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ORIGINAL ARTICLE

Effect of n-3 fatty acids on carotid atherosclerosis and haemostasis in patients with combined hyperlipoproteinemia: A double-blind pilot study in primary prevention

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Abstract

Background. Intake of n-3 polyunsaturated fatty acids (n-3 PUFA) either from natural sources or dietary supplementation is inversely associated with atherothrombosis.

Aim. A double-blind pilot study was designed to address the impact of n-3 PUFA on atherosclerosis, haemostasis and vascular status in patients with combined hyperlipoproteinemia.

Methods. Carotid intima-media thickness (C-IMT), texture of intima-media complex (T-IMC), lipids and platelet function were evaluated in 64 patients with combined hyperlipoproteinemia who received placebo or n-3 PUFA (6 g/day) for 2 years. C-IMT and T-IMC were assessed by B-mode ultrasound. Lipids and platelet function were determined by validated methods.

Results. C-IMT increased in placebo, but not in n-3 PUFA group with respect to baseline. In contrast T-IMC decreased in n-3 PUFA, but not in placebo; in both cases, however, treatment effect did not reach statistical significance. A fall of triglycerides, concomitant to a rise of high- and low-density lipoprotein cholesterol (HDL and LDL), was observed in the active treated group. Platelet function was significantly reduced by n-3 PUFA.

Conclusions. Results show a favourable effectiveness of n-3 PUFA on IMT progression and T-IMC that deserves to be confirmed in larger studies. Despite the small sample size, the beneficial effect of n-3 PUFA on platelet function, triglycerides and HDL-C is clearly highlighted.

Key words: *Atherosclerosis, carotid ultrasound, hyperlipidemic patients, imaging, lipoproteins, platelet function*

Introduction

Elevated plasma triglyceride levels affect millions of people in the world. Although associated with an increased risk of coronary artery disease (CAD) (1,2), the contribution of hypertriglyceridemia to the progression of atherosclerosis remains to be defined (3).

Carotid intima-media thickness (C-IMT) is a widely used surrogate index of the atherosclerotic burden. Besides the correlation with the presence of carotid plaque (4), C-IMT is directly associated with the presence and extent of CAD (5) and with the incidence of new vascular events (6,7). C-IMT is also largely accepted to assess the impact of pharmacologic/dietary interventions on atherosclerosis (8).

Although the relationship between C-IMT and conventional risk factors (e.g. hypercholesterolemia, hypertension etc) is well defined (9,10), the one with hypertriglyceridemia is not so clear; elevated triglyceride concentrations are, in fact, often associated with alterations of other lipid variables, mainly high density lipoprotein (HDL) (11,12). Even less information is available on the effects of triglyceride-lowering on IMT progression, particularly in asymptomatic patients.

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Abbreviations

n-3 PUFA	n-3 polyunsaturated fatty acids
C-IMT	carotid intima-media thickness
T-IMC	texture of intima-media complex
CAD	coronary artery disease
EPA	eicosapentaenoic acid
DHA	docosahexaenoic acid
CC	common carotid
Bif	bifurcation
ICA	internal carotid artery
Mean-IMT	mean intima-media thickness of the whole carotid tree
ROI	region of interest
AC_{50}	collagen concentration inducing 50% decrease of optical density in platelet-rich plasma

Key messages

- In patients with combined hyperlipoproteinemia, n-3 polyunsaturated fatty acids (PUFA) exert a beneficial effect on carotid intima-media thickness progression and texture of intima-media complex that, however, deserves to be confirmed in larger scale studies.
- The beneficial effect of n-3 PUFA on platelet function, triglycerides and high-density lipoprotein cholesterol (HDL-C) is clearly highlighted in patients with combined hyperlipoproteinemia.

It is well known that n-3 polyunsaturated fatty acids (n-3 PUFA), from either natural sources or dietary supplementation, have a consistent triglyceride-lowering effect (13); however, no long-term prospective studies on the efficacy of these agents on IMT progression in primary prevention exist (14). Indeed, although two epidemiological cross-sectional studies documented an inverse relationship between the intake of n-3 PUFA from fish and C-IMT (15,16), only one secondary prevention study addressed their effect when supplemented by capsules (17). IMT also correlates with some haemostatic variables, such as platelet aggregation (18), that have been reported to be influenced by n-3 PUFA (19). Finally, n-3 PUFA can reduce the incidence of vascular events by enhancing atherosclerotic plaque stability (20).

