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## n-3 Fatty Acid Ethyl Ester Administration to Healthy Subjects and to Hypertriglyceridemic Patients Reduces Tissue Factor Activity in Adherent Monocytes

Elena Tremoli, Sonia Eligini, Susanna Colli, Paola Maderna, Patrizia Risè, Franco Pazzucconi, Franca Marangoni, Cesare R. Sirtori, Claudio Galli

**Abstract** n-3 Fatty acids are known to influence several functions of monocytes, including adhesion, cytokine synthesis, and superoxide generation. Monocytes express tissue factor, a membrane-bound glycoprotein, that acts as a catalyst in the coagulation cascade. In this study we evaluated the effects of administration of n-3 fatty acid ethyl esters to healthy volunteers and to hypertriglyceridemic patients on tissue factor activity (TF activity) in adherent monocytes. n-3 Fatty acids containing 75% eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) (ratio of EPA to DHA, 1.34) were administered (3 g/d) to normal volunteers for 18 weeks. In addition, the effects of this treatment were evaluated in 30 hypertriglyceridemic patients for 24 weeks by using a double-blind, placebo-controlled study. TF activity in adherent monocytes was evaluated with a one-stage clotting assay. Plasma and monocyte fatty acid compositions were determined by gas-liquid chromatography. In healthy volunteers, n-3 fatty acids significantly reduced TF activity in adherent monocytes either

in the unstimulated condition or after exposure to endotoxin. The inhibitory effect was observed after 12 weeks of treatment and was more pronounced after 18 weeks (>70%,  $P < .001$  versus baseline). Concomitantly, levels of EPA and DHA increased in plasma and monocyte lipids. Interestingly, after stopping treatment, monocyte TF activity remained inhibited for at least 14 weeks. Treatment with n-3 fatty acids for 24 weeks also resulted in a significant reduction of TF activity in adherent monocytes from hypertriglyceridemic patients (-31% and -40% in unstimulated and endotoxin-stimulated cells;  $P < .05$  versus baseline). The described effect of n-3 fatty acids on TF activity in monocytes from healthy subjects and hypertriglyceridemic patients represents a novel mechanism by which these fatty acids may exert their antithrombotic activity. (*Arterioscler Thromb.* 1994;14:1600-1608.)

**Key Words** • eicosapentaenoic acid • docosahexaenoic acid • procoagulant activity • thrombosis

**E**pidemiological and interventional studies suggest that consumption of fish or fish oil containing elevated levels of n-3 polyunsaturated fatty acids confers protection against ischemic heart disease.<sup>1,2</sup> In addition, it has been shown that n-3 fatty acids exert beneficial effects in humans and animals with atherosclerotic disease.<sup>3,4</sup> Some of these effects may be attributed to the activity of n-3 fatty acids on platelet activation, as well as on changes in the behavior of cells of the vessel wall.<sup>5,6</sup> Moreover, n-3 fatty acids inhibit several functions of monocytes, ie, adhesion,<sup>7</sup> chemotaxis,<sup>8</sup> cytokine synthesis,<sup>9</sup> leukotriene generation,<sup>10</sup> and expression of mRNA for platelet-derived growth factor.<sup>11</sup>

Tissue factor (TF) is a membrane-bound glycoprotein that functions in the extrinsic pathway of blood coagulation by acting as a cofactor for factor VII.<sup>12,13</sup> TF binds the plasma serine protease, factor VIIa; the resulting factor VIIa-TF complex acts as a catalyst for conversion of factors IX to IXa and X to Xa, leading to the

formation of thrombin.<sup>14</sup> TF activity has been identified in several different tissues and, among blood cells, in monocytes.<sup>15</sup> Monocyte TF activity has been shown to be increased in various animal models after endotoxin shock.<sup>16,17</sup> In addition, elevated levels of TF activity have been reported during extracorporeal circulation in patients undergoing aortocoronary bypass,<sup>18</sup> possibly due to monocyte activation consequent to surgical maneuvers and during the outburst of unstable angina, as a result of lymphocyte activation from yet-undiscovered factor(s).<sup>19</sup> Moreover, TF has been found in the matrix of necrotic cores of atherosclerotic plaques.<sup>20</sup>

Factor VII coagulant has been described to correlate with the risk of future coronary events, and serum triglycerides have been shown to be associated with factor VII coagulant levels.<sup>21,22</sup> Factor VII circulates in the plasma as an inert precursor (zymogen) that, during clotting, is converted to the activated form (factor VIIa) by cleavage of a single peptide bond. Factor VIIa is inactive unless it binds to its essential cofactor, TF, and it has been proposed that low levels of factor VIIa serve as a primer, through the formation of a binary complex with TF, in initiating the clotting cascade.<sup>23</sup>

Recently it has been demonstrated that dietary administration of fish oil to nonhuman primates impairs TF expression by endotoxin-stimulated mononuclear cells.<sup>24</sup> In this study we demonstrate that supplementa-

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tion with n-3 fatty acids enriched in eicosapentaenoic (EPA) and docosahexaenoic (DHA) acids to healthy volunteers and to hypertriglyceridemic patients reduces TF activity generation in adherent monocytes in the unstimulated condition as well as after stimulation with endotoxin.

## Methods

### Materials

Plastic culture dishes were from Costar; phosphate-buffered saline (PBS) and RPMI-1640 were from Flow; bovine serum albumin was from Sigma Chemical Co; Ficoll-Paque was from Pharmacia; penicillin, streptomycin, and amphotericin B (Fungizone) were from GIBCO; endotoxin W from *Escherichia coli* 0111:B4 was from DIFCO; methanolic HCl and gas-liquid chromatography columns were from Supelco; human placental thromboplastin was from Behring; and monoclonal antibody against TF activity was from American Diagnostica.

