0.06
Plasma fatty acids (g/100 g total fatty acids)																
Total saturated	29.0 ^{ab}	0.3	27.3 ^a	0.7	28.8 ^{ab}	0.3	27.9 ^a	0.5	29.5 ^b	0.5	28.4 ^{ab}	0.6	28.4 ^{ab}	0.6	28.4 ^{ab}	0.6
Total monounsaturated	28.8 ^a	1.0	30.7 ^{ab}	1.0	30.7 ^{ab}	1.0	32.1 ^b	0.7	30.7 ^{ab}	0.8	32.7 ^b	0.8	32.7 ^b	0.8	32.7 ^b	0.8
Total polyunsaturated	42.1	1.3	40.7	1.4	40.2	0.9	40.0	0.8	40.0	0.8	39.3	0.9	39.3	0.9	39.3	0.9
20:5n-3	0.73 ^a	0.06	0.86 ^a	0.20	0.87 ^a	0.07	0.86 ^a	0.08	0.85 ^a	0.10	2.04 ^b	0.06	2.04 ^b	0.06	2.04 ^b	0.06
22:6n-3	2.6 ^a	0.13	2.5 ^a	0.17	2.9 ^a	0.16	2.7 ^a	0.13	2.7 ^a	0.13	3.4 ^b	0.16	3.4 ^b	0.16	3.4 ^b	0.16

^{a,b} Mean values within a row not sharing a common superscript letter were significantly different, $P < 0.05$.

* For details of diets and procedures, see Table 1 and pp. 32–34.

† R, healthy reference group; C, control group consisting of patients with peripheral vascular disease; O, group receiving olive oil; OF, group receiving olive oil plus fish oil.

observed among the groups. The intake of the fish-oil supplement significantly increased plasma *n*-3 PUFA levels, particularly those of EPA (20:5n-3) and DHA (22:6n-3) in the OF group compared with those of the baseline, O and C groups. Patients receiving olive oil registered higher percentages of total plasma MUFA.

After dietary intervention, only *n*-3 PUFA, that is EPA and DHA levels, were elevated in the OF group in comparison with the O, C, and R groups (Table 3). MUFA increased in the O and OF groups. These results paralleled those obtained in total plasma. The LDL α -tocopherol, β -carotene and retinol levels were significantly lower in patients from the C, O and OF groups than those of subjects from the R group. The patients receiving either olive oil or

olive oil and fish oil had higher LDL α -tocopherol concentrations than did the C group; however, no major differences were found for β -carotene and retinol. Furthermore, no significant differences in α -tocopherol were detected between O and OF groups (Table 4).

The lag phase of Cu-mediated LDL oxidation was longer in the OF group than in the O and C groups (Table 5). Moreover, the relative electrophoretic mobility of LDL (Table 5) was lower and the uptake of LDL macrophages was significantly reduced in the OF group as compared with the C group (Fig. 1). The O group exhibited the lowest values for LDL oxidation in the presence of Cu although the lag phase did not increase in this group (Table 5).

Table 3. Fatty acid composition (g/100 g total fatty acids) of plasma LDL in free-living male patients with peripheral vascular disease after dietary intervention with olive oil or olive plus fish oils as the main dietary lipid source for 3 months*