A double-blind 2-year single centre pilot study was therefore carried out, in a group of asymptomatic patients with combined hyperlipoproteinemia, to explore the potential effects of n-3 PUFA administration on (i) IMT progression, (ii) texture of intima-media complex (T-IMC), (iii) lipids and haemostasis.

Patients and methods

Patients

Males and post-menopausal females without history of cardiovascular events were selected. Patients aged 45–75 years, with at least one carotid lesion ≥1.3 mm, low density lipoprotein (LDL) cholesterol levels <4.93 mmol/L, triglyceride levels between 1.93 and 4.55 mmol/L and a 10-year cardiovascular risk <20% (according to Framingham algorithm) (21), were eligible. The triglyceride cutpoint of 1.7 mmol/L, suggested by ATPIII guidelines (3) to classify hypertriglyceridemia, was raised to 1.93 mmol/L to increase the probability that the condition of hypertriglyceridemia could be satisfied even after 2 months of prudent diet.

Patients with 'very high' LDL cholesterol levels (>4.93 mmol/L) according to ATPIII guidelines, were excluded for ethical reasons because n-3 PUFA were not expected to exert a positive effect on LDL cholesterol. Patients with 'high' LDL cholesterol (4.14–4.89 mmol/L) were considered as acceptable on condition that their 10-year cardiovascular risk was lower than 20%.

Exclusion criteria were: blood pressure >135/90 mmHg, diabetes, renal insufficiency or other endocrine syndromes, excessive alcohol consumption, heavy cigarette smoking (>10 cigarettes/day), and treatment with lipid-lowering drugs, anti-hypertensives, or hormone replacement therapy. Each patient signed an informed consent prior to randomization. The study was approved by the Ethical Committee of Niguarda Hospital (Milan, Italy).

Intervention

At baseline, patients who satisfied the inclusion criteria received a low fat content prudent diet (26% fats, 22% proteins, 52% carbohydrates; polyunsaturated/saturated fat ratio: 0.43), as recommended by the European Atherosclerosis Society. The presence of hypertriglyceridemia even after dietary intervention was confirmed 2 months later. Patients were thereafter randomized to placebo or n-3 PUFA. An appropriate software was used to obtain two groups balanced for sex, age and smoking. Each n-3 PUFA capsule contained 1 g of fatty acid mixture (19% eicosapentaenoic acid (EPA), 13% docosahexaenoic acid (DHA), 19% palmitic acid, 18% oleic acid, 2%

linoleic acid and 29% other minor components). Six capsules, corresponding to a daily intake of 1.08 g EPA, 0.72 g DHA, 0.01 g tocopherol acetate, were prescribed. The placebo group received 6 g/day olive oil prepared in opaque, identical-looking soft gelatine capsules. Patients were instructed to divide capsules among breakfast, lunch and dinner.

Patients were examined as outpatients at month 0, 3, 6, 12, 18, and 24. Compliance was assessed by counting returned capsules at each visit and by measuring EPA and DHA levels at month 24.

Carotid ultrasound

Carotid ultrasound was carried out at month 0, 6, 12, and 24 by a single operator (author M.A.) using an Esaote AU4 system, equipped with a 10–13 MHz linear array probe and recorded on sVHS videotapes. The far wall of the whole common carotid (CC), of the bifurcation (Bif) and of the first proximal centimetre of the internal carotid artery (ICA) was visualized in anterior, lateral and posterior projections.

Ultrasonic measurements were performed by a single operator (author B.F.) with appropriate software (Eurequa System) (22), which allows the automatic edge detection of echogenic lines limiting the intima-media complex. Progressions of C-IMTs were estimated by assuming a linear trend with time.

The ultrasonic scans performed at months 0 and 24 were repeated within 2 weeks; the mean of the two independent C-IMT determinations was adopted for statistical analyses and to assess reproducibility. The absolute differences (mean $\pm$ SD) between replicate scans at baseline were 0.024 ± 0.019 mm, 0.072 ± 0.060 mm, 0.052 ± 0.045 mm and 0.039 ± 0.034 mm for CC-IMT, Bif-IMT, ICA-IMT and Mean-IMT, respectively. The same absolute differences at month 24 were 0.024 ± 0.017 mm, 0.048 ± 0.033 mm, 0.039 ± 0.033 mm, 0.026 ± 0.019 mm, respectively.