The capsules of n-3 fatty acids (1 g each) containing 75% EPA plus DHA (430 mg EPA and 320 mg DHA; EPA to DHA ratio, 1.34) and 25% other n=3 fatty acids and the olive oil capsules (1 g each of olive oil, containing 78% oleic acid) were from Pharmacia-Farmitalia Carlo Erba srl.

### Subjects and Patients

A preliminary study was carried out in eight healthy volunteers (4 men and 4 women; age range, 23 to 39 years) selected from the staff attending the E. Grossi Paoletti Center for the Study of Metabolic Diseases (Niguarda Hospital, Milan, Italy). The subjects were disease-free and refrained from taking any medication during the course of the study. After the preliminary study was completed, a double-blind, placebo-controlled study was carried out in hypertriglyceridemic patients. Thirty hypertriglyceridemic patients were selected at the E. Grossi Paoletti Center. Patient selection was based on diagnosis of type IV hyperlipoproteinemia according to World Health Organization (WHO) criteria.<sup>25</sup> None of the patients had clinical signs of atherosclerotic disease, nor were they hypertensive or diabetic. Mean plasma triglyceride level was  $3.72 \pm 0.42$  and  $4.20 \pm 0.45$  mmol/L in patients assigned to n-3 fatty acid and olive oil treatments, respectively. Mean total cholesterol levels were  $6.26 \pm 0.24$  and  $5.78 \pm 0.27$  mmol/L, respectively, for the two groups. None of the patients was taking hormonal therapies or other drugs known to interfere with lipid or coagulation parameters.

During these studies healthy subjects and patients were asked to continue their usual diet and to refrain from consuming foods rich in n-3 fatty acids, eg, salmon, herring, and tuna fish. The diet of healthy subjects contained 40% fat: 14% saturated, 22% monounsaturated, and 3% to 4% polyunsaturated, with a polyunsaturated to saturated fat ratio of 0.3. From at least 1 month before the beginning of the study, the patients were on a low lipid/carbohydrate diet according to established guidelines in Italy: 40% fat, with a polyunsaturated to saturated fat ratio >1.8, 38% to 40% carbohydrate, and 20% protein.<sup>26</sup> From the actual lipid contents and fatty acid composition of the diets, it was calculated that daily consumption of EPA and DHA by patients and control subjects was <50 mg (DHA >> EPA).

The studies were performed according to the Declaration of Helsinki. The project was approved by the Ethics Committee of the E. Grossi Paoletti Center. All participants were carefully informed of the end points of the study. Informed consent was obtained from all subjects entering the study.

### Study Design

In the preliminary study, healthy subjects were administered 3 g/d n-3 fatty acids (three capsules, 1 g each) for 18 weeks. Blood sampling was performed at weeks 0 (baseline), 6, 12,

and 18 during treatment and 14 weeks after treatment withdrawal. At these times plasma lipids, monocyte TF activity, and plasma and monocyte fatty acid composition were determined. At week 14 after treatment withdrawal, TF activity was also determined in monocytes isolated from a group (n=6) of normal donors not exposed to treatment with n-3 fatty acids and who were matched for age and sex to the study subjects.

After the preliminary study was completed, a double-blind, placebo-controlled study was carried out in hypertriglyceridemic patients. Hypertriglyceridemic patients were randomly assigned to receive either 3 g/d n-3 fatty acids or 3 g/d olive oil for 24 weeks. Blood was drawn at time 0 (baseline) and at 12, 18, and 24 weeks after starting the treatments for determination of plasma fatty acid composition and monocyte TF activity. Before and at the end of treatment, hematocrit; erythrocyte, leukocyte, and platelet counts; and hepatic and renal function tests were performed.

### Monocyte Isolation and Stimulation

All procedures for monocyte isolation were performed under sterile conditions. Blood, anticoagulated with 3.8% sodium citrate (9:1, vol/vol), was centrifuged at 150g for 18 minutes to obtain platelet-rich plasma, which was discarded. The residue was processed for mononuclear cell isolation by adding an equal volume of PBS containing 0.5% bovine serum albumin and 0.1% glucose. Mononuclear cells were separated by centrifugation on Ficoll-Paque. For monocyte purification, mononuclear cells were washed twice with PBS containing 5 mmol/L EDTA to eliminate platelet contamination and once with PBS containing 0.5% bovine serum albumin. Cells were then resuspended in RPMI-1640 plus 50 U/mL penicillin, 50  $\mu$ g/mL streptomycin, 0.125  $\mu$ g/mL amphotericin B (Fungizone), and 10% autologous serum. Cells were placed in 16-mm multiwell tissue-culture plates at  $2 \times 10^6$  cells per well and kept at 37°C in a 5% CO<sub>2</sub> humid atmosphere for 2 hours. Supernatants containing nonadherent cells were removed, and adherent monocytes were washed twice with prewarmed RPMI-1640. The purity of monocyte preparations was >90%, as defined by cytochemical reactivity for  $\alpha$ -naphthyl acetate esterase.<sup>27</sup> Cell viability was determined by trypan blue dye exclusion. For fatty acid analyses, adherent monocytes were gently scraped off and subjected to osmotic lysis with distilled water, and the final pellets were stored at -80°C until assayed. For TF activity determination, adherent monocytes were incubated for 4 hours at 37°C in a 5% CO<sub>2</sub> humid atmosphere in the absence or presence of 10  $\mu$ g/mL endotoxin. At the end of incubation, cells were scraped off and subjected to three cycles of freezing and thawing. Each well was then examined by microscopy to ensure that most of the cells had been scraped from the plates. Monocyte TF activity was determined in cell extracts as described below.

In preliminary experiments, adherent cells were detached from wells by incubation with PBS containing 5 mmol/L EDTA (pH 7.4) and counted by phase-contrast microscopy, and cell protein content was evaluated in the same samples with the Bio-Rad protein assay.<sup>28</sup> A linear relation between cell count and protein concentration in cell extracts was found ( $r=1$ ). Therefore, to minimize intrasubject and intersubject variations in the number of adherent cells, monocyte TF activity was determined in cell extracts and expressed as units of TF activity per microgram of protein.