Experimental group† . . .	(Mean values with their standard errors)															
	R (n 20)				C (n 13)				O (n 24)				OF (n 24)			
	Baseline		3 months		Baseline		3 months		Baseline		3 months		Baseline		3 months	
Fatty acid	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM
18:1n-9	19.9 ^{ab}	0.7	21.8 ^{ab}	0.8	19.9 ^a	0.7	22.0 ^b	0.6	20.5 ^{ab}	0.6	21.4 ^{ab}	0.6	21.4 ^{ab}	0.6	21.4 ^{ab}	0.6
18:2n-6	32.0 ^b	1.0	27.9 ^a	0.9	30.5 ^{ab}	0.8	28.5 ^a	0.9	30.0 ^{ab}	0.9	27.9 ^a	0.7	27.9 ^a	0.7	27.9 ^a	0.7
18:3n-3	0.27 ^b	0.03	0.19 ^a	0.01	0.27 ^b	0.02	0.26 ^{ab}	0.02	0.27 ^b	0.02	0.25 ^{ab}	0.01	0.25 ^{ab}	0.01	0.25 ^{ab}	0.01
20:4n-6	6.19 ^{ab}	0.44	6.52 ^{ab}	0.50	7.27 ^b	0.61	6.56 ^{ab}	0.42	7.03 ^{ab}	0.45	6.09 ^a	0.26	6.09 ^a	0.26	6.09 ^a	0.26
20:5n-3	0.70 ^a	0.07	0.72 ^a	0.09	0.95 ^a	0.10	0.64 ^a	0.18	0.96 ^a	0.12	2.18 ^b	0.22	2.18 ^b	0.22	2.18 ^b	0.22
22:6n-3	2.16 ^a	0.16	2.15 ^a	0.13	2.15 ^a	0.13	2.25 ^a	0.13	2.26 ^a	0.14	2.96 ^b	0.14	2.96 ^b	0.14	2.96 ^b	0.14
Total saturated	30.0	0.83	31.0	0.8	28.9	0.3	30.3	0.8	29.2	0.5	29.5	0.4	29.5	0.4	29.5	0.4
Total monounsaturated	25.0 ^a	0.8	27.0 ^{ab}	0.9	25.6 ^a	0.8	29.0 ^b	1.2	26.2 ^a	0.8	27.3 ^{ab}	0.6	27.3 ^{ab}	0.6	27.3 ^{ab}	0.6
Total polyunsaturated	45.3 ^b	1.3	41.4 ^{ab}	1.3	45.4 ^b	0.9	40.4 ^a	1.6	44.5 ^b	0.1	43.1 ^{ab}	0.6	43.1 ^{ab}	0.6	43.1 ^{ab}	0.6

^{a,b} Mean values within a row not sharing a common superscript letter were significantly different, $P < 0.05$.

* For details of diets and procedures, see Table 1 and pp. 32–34.

† R, healthy reference group; C, control group consisting of patients with peripheral vascular disease; O, group receiving olive oil; OF, group receiving olive oil plus fish oil.

Table 4. α -Tocopherol, β -carotene and retinol contents of LDL in free-living male patients with peripheral vascular disease after dietary intervention with olive oil or olive plus fish oils as the main dietary lipid source for 3 months*
(Mean values with their standard errors)

Experimental group† . . .	R (n 20)		C (n 13)		O (3 months)‡ (n 24)		OF (3 months)‡ (n 24)	
	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM
α -Tocopherol (nmol/mg LDL protein)	33.7 ^c	6.6	11.4 ^a	2.1	15.6 ^b	1.9	16.9 ^b	2.4
β -Carotene (nmol/mg LDL protein)	5.1 ^b	1.2	1.9 ^a	0.6	2.5 ^{ab}	0.4	2.8 ^a	0.6
Retinol (nmol/mg LDL protein)	0.71 ^b	0.02	0.21 ^a	0.06	0.25 ^a	0.02	0.22 ^a	0.02

^{a,b,c}Mean values within a row not sharing a common superscript letter were significantly different, $P < 0.05$.

* For details of diets and procedures see Table 1 and pp. 32–34.

† R, healthy reference group; C, control group consisting of patients with peripheral vascular disease; O, group receiving olive oil; OF, group receiving olive oil plus fish oil.

‡ Baseline samples for groups O and OF were lost.

Discussion

Nutritional intervention has been demonstrated to be highly useful in the prevention of CVD. It has been reported that both the quality and quantity of dietary lipids have a direct effect on the development and progression of the atheromatous plaque, modifying its structure and lipid content, which in turn changes its susceptibility to breakage (Gartside & Glueck, 1995).

All patients adhered to the general recommendations but no differences were found for dietary intake among the considered groups. Mean values for the entire group were 11 483 kJ/d total energy, 12.6 % energy from protein, 42.2 % energy from carbohydrates and 38.1 % energy from fat. Fat from the fish-oil capsules was not included in the energy and fat values.