Texture of intima-media complex (T-IMC) by video-densitometric analysis

CC-IMT video-densitometric analysis (23) was performed at months 0 and 24. Briefly, two bi-dimensional optimal images of the far wall of both CC arteries were digitized in longitudinal projection, with a resolution of 576×768 pixels, and 256-degree grey-scale per pixel.

A region of interest (ROI) of 10 mm, including the intima-media complex, was selected 10 mm below the bifurcation. To adjust for different ultrasound attenuation and different gain settings in different subjects, two calibration steps were introduced into the analysis: the grey-scale value of each ROI was adjusted linearly, so that the mean value of blood was 0 and that of adventitia was 256. All recordings were evaluated by a single reader (author M.K.). A computer-driven image analysis system (Medical Imaging Processing) was used to analyse the ROIs by first- and second-order parameters. First-order parameters, reflecting IMT mean grey level, are arithmetic mean and standard deviation. Mean grey levels are positively related to the content of smooth muscle cells (SMCs) and negatively to that of macrophages (23).

Second-order parameters, reflecting IMT heterogeneity, are entropy, contrast and second angular moment. Entropy and contrast correlate positively, whereas second angular moment correlates negatively, with the prevalence of SMCs over macrophages (23).

Plasma lipids

Lipids were measured two months and one week before the start of the study and thereafter at months 3, 6, 12, 18, and 24. Total and HDL cholesterol and triglycerides were determined by enzyme methods in serum, after an overnight fast (24,25). Serum LDL was calculated by Friedewald's formula (26) in samples with triglyceride levels < 4.55 mmol/L (400 mg/dL); otherwise measurement was performed after separation of LDL fraction by ultracentrifugation.

Platelet function

Platelet aggregation was carried out in platelet-rich plasma at months 0, 6, 12, and 24 using collagen (Mascia Brunelli, Milano, Italy) as stimulus. For each patient, a collagen concentration that induces 50% decrease of optical density (AC_{50}) was calculated on the basis of a linear regression between collagen concentrations and corresponding percentages of aggregation (27), measured 5 minutes after collagen addition. Platelet thromboxane B_2 was determined by enzyme-linked immuno-assay (Cayman Chemical, Spi Bio, Massy Cedex France) in platelet-rich plasma samples (3×10^{11} platelets/L) incubated for 5 min (1,000 rpm stirring) with collagen (5 μg/mL).

Plasma fatty acid analysis

Plasma fatty acid analysis was performed as described (28). Briefly, fatty acid methyl esters were prepared by acid trans-methylation from total lipid

extracts and separated by high-pressure liquid chromatography. Peaks were identified using pure reference compounds and quantified by an internal standard (C19:0), added to the lipid extract before methylation. Data are reported as percentages of total fatty acids.

Statistical methods

The ultrasonic variables considered for statistical analyses were the mean IMT of common carotids (CC-IMT), of bifurcations (Bif-IMT), of ICAs (ICA-IMT) and of the whole carotid tree (Mean-IMT). C-IMT progression was estimated by weighted linear regression of up to eight serial measurements versus time; weights were proportional to the number of visualised arterial walls used to compute C-IMTs.

Treatment efficacy was evaluated by comparing the slope of the progression lines IMT/time in placebo and active treated group. Mean C-IMT progressions of the two groups were compared by analysis of covariance (ANCOVA). Progression rates were also weighted proportionally to the inverse of the estimated variance of progression slope. Baseline C-IMTs were used as covariates.

The individual extent of lipid-lowering was calculated as differences between baseline and the mean of all on-trial measurements obtained between months 6 and 24. All statistical tests were two-tailed with a significance level of $P<0.05$. Comparison versus baseline (within-group analysis) was performed by ANOVA completed by Dunnett's test. ANOVA for repeated measures was carried out to compare the two treatment groups over time (between-group analysis). Version 6.12 Windows of SAS software was used to perform statistical analyses.

Results

Treatment

Six hundred and forty-two patients were screened, and 82 matched the inclusion/exclusion criteria. After 2 months of prudent diet, hypertriglyceridemia was confirmed in 64 patients who were therefore randomized for the treatments (32/group). One patient left the study after 3 months because he moved to another city and was therefore excluded from statistical analyses. Two patients were excluded because of major deviation from the protocol during the follow-up (anti-hypertensive assumption) and four because of non-compliance on the basis of returning capsules (compliance <70%). The final analysed group included 57 patients (30 on active treatment).