### Plasma Lipids

Serum total cholesterol and triglycerides were determined by enzymatic methods.<sup>29,30</sup> Lipid determinations were standardized within the WHO Quality Control Program, the coefficients of variation for total cholesterol and triglycerides being 1.5% and 2.3%, respectively.

### Fatty Acid Analyses

For measurement of plasma fatty acids, blood was collected in EDTA (1 mg/mL of blood) and centrifuged for 20 minutes at 800g. The plasma was removed and frozen at  $-80^{\circ}\text{C}$  until analyzed. Lipids were extracted from plasma by the stepwise addition (to 1 volume of plasma) of 2 volumes distilled water, 4 mL methanol, and 8 mL chloroform containing 5  $\mu\text{g/mL}$  of the antioxidant butylated hydroxytoluene. After phase separation at low temperature, the aqueous phase was collected and, after solvent evaporation, redissolved in chloroform/methanol, 2:1, vol/vol. Lipids from monocyte membranes were extracted with chloroform/methanol (2:1, vol/vol) containing 5  $\mu\text{g/mL}$  butylated hydroxytoluene.<sup>31,32</sup> The contents of extracts were determined from aliquots by microgravimetry after solvent evaporation. Total phospholipids were isolated from lipid extracts by thin-layer chromatography with hexane/diethyl ether/acetic acid, 80:20:1, vol/vol/vol, as developing agents. The zones containing total phospholipids were scraped off. Fatty acid methyl esters were prepared by transmethylation with methanolic HCl. The methyl esters were separated by gas-liquid chromatography on capillary columns (Supelcowax 10: fused silica, 30 m, 0.32-mm internal diameter, 0.25-mm film thickness) at a programmed temperature ( $140^{\circ}\text{C}$  to  $210^{\circ}\text{C}$  in  $2.5^{\circ}\text{C}/\text{min}$  increments). Quantification of fatty acids was carried out by the use of the internal standard nonadecane and calibration curves obtained with reference compounds.

### TF Activity Determination

Total cellular content of TF activity was determined by a one-stage clotting assay on disrupted monocytes.<sup>33</sup> The assay mixture contained 0.1 mL cell lysates; 0.1 mL citrated, pooled, normal plasma; and 0.1 mL of 25 mmol/L  $\text{CaCl}_2$ . Clotting times were determined at  $37^{\circ}\text{C}$  and results were expressed in arbitrary units by comparison with a standard curve of clotting times, which were obtained by serial dilutions of a preparation of human placental thromboplastin to which an arbitrary value of 2000 U was assigned. Assays performed with plasma from donors who were congenitally deficient in factor VII consistently demonstrated no TF activity. TF activity was expressed as units per microgram of protein. The specificity of this assay for TF activity was shown by preincubation of cells with an inhibitory monoclonal antibody against TF that blocked the activity by  $>90\%$ .

### Statistical Analysis

The results are presented as mean  $\pm$  SEM. Two-way ANOVA for paired data was used to evaluate the effect of n-3 fatty acids in healthy subjects. Two-way ANOVA with a two-factor mixed design (split-plot design) was used to compare treatment conditions in hypertriglyceridemic patients. Statistical significance was analyzed by Tukey's test for multiple comparisons; probability values greater than 0.05 were not considered significant.

### Results

All subjects (healthy volunteers and hypertriglyceridemic patients) completed the study without major adverse effects, with the exception of one hypertriglyceridemic patient who left the study because of gastrointestinal disturbances. In both volunteers and patients, body weight as well as other indices, eg, pulse and blood pressure, did not change during the study. In addition, there were no alterations in major hematologic and biochemical parameters with the exception of plasma lipids.

Treatment with n-3 fatty acids reduced triglyceride levels in healthy subjects (from  $0.88 \pm 0.10$  mmol/L at baseline to  $0.69 \pm 0.11$  mmol/L after 18 weeks of treat-

ment; NS) and in hypertriglyceridemic patients (from  $3.72 \pm 0.42$  mmol/L at baseline to  $2.2 \pm 0.13$  and  $2.5 \pm 0.12$  mmol/L at 18 and 24 weeks of treatment, respectively;  $P < .01$ ). No significant change in triglyceride levels was observed in the olive oil group during treatment ( $4.20 \pm 0.45$  and  $3.90 \pm 0.29$  mmol/L at baseline and at week 24, respectively; NS). No changes in plasma cholesterol levels were recorded in normal subjects during treatment ( $4.87 \pm 0.10$  and  $4.68 \pm 0.16$  mmol/L at baseline and after 18 weeks of n-3 fatty acids, respectively; NS). In hypertriglyceridemic patients, plasma cholesterol levels tended to increase during the study (from  $5.77 \pm 0.27$  mmol/L at baseline to  $6.29 \pm 0.29$  mmol/L after 24 weeks for the placebo group [ $P < .01$ ] and from  $6.27 \pm 0.25$  mmol/L at baseline to  $6.70 \pm 0.24$  mmol/L after 24 weeks for the n-3 fatty acid group [ $P < .05$ ]). At 24 weeks, however, no significant difference was observed in the two treatment groups.

### Preliminary Study: Effects of n-3 Fatty Acid Administration to Healthy Volunteers

In this study, TF activity was determined in adherent monocytes incubated for 4 hours with medium alone or with medium containing 10  $\mu\text{g/mL}$  endotoxin. TF activity, as determined in freshly isolated mononuclear cells of healthy subjects, was below the detection limit of the assay (clotting times  $>7$  minutes). As has already been observed by others,<sup>34,35</sup> appreciable amounts of TF activity are detectable after adherence of monocytes to tissue-culture plates. A further increase in TF activity was observed after a 4-hour incubation of adherent cells with medium alone at  $37^{\circ}\text{C}$  (5%  $\text{CO}_2$  and a humid atmosphere).