Intakes of vitamin A (retinol plus β -carotene) were the same in the three patient groups (mean overall value 637 μ g/d.

The daily intake of α -tocopherol was significantly higher ($P < 0.05$) in group OF (12.1 (SE 1.2) mg from diet plus 8 mg vitamin E from the fish-oil capsules) than in group C (12.7 (SE 1.2) mg) or group O (12.1 (SE 1.2) mg) because 0.5 g α -tocopherol/kg was added to avoid *n*-3 PUFA oxidation. During the study all patients from groups O and OF tried to decrease the number of cigarettes smoked daily. Overall, 49 % of the patients did not smoke, 38 % of them smoked 2–8 cigarettes/d, and only 13 % smoked one or more packs of cigarettes daily. However, in the control group 46 % did not smoke, 24 % smoked 2–8 cigarettes/d ($P < 0.05$ v. group O and OF), and 30 % smoked more than one pack of cigarettes daily ($P < 0.05$ v. groups O and OF). Moreover, there were no differences in physical activity between patients during the O and OF periods although this group exercised significantly ($P < 0.05$) more than group C. Overall, in the O and OF periods 16.8 % patients walked less than 1 km/d, 27.6 % walked about 1–3 km/d and 55.6 %

Table 5. Cu^{2+} -mediated LDL oxidation in free-living male patients with peripheral vascular disease after dietary intervention with olive oil or olive plus fish oils as the main dietary source for 3 months*
(Mean values with their standard errors)

Experimental group† . . .	R (n 20)		C (n 13)		O (n 24)				OF (n 24)			
					Baseline		3 months		Baseline		3 months	
	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM
24 h time-course Cu^{2+} oxidation slope (nmol TBARS/mg protein per h)	21.4 ^c	2.8	18.1 ^b	2.2	17.1 ^{ab}	2.2	14.8 ^a	1.6	17.3 ^{ab}	2.1	16.1 ^{ab}	2.4
24 h time-course Cu^{2+} oxidation lag phase (min)	66.0 ^a	5.3	60.6 ^{ab}	25.2	65.1 ^{ab}	21.7	64.3 ^{ab}	12.5	71.6 ^{ab}	14.0	82.0 ^b	13.0
0–20 μ mol/l Cu^{2+} oxidation increase (nmol TBARS/mg protein)	25.2 ^c	2.0	16.7 ^b	3.0	16.6 ^b	2.0	10.1 ^a	1.1	14.3 ^{ab}	1.5	15.6 ^b	1.8
LDL oxidized mobility (oxidized: non-oxidized LDL ratio)‡	1.17 ^b	0.005	1.15 ^{ab}	0.012	–	–	1.13 ^a	0.001	–	–	1.12 ^a	0.010

TBARS, thiobarbituric acid-reactive substances.

^{a,b,c}Mean values within a row not sharing a common superscript letter were significantly different, $P < 0.05$.

* For details of diets and procedures see Table 1 and pp. 32–34.

† R, healthy reference group; C, control group consisting of patients with peripheral vascular disease; O, group receiving olive oil; OF, group receiving olive oil plus fish oil.

‡ Baseline samples for groups O and OF were lost.

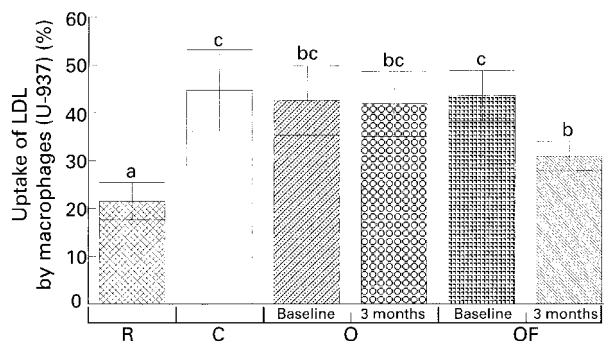


Fig. 1. Uptake by macrophages of oxidized LDL from the plasma of free-living patients with peripheral vascular disease after dietary intervention with olive oil or olive oil plus fish oil as the main dietary lipid source for 3 months. Experimental groups were: R, healthy reference group (n 20); C, control group consisting of patients with peripheral vascular disease (n 13); O, group receiving olive oil (n 24); OF, group receiving olive oil plus fish oil (n 24). Values are means with their standard errors represented by vertical bars. ^{a,b,c} Mean values not sharing a common superscript letter were significantly different, $P < 0.05$. For details of diets and procedures, see Table 1 and pp. 32–34.