Table I shows the clinical characteristics of the patients. The two groups were fully comparable; no significant differences were, indeed, observed for any variable considered.

The adherence to treatment was documented by the different frequency distribution of plasma EPA and DHA in the two groups at the end of the study

Table I. Characteristics of the patients at baseline.

	Placebo (n=32)	n-3 PUFA (n=32)
Males (%)	29 (90.6)	29 (90.6)
Age (y)	53.7±6.9	53.7±7.2
Weight (kg)	72.6±9.6	72.1±7.6
Body mass index (kg/m^2)	25.1±2.3	25.0±2.4
Smokers (%)	9 (28.1)	9 (28.1)
No-smokers (%)	9 (28.1)	9 (28.1)
Former smokers (%)	14 (43.8)	14 (43.8)
Systolic blood pressure (mmHg)	129.0±13.1	128.7±13.8
Diastolic blood pressure (mmHg)	76.3±8.3	78.4±7.2
Heart rate (b/min)	67.6±5.7	67.7±6.8
Total cholesterol (mmol/L)	6.47±0.64	6.32±0.53
LDL (mmol/L)	4.0±0.66	3.89±0.58
HDL (mmol/L)	1.05±0.15	1.06±0.24
Triglycerides (mmol/L)	3.12±0.85	3.06±0.77
Platelet aggregation (AC_{50}; μg/mL)	0.37±0.26	0.30±0.17
Platelet thromboxane B_2 (ng/mL)	194.7±68.4	214.3±69.6
CC-IMT (mm)	0.65±0.09	0.67±0.01
Bif-IMT (mm)	0.98±0.28	0.91±0.25
ICA-IMT (mm)	0.76±0.21	0.73±0.17
Mean-IMT (mm)	0.83±0.16	0.79±0.15

Data are reported as means±SD. No difference between groups has been observed by Student's t-test (normally distributed variables) or by Wilcoxon's test. (CC=common carotid; Bif=bifurcations; ICA=internal carotid artery; IMT=intima-media thickness)