Before treatment, TF activity as determined in monocytes incubated with medium alone was  $7.0 \pm 1.1$  U/ $\mu\text{g}$  protein (range, 4.0 to 13.9 U/ $\mu\text{g}$  protein,  $n=8$ ). Incubation of adherent monocytes with 10  $\mu\text{g/mL}$  endotoxin induced an almost 100% increase in monocyte TF activity compared with that in the unstimulated condition ( $13.1 \pm 1.8$  U/ $\mu\text{g}$  protein; range, 8.7 to 23.2 U/ $\mu\text{g}$  protein,  $n=8$ ).

Levels of TF activity in unstimulated, adherent monocytes at baseline and during n-3 fatty acid treatment are presented in Fig 1A. Six-week treatment with n-3 fatty acids did not influence TF activity in unstimulated cells, whereas after 12 weeks, a dramatic reduction in TF activity was observed. At this time, unstimulated monocytes formed  $2.7 \pm 0.6$  U TF activity per microgram of protein (range, 0.7 to 4.8 U/ $\mu\text{g}$  protein;  $P < .001$  versus baseline and 6 weeks). A further reduction of monocyte TF activity compared with values obtained at 12 weeks was observed after 18 weeks of treatment (mean levels of TF activity were  $2.0 \pm 0.12$  U/ $\mu\text{g}$  protein; range, 1.3 to 2.3 U/ $\mu\text{g}$  protein;  $P < .001$  versus baseline and 6 weeks).

n-3 Fatty acids influenced TF activity in endotoxin-stimulated monocytes (Fig 1B) in a fashion comparable to that observed in unstimulated cells. Six-week treatment failed to influence TF activity in endotoxin-stimulated cells, and inhibition of TF activity compared with baseline values was observed only after 12 and 18 weeks of treatment with n-3 fatty acids. TF activity levels in endotoxin-stimulated monocytes at 12 and 18 weeks averaged  $5.0 \pm 0.4$  and  $2.6 \pm 0.2$  U/ $\mu\text{g}$  protein, respectively ( $P < .001$  versus baseline and 6 weeks). After 18

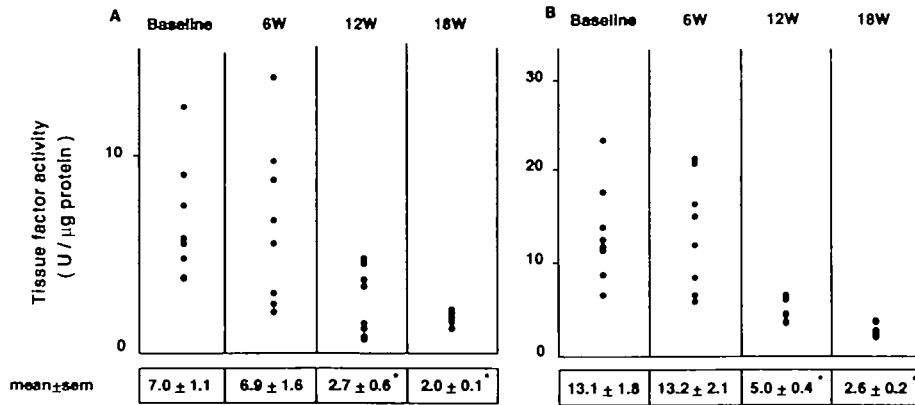


Fig 1. Bar graphs of tissue factor activity in monocytes isolated from healthy subjects during treatment with n-3 fatty acid ethyl esters (3 g/d) for 18 weeks. Each point represents the value for each subject at each time. Values at 12 and 18 weeks (W) were significantly (\*) different ( $P < .001$ ) from values at baseline and after 6 weeks of treatment in both unstimulated (A) and endotoxin-stimulated (B) cells. Mean  $\pm$  SEM values of tissue factor activity are also shown at each time.

weeks of treatment, not only were the absolute amounts of TF activity in both unstimulated and stimulated cells significantly reduced but the percent of stimulation induced by endotoxin was also reduced (30% only versus 100% observed at baseline).

Mean levels of fatty acids in the plasma of healthy subjects at baseline and during treatment are presented in Table 1. During treatment the major changes in plasma fatty acids were related to n-3 polyunsaturates (20:5, 22:5, and 22:6). EPA (20:5 n-3) and DHA (22:6 n-3) accumulated in plasma lipids after 6 weeks, with maximal accumulation occurring after 18 weeks of treatment. So far as n-6 fatty acids are concerned, levels of arachidonic acid (20:4 n-6) in plasma decreased only slightly during treatment.

Changes in monocyte fatty acid composition essentially reflected those observed in plasma (Table 2), with marked increases in n-3 fatty acids and a slight reduc-

tion in n-6 fatty acids, a change confined to arachidonic acid. As a consequence of changes in plasma and monocyte fatty acids, the 20:4 n-6 to 20:5 n-3 ratio decreased significantly (Tables 1 and 2).

At 14 weeks after treatment withdrawal, TF activity was simultaneously evaluated in eight subjects who completed the study and in six normal donors who had not been exposed to treatment with n-3 fatty acids. TF activity levels in unstimulated and endotoxin-stimulated monocytes from untreated donors were  $8.5 \pm 2.0$  and  $13.1 \pm 1.9$  U/ $\mu$ g protein, respectively ( $n=6$ ). These values are fully comparable with those determined at baseline in monocytes from healthy volunteers who completed treatment with n-3 fatty acids. In the latter group, in contrast, TF activity levels measured 14 weeks after treatment withdrawal were still significantly lower than at baseline (mean levels of TF activity were  $2.0 \pm 0.5$  and  $4.9 \pm 0.8$  U/ $\mu$ g protein for unstimulated and endotoxin-stimulated

TABLE 1. Fatty Acid Composition of Plasma of Healthy Subjects Before, During, and After n-3 Fatty Acid Administration