walked more than 3 km/d. Of the C group, 38% walked less than 1 km/d, 46% about 1–3 km/d, and only 16% walked more than 3 km/d. We observed that the patients' adherence to the diet, physical activity and smoking behaviour improved in this study, since patients with PVD characteristically smoke abundantly, eat unbalanced diets and do not exercise regularly as shown in group C.

The present study was designed to evaluate the effects of the intake of olive oil and fish oil on the plasma lipoprotein composition of free-living patients with PVD. Although Yao indices did not change with dietary intervention, changes in plasma composition (fatty acids and lipoproteins) suggested a biochemical improvement in these patients as a result of the dietary intervention. A lack of change in the Yao index does not necessarily mean that claudicometry is unchanged. It is generally accepted that a Yao index value closer to 1 parallels the longest run in a treadmill. However, some individuals with low Yao indices, surprisingly, are able to run longer than expected. In those cases subjects usually walk regularly. We believe that claudicometry could change significantly in a lifestyle and nutritional intervention study provided the period of intervention was sufficiently long. The present study was probably of insufficient length to detect statistical differences in claudicometry although patients tended ($P < 0.1$) to run further during the OF period than they did during the O period, and further than the C group.

After considering the results of the present study, we think that a change in the Yao index would require a longer intervention period since vascular stenosis and atheromatous plaques in peripheral members, responsible for low Yao indices, do not regress easily. The daily intake of 16 g fish oil resulted in major changes in plasma and LDL fatty acid patterns characterized by increases in both EPA and DHA. This is in agreement with many studies with normal subjects and patients suffering CVD which have

demonstrated that the intake of n -3 long-chain PUFA leads to rapid alterations of plasma fatty acid profiles with higher concentrations of n -3 and lower concentrations of n -6 PUFA. It has been shown that a daily intake of 5.2 g fish oil for 4 weeks significantly increases EPA and DHA concentrations and decreases the arachidonic acid content of platelet phospholipids and of plasma in patients with vascular disease (Mori *et al.* 1992). Relatively high plasma levels of EPA and DHA are considered beneficial for vascular patients, since the former fatty acid inhibits platelet cyclooxygenase (EC 1.14.99.1) and macrophage lipoxygenase (EC 1.13.11.12), thereby depressing the synthesis of eicosanoids, namely platelet thromboxane A_2 and macrophage leukotriene B_4 , with a parallel increase in the production of prostaglandin I_3 by the artery endothelium, resulting in a delay of plaque development, and thromboxane A_3 and leukotriene B_5 which have a markedly lower platelet aggregant capacity (Flaten *et al.* 1990). Since we observed increased levels of EPA and DHA in our study, we hypothesize that both fatty acids could help to lower the risk of thrombosis, a common problem in patients with PVD.