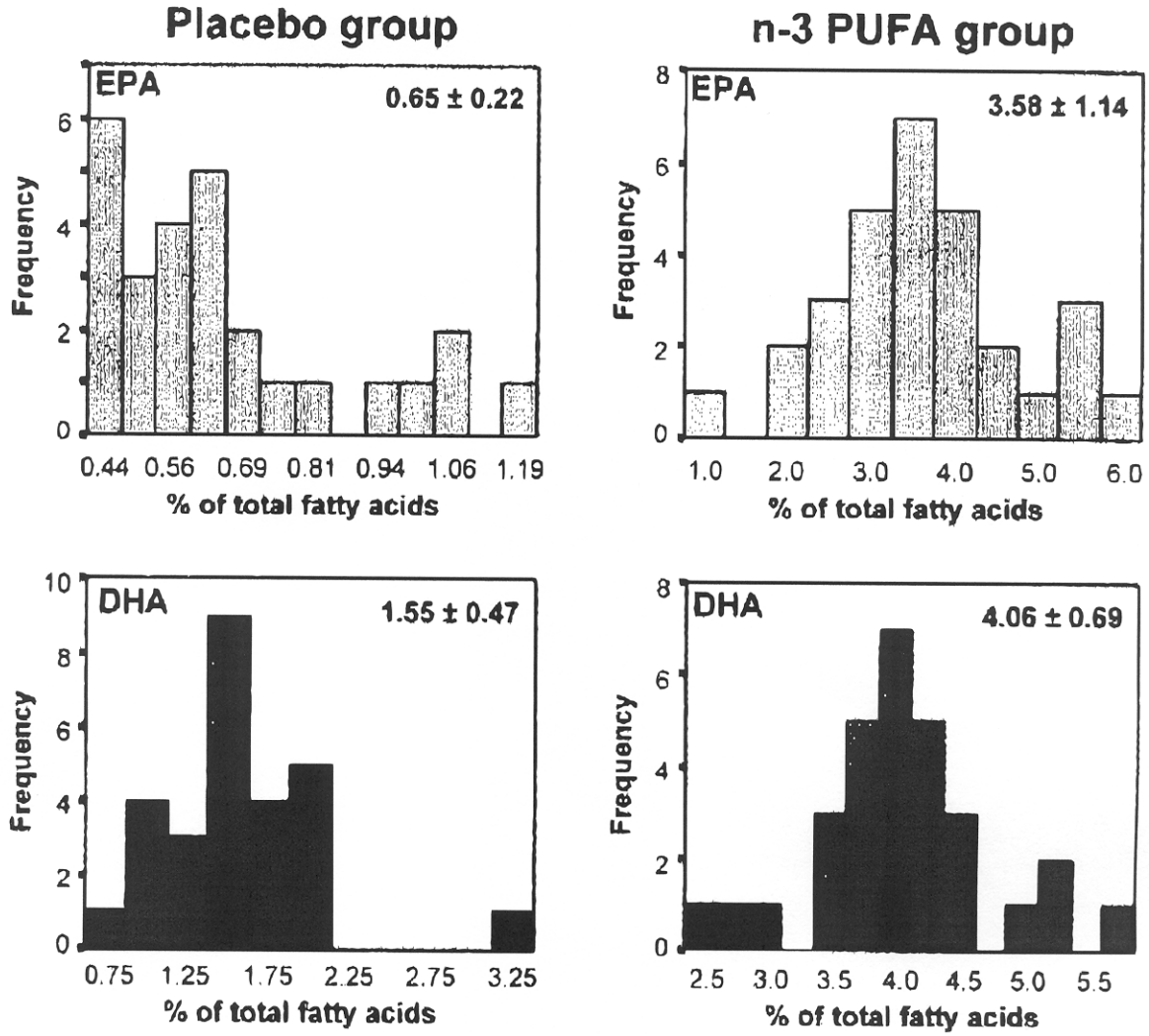

Figure 1. Frequency distribution of plasma eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) after 24 months of treatment.

(Figure 1). In the active treatment group one case of myocardial infarction occurred. Four cases of newly diagnosed hypertension were recorded; two in the n-3 PUFA and two in the placebo treated group. None of the adverse events were considered by physician as attributable to treatment.

A rise in body weight (+3%, $P<0.01$) was observed at the end of the study in both groups. Blood pressure and heart rate were unchanged throughout the study.

Effects on plasma lipids/lipoproteins

A significant reduction of triglycerides, ranging from −20% at the 3rd month to −30% at the 24th month, was observed in the n-3 PUFA-treated group. Triglyceridemia was essentially unchanged until month 18 in the placebo group with a significant increase observed at month 24 (+13.1%; $P<0.05$). n-3 PUFA significantly reduced plasma triglycerides at each time point considered (P values between-group ranging from 0.005 to 0.0001).

The levels of LDL significantly increased in the n-3 PUFA group; the rise approximated 10% ($P=0.002$) and 16.4% ($P=0.005$) after month 3 and 24, respectively. The treatment effect was significant at month 18 and 24 ($P=0.01$ and $P=0.003$, respectively). HDL increased significantly in the active treated group, reaching a 7% rise at month 6 ($P=0.003$) that persisted throughout the study. No change versus baseline was observed in the placebo group. The between-group analysis did

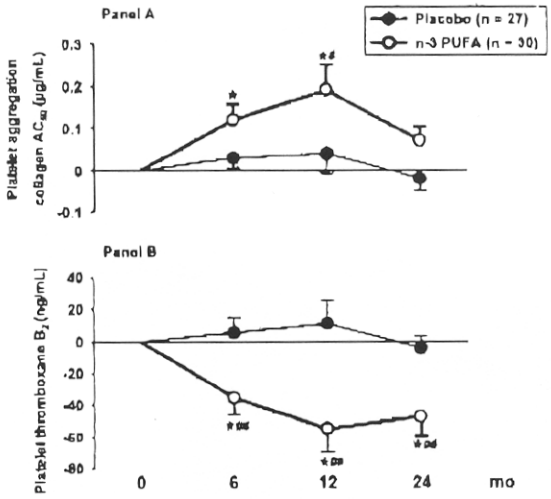

Figure 2. Effects of n-3 polyunsaturated fatty acids (n-3 PUFA) on platelet aggregation (Panel A) and platelet thromboxane B_2 synthesis (Panel B). Data are expressed as changes with respect to baseline. (*$P_{within}<0.01$; #$P_{between}<0.05$, ##$P_{between}<0.01$).

not reach statistical significance at any time point considered.