Fatty Acids*	Weeks of Study				After 14 Weeks of Washout
	0 (Baseline)	6	12	18	
16:0	21.7 $\pm$ 1.0	23.3 $\pm$ 1.0	28.0 $\pm$ 1.0†	24.2 $\pm$ 1.2	23.4 $\pm$ 0.6
18:0	8.8 $\pm$ 0.26	9.7 $\pm$ 0.41	8.5 $\pm$ 0.3	8.4 $\pm$ 0.4	7.4 $\pm$ 0.2†
16:1	1.2 $\pm$ 0.2	1.0 $\pm$ 0.1	1.2 $\pm$ 0.3	1.3 $\pm$ 0.1	1.5 $\pm$ 0.2
18:1	21.8 $\pm$ 1.7	18.9 $\pm$ 1.1†	17.0 $\pm$ 1.6‡	18.7 $\pm$ 1.1†	20.2 $\pm$ 1.5
24:1	0.4 $\pm$ 0.04	0.3 $\pm$ 0.04	0.3 $\pm$ 0.09	1.3 $\pm$ 0.2‡	0.8 $\pm$ 0.1
18:2 n-6	34.5 $\pm$ 2.0	32.4 $\pm$ 1.8	34.1 $\pm$ 2.3	29.1 $\pm$ 0.8	34.0 $\pm$ 2.3
20:3 n-6	1.9 $\pm$ 0.15	1.0 $\pm$ 0.1‡	0.9 $\pm$ 0.1	1.0 $\pm$ 0.1	1.5 $\pm$ 0.1†
20:4 n-6	7.0 $\pm$ 0.3	6.5 $\pm$ 0.8	4.7 $\pm$ 0.4‡	6.4 $\pm$ 0.3	6.9 $\pm$ 0.3
22:4 n-6	0.16 $\pm$ 0.01	0.13 $\pm$ 0.04	0.11 $\pm$ 0.02	0.30 $\pm$ 0.03	0.30 $\pm$ 0.05
22:5 n-6	0.12 $\pm$ 0.01	0.08 $\pm$ 0.02	0.08 $\pm$ 0.02	0.17 $\pm$ 0.04	0.30 $\pm$ 0.09
20:5 n-3	0.64 $\pm$ 0.08	3.85 $\pm$ 0.4‡	3.30 $\pm$ 0.23‡	4.40 $\pm$ 0.37‡	0.80 $\pm$ 0.13
22:5 n-3	0.31 $\pm$ 0.04	0.50 $\pm$ 0.06	0.40 $\pm$ 0.03	0.90 $\pm$ 0.07‡	0.60 $\pm$ 0.09‡
22:6 n-3	1.4 $\pm$ 0.1	2.3 $\pm$ 0.19‡	1.9 $\pm$ 0.19‡	3.9 $\pm$ 0.26‡	2.4 $\pm$ 0.22†
20:4 n-6/20:5 n-3 ratio	11.9 $\pm$ 1.4	1.7 $\pm$ 0.1‡	1.4 $\pm$ 0.1‡	1.5 $\pm$ 0.1‡	9.8 $\pm$ 1.6

\*Values are percentages of total fatty acids and are expressed as mean  $\pm$  SEM. Statistical analysis was performed as described in "Methods."

† $P < .05$ , ‡ $P < .01$  versus baseline.

**TABLE 2. Fatty Acid Composition of Monocytes in Healthy Subjects Before, During, and After n-3 Fatty Acid Administration**

Fatty Acids*	Weeks of Study				After 14 Weeks of Washout
	0 (Baseline)	6	12	18	
16:0	25.9±1.0	24.5±0.85	25.3±1.1	24.7±1.8	25.1±1.4
18:0	20.6±0.9	21.2±0.2	22.5±0.5	22.8±0.4	23.2±0.5
16:1	0.85±0.2	0.45±0.03	1.80±0.5	0.50±0.04	0.62±0.05
18:1	15.9±0.5	15.4±0.5	17.3±0.9	15.7±0.8	16.8±0.6
24:1	1.3±0.2	1.3±0.1	1.3±0.2	1.3±0.1	1.2±0.1
18:2 n-6	10.1±0.6	9.8±0.6	8.6±0.6	9.6±1	7.9±0.4
20:3 n-6	1.6±0.22	1.5±0.08	1.3±0.09	1.6±0.14	1.9±0.09
20:4 n-6	18.0±0.5	16.0±0.6	13.3±0.7‡	14.5±0.8‡	15.8±1.2
22:4 n-6	1.8±0.2	1.0±0.07†	1.3±0.1	1.2±0.1	1.8±0.1
22:5 n-6	0.26±0.05	0.17±0.04	0.27±0.03	0.16±0.02	0.38±0.13
20:5 n-3	0.34±0.1	1.8±0.17‡	1.4±0.13‡	1.7±0.13‡	0.58±0.08
22:5 n-3	1.4±0.2	3.3±0.2‡	2.6±0.2‡	2.8±0.2‡	1.8±0.1
22:6 n-3	1.9±0.26	3.5±0.22‡	3.0±0.29‡	3.3±0.29‡	2.8±0.3†
20:4 n-6/20:5 n-3 ratio	54.6±7.8	9.4±0.8‡	9.8±1.0‡	8.3±0.6‡	28.0±3.1‡

\*Values are percentages of total fatty acids and are expressed as mean±SEM. Statistical analysis was performed as described in "Methods."

† $P < .05$ , ‡ $P < .01$  versus baseline.

cells, respectively;  $P < .01$  versus baseline and 6 weeks). Despite treatment withdrawal, absolute amounts of TF activity were still lower compared with those at baseline; however, endotoxin stimulation resulted in a consistent increase of TF activity in monocytes from all subjects (mean percent increase, 140).