A higher concentration of long-chain PUFA in plasma and particularly in LDL may contribute to a higher susceptibility of the lipoprotein particles to oxidation. Some studies have shown no effect of n -3 PUFA treatment on *in vitro* and *in vivo* LDL oxidation in human subjects (Bonanome *et al.* 1996) whereas others have shown that fish-oil treatment accelerates LDL oxidation (Suzukawa *et al.* 1995a). It has been reported that n -3 fatty acid-enriched macrophages are able to oxidize LDL, while the antioxidant vitamins (vitamins E and C) inhibit the enhanced capacity to oxidize LDL (Suzukawa *et al.* 1996). In addition, other studies have shown that n -3 fatty acid incorporation into LDL particles renders them more susceptible to oxidation *in vitro* but not necessarily more atherogenic *in vivo* (Whitman *et al.* 1994). However, under our experimental conditions, simultaneous intake of a fish-oil supplement and olive oil did not increase the susceptibility of LDL to oxidation in the presence of Cu^{2+} but increased the lag phase of LDL oxidation in comparison with the other groups. These results seem to indicate that LDL from patients with PVD taking fish oil and olive oil simultaneously are more resistant to LDL oxidation in the presence of transition metals; however once the LDL particle starts to be oxidized, the rate of oxidation is higher than in patients taking olive oil only. These findings are consistent with those reported by other investigators for normal subjects. It has been well documented that tocopherols confer major protection against the development and progression of atherosclerosis by lowering the potential of LDL to oxidation (Eritsland *et al.* 1995; Suzukawa *et al.* 1995b). The natural antioxidants in the unrefined olive oil help to prevent lipid oxidation (i.e. LDL oxidation) thus retarding the formation of atherosclerotic plaques (Visioli *et al.* 1995; Sola *et al.* 1997). In our present study, the α -tocopherol, β -carotene and retinol contents of LDL were higher in O and OF groups than in the C group. Despite the fact that the OF group had a higher intake of vitamin E than the other groups to avoid n -3 PUFA oxidation, the LDL vitamin E concentration was not

significantly different between the O and OF groups. These results suggest that LDL α -tocopherol is oxidized at a higher rate, thus preventing the oxidation of LDL particles with a higher degree of unsaturation. α -Tocopherol has been demonstrated to be the first antioxidant involved in the protection against plasma LDL oxidation (Norman, 1993). The lower susceptibility to oxidation of LDL enriched with *n*-3 PUFA found in our study accounts for the lower uptake of LDL by macrophages as well as the lower LDL relative electrophoretic mobility. Thus, an adequate intake of fish oil with olive oil appears to decrease the oxidation rate of plasma LDL compared with LDL rich in *n*-3 PUFA, and therefore the simultaneous intake of both oils could be useful in the management of vascular disease.

The effects of diets enriched in MUFA on plasma lipids have been contradictory. Many studies have found no effect of MUFA on plasma lipids. Flaten *et al.* (1990) contended that olive oil (rich in MUFA) was not a lipid-lowering agent. Thus, no significant changes were observed in total plasma lipid concentrations after olive-oil ingestion (Green *et al.* 1990). The findings of others suggest that MUFA may have favourable effects on blood lipid concentration as well as on the risk of CHD. Bonanome *et al.* (1992) reported that diets rich in MUFA (olive oil) raise the resistance of plasma LDL to oxidative modification. Visioli *et al.* (1995) found that natural antioxidants (polyphenolic compounds) contained in olive oil inhibited the formation of cytotoxic products such as LDL lipid peroxides, thus retarding the onset of the atherosclerotic damage. Recently, Sola *et al.* (1997) reported that HDL rich in oleic acid was less easily oxidized regardless of the content of antioxidants such as vitamins A and E. Therefore, diets rich in MUFA prevent the oxidative alteration of lipoproteins. In our present study, we found that olive oil intake resulted in the lowest value for LDL oxidation, but the lag phase was not prolonged. Perhaps the antioxidants present in olive oil protected the lipoperoxidation at other stages of the reaction, rather than at the beginning.

The most reproducible effect of dietary fish oil on serum lipids is a decrease in triacylglycerol concentrations both in healthy volunteers and in subjects with vascular disease (Mori *et al.* 1992; Sacks *et al.* 1995). This effect was reproduced in the present study when a decrease in plasma triacylglycerol concentration was observed in patients with CVD following fish-oil treatment.

In conclusion, intake of olive oil together with a dietary supplement of fish oil may be useful in the nutritional management of patients suffering PVD in that it increases plasma *n*-3 long-chain PUFA and decreases the susceptibility of LDL to oxidation.

Acknowledgements

This work was supported by a grant from the CICYT, Ministerio de Educacion y Ciencia, Spain, ALI91-1113-03. We thank the Research and Development Department of Abbott Laboratories, Granada, Spain, for providing the fish-oil supplement, the professional English speaker translator (Davis Nesbitt) who corrected the manuscript and the dietitians (Dr Urbano, Dr López-Jurado and Dr Nestares) for their nutritional support.

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