Effect on platelets

Changes of platelet aggregation compared to baseline are shown in Figure 2 (Panel A). Collagen AC_{50} increased significantly at month 6 and 12 in the n-3 PUFA group. The effect of treatment (between-group analysis) reached statistical significance ($P=0.03$) at month 12.

The effect of n-3 PUFA on platelet aggregation was concomitant with the inhibition of thromboxane B_2 synthesis in platelet-rich plasma (Figure 2, Panel B). As for platelet aggregation, the highest treatment effect was observed at month 12 ($P=0.002$).

Effect on intima-media thickness

All ultrasonic variables increased significantly versus baseline in the placebo group (Table II). In contrast, no IMT progression was observed in n-3 PUFA group, except for CC-IMT. Despite these differences, the effect of n-3 PUFA did not reach statistical significance when the between-group analysis was performed.

Effect on IMT composition

Table III shows the effect of n-3 PUFA on IMT video-densitometric variables. No change versus baseline was observed in the placebo group when mean grey levels (first-order parameters) were considered. Among second-order parameters that reflect IMT heterogeneity, only entropy was significantly reduced. In contrast, patients included in the n-3 PUFA group showed a significant reduction of almost all the video-densitometric variables, with the exception of the second angular moment that increased significantly. The effect of n-3 PUFA treatment did not reach statistical significance when the between-group analysis was performed. Similar

Table II. Intima-media thickness (IMT) progression in n-3 polyunsaturated fatty acids (n-3 PUFA) and placebo groups.

	Placebo (n=27)		n-3 PUFA (n=30)		Treatment effect	
	Slope	P_{within}	Slope	P_{within}	Change	$P_{between}$
CC-IMT (µm/y)	1.4±0.4	0.002	0.9±0.4	0.02	−0.5	ns
Bif-IMT (µm/y)	2.2±0.9	0.02	0.3±0.9	ns	−1.9	ns
ICA-IMT (µm/y)	1.7±0.7	0.05	1.3±0.7	ns	−0.4	ns
Mean-IMT (µm/y)	1.6±0.6	0.01	0.9±0.6	ns	−0.7	ns

Data are reported as means±SEM. Between groups differences are analysed by covariance analysis. (CC=common carotid; Bif=bifurcation; ICA=internal carotid artery; P_{within}=statistical significance versus the baseline values; $P_{between}$=statistical significance between the two groups evaluated after data adjustment for baseline.)

Table III. Effect of n-3 polyunsaturated fatty acids (n-3 PUFA) on ultrasound video-densitometry of right common carotid intima-media thickness (CC-IMT) segments.

	Placebo (n=27)		n-3 PUFA (n=30)		Treatment effect		
	Differences between 24th month and baseline	P_{within}	Differences between 24th month and baseline	P_{within}	Change	$P_{between}$	$P_{between}$[a]
First-order parameters (IMT mean grey level)							
Arithmetic mean	2.65±4.31	0.544	−6.80±4.95	0.180	−9.45	0.111	0.105
Std deviation	−2.19±2.05	0.293	−6.87±2.24	0.005	−4.68	0.092	0.319
Second-order parameters (IMT heterogeneity)							
Entropy	−0.34±0.20	0.038	−0.34±0.11	0.004	0.00	0.668	0.420
Contrast	−76.9±178	0.668	−480±238	0.050	403	0.131	0.603
Second angular moment (10^{-2})	−0.03±0.07	0.068	0.03±0.01	0.011	0.06	0.508	0.302

Data are reported as means ± SEM. The treatment effect was analysed with analysis of covariance. (P_{within}=statistical significance versus the baseline values; $P_{between}$=treatment effect.) [a]Significances evaluated after data adjustment for baseline values.

results were obtained when an analogue segment was analysed on the left CC (data not shown).

Discussion

Firstly, results from this study show that carotid IMT progression in patients with combined hyperlipoproteinemia can be appreciated as significant even after a relatively short period of follow-up (2 years), notwithstanding IMT changes are smaller than those observed in patients with other vascular risk factors (10). In addition, despite the lack of significance of the between-group analysis, likely due to the small sample size, the within-group analysis clearly shows that IMT progression is blunted in the n-3 PUFA group but not in placebo. These results encourage the design of a larger trial to confirm the potential favourable effectiveness of these agents on IMT progression observed in this pilot study.