After treatment withdrawal, levels of 20:5 n-3 in plasma returned toward baseline values ( $0.8 \pm 0.18\%$  of total fatty acids; not significant versus baseline), whereas those of 22:6 n-3 remained higher than at baseline ( $2.4 \pm 0.22\%$  of total fatty acids;  $P < .01$  versus baseline). A similar trend was also observed in monocytes. Levels of 20:4 n-6 in both plasma and monocytes were not different from baseline values. The 20:4 n-6 to 20:5 n-3 ratio tended to return toward baseline in plasma (Table 1), whereas it remained significantly lower in monocytes (Table 2).

#### Effects of n-3 Fatty Acid Administration to Hypertriglyceridemic Patients

The results of the preliminary study carried out in healthy subjects indicated that n-3 fatty acids profoundly affected the capacity of monocytes to express TF activity and that this effect lasted more than 14 weeks after treatment withdrawal. That study, however, did not include a placebo group, and this factor could represent a substantial drawback for its significance. Therefore, a double-blind, placebo-controlled study with either 3 g/d n-3 fatty acids or olive oil was carried out in hypertriglyceridemic patients. A crossover design was not feasible because of the prolonged carryover effect of n-3 fatty acids that we observed; therefore, the study was conducted in two parallel groups of patients randomly assigned to n-3 fatty acids or olive oil treatment.

At baseline, values of TF activity in adherent monocytes that had been isolated from hypertriglyceridemic patients were similar to those detected in healthy subjects ( $7.3 \pm 0.88$  and  $12.04 \pm 1.19$  U/ $\mu$ g protein for unstimulated and endotoxin-stimulated adherent monocytes, respectively;  $n = 29$ ). After 18 weeks of treatment with 3 g/d n-3 fatty acids, TF activity in unstimulated and endotoxin-stimulated monocytes was significantly decreased (Fig 2). At this time, monocytes incubated in medium alone produced only 4.6 U/ $\mu$ g protein compared with a baseline level of 7.1 U/ $\mu$ g protein ( $-35\%$ ). Similarly, monocytes stimulated with 10  $\mu$ g endotoxin per milliliter produced 7.1 U/ $\mu$ g protein compared with a baseline level of 12.2 U/ $\mu$ g protein ( $-41\%$ ). These reductions remained constant for 6 weeks thereafter. Administration of olive oil for 24 weeks did not significantly influence monocyte TF activity in the hypertriglyceridemic patients. When the n-3 fatty acid-treated group was compared with the olive oil-treated group, it appeared that TF activity levels in both unstimulated and stimulated monocytes differed significantly at the 14th week (Fig 2;  $P < .05$ ).

Compliance with n-3 FA treatment was confirmed by changes in n-3 and n-6 fatty acids in plasma. Specifically, 20:5 n-3 in plasma was raised from  $0.72 \pm 0.18\%$  of total fatty acids at baseline to  $3.7 \pm 0.26\%$  after 24 weeks of treatment ( $P < .001$ ). Plasma levels of 22:6 n-3 increased from  $1.53 \pm 0.54\%$  to  $4.14 \pm 0.24\%$  of total fatty acids (Table 3;  $P < .001$ ). These levels are fully comparable with those recorded in healthy subjects after administration of the same dose of n-3 fatty acids. So far as n-6 fatty acids are concerned, 20:4 n-6 levels in

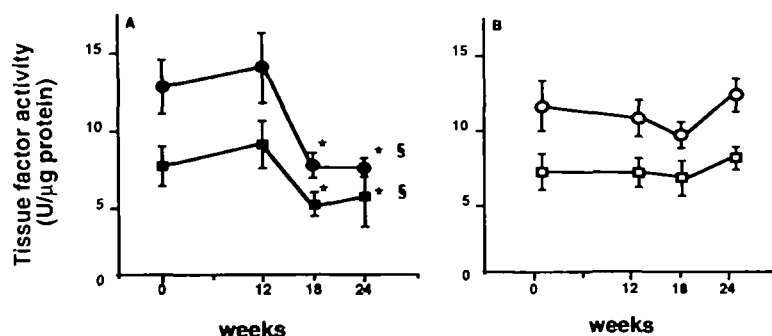


FIG 2. Line plots of tissue factor activity in monocytes isolated from hypertriglyceridemic patients at baseline (0) and during treatment with n-3 fatty acid ethyl esters (3 g/d; A) or with olive oil (3 g/d; B) for 24 weeks. Each point represents the mean  $\pm$  SEM value for 14 patients in the n-3 fatty acid group and 15 patients in the olive oil group at each time.  $\square$ ,  $\blacksquare$  indicate unstimulated monocytes;  $\circ$ ,  $\bullet$ , endotoxin-stimulated monocytes. \*Values significantly different from corresponding values at baseline and 12 weeks ( $P < .05$ ). §Values significantly different from corresponding values of the olive oil group ( $P < .05$ ).

plasma decreased slightly in the hypertriglyceridemic patients who received n-3 fatty acids, whereas levels of 18:2 n-6 remained unchanged (Table 3). The 20:4 n-6 to 20:5 n-3 ratio was reduced from 8 at baseline to 1.5 at 24 weeks.

Levels of polyunsaturated fatty acids did not change in the olive oil group during treatment (levels of 20:5 n-3 were  $0.70 \pm 0.07\%$  and  $0.72 \pm 0.08\%$  and of 22:6 n-3 were  $1.60 \pm 0.12\%$  and  $1.74 \pm 0.17\%$  of total fatty acids at baseline and after 24 weeks of treatment, respectively). Administration of 3 g/d of olive oil did not affect plasma levels of 18:1 n-9, nor did it influence 18:2 n-6 or 20:4 n-6 levels in plasma.

### Discussion

These data indicate that relatively prolonged administration of a moderate dose of n-3 fatty acids to healthy subjects and to patients with hypertriglyceridemia reduces TF activity in unstimulated and endotoxin-stimulated adherent monocytes.

Data from previous studies on the effects of n-3 fatty acids on TF activity are conflicting. Inhibition of TF activity in human mononuclear cells after fish oil administration to healthy subjects was previously reported by Hansen et al.<sup>36</sup> This observation, however, was not confirmed by others.<sup>37,38</sup> These latter studies were performed with high doses of n-3 fatty acids (5 g/d) administered as fish oil for relatively short periods of time (4 to 8 weeks). Moreover, it has recently been reported that n-3 fatty acids (5 g/d EPA and DHA) administered to patients undergoing coronary artery bypass surgery have no effect or even increase thromboplastin generation by monocytes isolated from whole blood stimulated with endotoxin.<sup>39</sup> On the other hand, administration of 1 g/kg body weight of an n-3 ethyl ester concentrate to nonhuman primates subjected to carotid endarterectomy has been shown to result in a dramatic impairment of mononuclear cell TF activity.<sup>24</sup>

In the studies cited above, TF activity determination was performed in a mixed population of lymphocytes

TABLE 3. Fatty Acid Composition of Plasma of Hypertriglyceridemic Patients Before, During, and After Treatments

Fatty Acids*	n-3 Fatty Acid Group (n=14)				Olive Oil Group (n=15)	
	0 Week (Baseline)	12 Weeks	18 Weeks	24 Weeks	0 Week (Baseline)	24 Weeks
16:0	27.0 $\pm$ 0.73	26.2 $\pm$ 1.18	26.2 $\pm$ 1.10	25.0 $\pm$ 0.83	26.0 $\pm$ 1.0	24.9 $\pm$ 1.0
18:0	6.8 $\pm$ 0.2	6.8 $\pm$ 0.22	7.1 $\pm$ 0.25	7.0 $\pm$ 0.18	6.6 $\pm$ 0.25	6.4 $\pm$ 0.19
16:1	3.0 $\pm$ 0.30	2.7 $\pm$ 0.37	2.4 $\pm$ 0.29	2.4 $\pm$ 0.28	2.2 $\pm$ 0.20	2.1 $\pm$ 0.18
18:1	27.6 $\pm$ 1.1	23.5 $\pm$ 1.1†	24.0 $\pm$ 1.1†	23.2 $\pm$ 1.2†	28.4 $\pm$ 1.3	26.4 $\pm$ 1.2
24:1	0.61 $\pm$ 0.05	0.80 $\pm$ 0.06	0.67 $\pm$ 0.05	0.85 $\pm$ 0.06	0.53 $\pm$ 0.04	0.56 $\pm$ 0.06
18:2 n-6	24.5 $\pm$ 1.0	25.1 $\pm$ 1.3	25.2 $\pm$ 1.3	25.9 $\pm$ 1.4	26.1 $\pm$ 1.3	28.9 $\pm$ 1.2
20:3 n-6	1.7 $\pm$ 0.41	1.3 $\pm$ 0.08†	1.3 $\pm$ 0.13†	1.3 $\pm$ 0.07†	1.5 $\pm$ 0.06	1.5 $\pm$ 0.09
20:4 n-6	5.6 $\pm$ 0.24	5.3 $\pm$ 0.27	5.2 $\pm$ 0.18	5.4 $\pm$ 0.21	5.5 $\pm$ 0.34	6.1 $\pm$ 0.42
22:4 n-6	0.32 $\pm$ 0.04	0.18 $\pm$ 0.02†	0.15 $\pm$ 0.01†	0.17 $\pm$ 0.01†	0.24 $\pm$ 0.02	0.22 $\pm$ 0.03
22:5 n-6	0.16 $\pm$ 0.05	0.11 $\pm$ 0.01†	0.10 $\pm$ 0.01†	0.11 $\pm$ 0.01†	0.15 $\pm$ 0.02	0.16 $\pm$ 0.02
20:5 n-3	0.72 $\pm$ 0.18	3.6 $\pm$ 0.27†	3.4 $\pm$ 0.23†	3.7 $\pm$ 0.26†	0.70 $\pm$ 0.07	0.72 $\pm$ 0.08
22:5 n-3	0.41 $\pm$ 0.07	0.81 $\pm$ 0.07†	0.70 $\pm$ 0.04†	0.89 $\pm$ 0.05†	0.40 $\pm$ 0.03	0.41 $\pm$ 0.05
22:6 n-3	1.5 $\pm$ 0.54	3.7 $\pm$ 0.34†	3.5 $\pm$ 0.19†	4.1 $\pm$ 0.24†	1.6 $\pm$ 0.12	1.7 $\pm$ 0.17
20:4 n-6/20:5 n-3 ratio	8.7 $\pm$ 0.8	1.6 $\pm$ 0.1†	1.6 $\pm$ 0.1†	1.5 $\pm$ 0.1†	8.8 $\pm$ 1.0	9.8 $\pm$ 1.3

\*Values are percentages of total fatty acids and are expressed as mean  $\pm$  SEM. Statistical analysis was performed as described in "Methods."

† $P < .01$  versus baseline; n indicates number of patients.

and monocytes. Although lymphocytes do not express TF activity, it is known that these cells cooperate with monocytes to amplify TF generation.<sup>40</sup> n-3 Fatty acids, however, are reported to influence several aspects of lymphocyte function<sup>41</sup>; thus, the effects of n-3 fatty acids observed on monocytes might reflect functional changes at the lymphocyte level. In our study, to assess whether these fatty acids directly influenced the monocyte, TF activity was evaluated in a virtually pure population of adherent monocytes. In addition, to minimize variations due to adhesion, we normalized the data by expressing TF activity per microgram of protein. The results indicate that n-3 fatty acids directly impair the capacity of adherent monocytes to generate TF activity both in the unstimulated condition and after stimulation with endotoxin.