Only few studies have so far addressed the effects of n-3 PUFA on carotid arteries. Results from a longitudinal (24 months' duration) placebo-controlled study in CAD patients, supplemented with a dose of n-3 PUFA similar to that adopted in our study, did not show any change in IMT progression (17). Of interest, an inverse relationship between fish consumption and carotid atherosclerosis has been reported by two Japanese epidemiological cross-sectional studies (15,16). Besides the different nature of the studies (prospective versus cross-sectional), it can be hypothesized that this discrepancy can be explained by a more efficient incorporation of n-3 PUFA in plasma lipids when ingested as fish rather than as capsules (29) or by a different efficacy of n-3 PUFA on early or advanced carotid atherosclerosis.

Our data from IMT video-densitometry may provide an additional explanation for this controversy. It has been reported that mean grey levels are positively related to the content of smooth muscle cells (SMCs) and negatively to that of macrophages, and that the prevalence of SMCs over macrophages correlates positively with entropy and contrast and negatively with the second angular moment (23). On this basis, our results suggest that, if an effect of n-3 PUFA on IMT exists, this is directed toward a reduction of SMC content rather than a reduction of macrophages. Although these results have to be interpreted with caution in consideration of the relatively small scale of the study, they may provide a further explanation for the positive effect of n-3 PUFA observed in population-based studies but not in CAD patients. Indeed, since ultrasound techniques do not distinguish between media and intima, an effect of n-3 PUFA on IMT in terms of reduction of SMCs can be more easily appreciated when evaluated in patients with early atherosclerosis in which the media contributes to IMT measurements much more than the intima (30). In contrast, in CAD patients, in whom the contribution of intima to IMT measurement is higher, this effect could be less appreciable. Of interest, a beneficial effect of EPA on the composition of atherosclerotic lesions has been reported in an animal model of the twice-injured carotid artery (31).

A clear-cut effect of n-3 PUFA emerges when coronary atherosclerosis progression and/or incidence of cardiovascular events are considered (32–35). The effect has been attributed to a favourable activity on lipid profile (36,37), to an anti-arrhythmic effect (38) and also to an anti-thrombotic potential (19). As already reported (39),

results from our study confirm that n-3 PUFA exert an antiplatelet effect both in terms of inhibition of platelet aggregation and thromboxane synthesis.

Of interest, our data show that at least one year of treatment is required to reach the maximal effect on platelet function. A trend toward baseline values for platelet aggregation was observed at month 24, concomitantly with the maximal increase of LDL, a finding explainable by the pro-aggregatory effect of LDL observed *in vitro* (40) and *ex vivo* (27,41).

The role of hypertriglyceridemia as a predictor of carotid atherosclerosis is also controversial. Several cross-sectional studies have identified a correlation between postprandial triglyceridemia and C-IMT (42). Less information is, however, available on the role of fasting triglyceridemia on C-IMT and even less on C-IMT progression. As expected (36), a reduction of triglycerides, accompanied by a significant increase of HDL and LDL in the active treated group, was observed also in our study. The increase of LDL was, however, even higher than that reported by others (34,35,43). In spite of this, IMT progression was clearly reduced in the n-3 PUFA group but not in placebo, suggesting that the rise of LDL, a well known determinant of IMT (44), does not completely obscure the effects of n-3 PUFA. A possible explanation is that the increase of LDL induced by n-3 PUFA is mainly due to a rise of LDL bigger in size, known to be less atherogenic (45). In addition, also the concomitant rise of HDL may have contributed to the inhibition of IMT progression observed in the n-3 PUFA group (46).

Taken together, our results may give grounds for the reported protection of n-3 PUFA against vascular events, and indicate that, in patients with combined hyperlipoproteinemia, the antiplatelet effect emerges more clearly than the anti-atherosclerotic one.

Results on the effect of n-3 PUFA on IMT progression herein described strongly encourage an adequately dimensioned primary prevention trial in hypertriglyceridemic patients, or in patients with combined hyperlipoproteinemia, to be performed in order to strengthen not only the potential benefit of n-3 PUFA on IMT progression but also their effect on SMC content as one of the mechanisms responsible for the reduction of IMT progression.

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