In healthy subjects, administration of 3 g/d of n-3 fatty acids (2.5 g/d EPA plus DHA) for periods longer than 6 weeks suffices to inhibit TF activity in adherent monocytes. Delay in the onset of effects of n-3 fatty acids on monocyte TF activity compared with those observed for other monocyte-derived inflammatory mediators, such as cytokines and leukotrienes,<sup>9,10</sup> suggests that TF activity regulation is not an immediate target for the fatty acids but that they probably act in concert with other as-yet-undefined mediators, possibly influencing gene expression. This hypothesis is consistent with the finding that n-3 fatty acids inhibit gene expression of platelet-derived growth factor A and B in monocytes.<sup>11</sup> The extent of TF activity inhibition in unstimulated and endotoxin-stimulated monocytes that we observed after 12 weeks of treatment with 3 g/d n-3 fatty acids to healthy volunteers was comparable with that described for mononuclear cells from nonhuman primates supplemented for 12 weeks with far higher amounts of n-3 fatty acids. During treatment, inhibition of TF activity was accompanied by a consistent accumulation of n-3 fatty acids in plasma and monocyte compartments. In addition, 20:4 n-6 levels were reduced during treatment, mostly in monocytes.

Impairment of TF activity persisted after withdrawal of treatment; plasma and monocyte fatty acid levels tended to return toward baseline, but the arachidonic acid to EPA ratio in monocytes still remained significantly lower than at baseline. Interestingly, levels of 22:6 n-3 remained consistently high as long as 14 weeks after treatment withdrawal. It is worth mentioning that during this study, the subjects did not modify their diet, nor did they increase their intake of food items containing n-3 fatty acids. On the other hand, a long-lasting effect of n-3 fatty acids was previously observed in platelets<sup>42</sup> and monocytes.<sup>9</sup> In particular, cytokine synthesis after fish oil administration was shown to be impaired for more than 10 weeks after stopping treatment.<sup>9</sup>

n-3 Fatty acids effectively also reduced TF activity in hypertriglyceridemic patients, both in unstimulated adherent monocytes and after cell stimulation with endotoxin. In hypertriglyceridemic patients, however, TF activity was reduced to a lesser extent than in normal subjects. This may be due to the abnormally elevated levels of triglyceride-rich lipoproteins in hypertriglyceridemic plasma that undergo complex interactions with n-3 fatty acids as well as with the monocyte.<sup>43</sup> Inhibition

of TF activity was accompanied by a consistent accumulation of EPA and DHA in the patients' plasma samples, which was comparable with that observed in healthy subjects.

TF activity in monocytes isolated from hypertriglyceridemic patients was found to be comparable with that observed in cells from healthy subjects. Thus, one might raise the question of the possible significance of attenuating TF activity in this pathological condition. Indeed, as previously discussed, a procoagulant state exists in hypertriglyceridemia due to elevated amounts of the essential cofactor for TF activity, ie, factor VII/VIIa in plasma.<sup>22</sup> Because formation of the TF-VIIa complex is the first step in triggering the clotting cascade and thus represents a key regulatory step in controlling activation of the clotting system, the attenuation of TF activity following n-3 fatty acid administration may be relevant to this process.

The molecular mechanism(s) underlying TF activity reduction after n-3 fatty acid intake is unknown. n-3 Fatty acids, when incorporated into cell membranes, are substrates for phospholipases and lead to the formation of prostanoids of the n-3 series, including EPA-derived leukotrienes and hydroxy fatty acids that possess reduced biological activities compared with corresponding arachidonate-derived products.<sup>10</sup> Changes in the capacity of monocytes to synthesize arachidonate products might thus influence monocyte TF activity. So far, among arachidonate-derived products, 12-hydroxyeicosatetraenoic acid, the major product of platelet 12-lipoxygenase, has been shown to be implicated in TF activity regulation.<sup>44</sup> The capacity of cells to generate arachidonate products, however, recovers within a short time after treatment withdrawal and follows the decline of EPA in cell lipids,<sup>10</sup> thus making it unlikely that the impairment in TF activity that we observed could be ascribed only to changes in the eicosanoid system. As previously discussed, n-3 fatty acids are known to impair monocyte cytokine synthesis. Cytokines induce TF expression in endothelial cells,<sup>45</sup> and interferon gamma and granulocyte-colony stimulating factor act synergistically with endotoxin to induce macrophage and endothelial cell-associated TF activity.<sup>46,47</sup> Thus, the observed reduction in monocyte TF activity exerted by n-3 fatty acids associated with their long-lasting effect might be ascribed to modifications in the synthesis of cytokines.

The overall picture of the mechanisms through which n-3 fatty acids may operate is rendered more complex if we consider that the effect on TF activity was observed on adherent monocytes, a condition that triggers several monocyte functions, including TF activity. Leukocyte adherence is known to be mediated through expression of the  $\beta_2$  integrin CD11b/CD18, which is involved in functional enhancement of macrophage TF response.<sup>48</sup> Moreover, n-3 fatty acids are known to attenuate leukocyte-endothelium interaction through the increase of mediators, such as prostacyclin and endothelium-derived-relaxing factor, which negatively influence the adhesion process.<sup>6,49</sup> Further studies, however, are needed to assess whether the effects of n-3 fatty acids on monocyte TF activity that we describe here are at least in part consequent to modifications of the adhesive machinery of the monocyte.



In conclusion, the effect of n-3 fatty acids on monocyte TF activity, ie, the reduction in the capacity to generate thrombin, should be added to other known activities of these fatty acids that are directed toward an impairment of monocyte functions. Monocyte-mediated thrombin generation might occur within the circulation as well as within the vessel wall in atherosclerotic plaques. Because our study indicates that n-3 fatty acids interfere with TF activity induced in monocytes following their adhesion, one might speculate that this mechanism is operative in the initial formation of atherosclerotic lesions, when the monocyte is the first cell that adheres to the vascular endothelium.

Thus, n-3 fatty acids that reduce basal and stimulated TF activity in adherent monocytes may interfere with in vivo activation of thrombin generation, influencing positively the prothrombotic risk in hypertriglyceridemic patients. This hypothesis needs to be further supported by ad hoc designed clinical studies.

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