Published Ahead of Print on May 10, 2008, as doi:10.3324/haematol.11845.

Original Article

Effect of the administration of n-3 polyunsaturated fatty acids on circulating levels of microparticles in patients with a previous myocardial infarction

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ABSTRACT

Background

Increased levels of microparticles exposing tissue factor circulate in the blood of patients with coronary heart disease, possibly disseminating their pro-thrombotic and pro-inflammatory potential. Because diets rich in n-3 (polyunsaturated) fatty acids have been associated with reduced incidence of coronary heart disease-related events, we investigated the *in vivo* effects of treatments with n-3 fatty acids on levels of circulating microparticles and their tissue factor-dependent procoagulant activity in patients with a previous myocardial infarction.

Design and Methods

Forty-six post-myocardial infarction patients were assigned to receive either 5.2 g of n-3 fatty acids daily (n=23) or an olive oil placebo (n=23) for 12 weeks. Circulating microparticles were isolated from peripheral blood. The number of microparticles, their cellular source and tissue factor antigen were determined by flow cytometry, and their procoagulant potential assayed by a fibrin generation test.

Results

The total number of microparticles, endothelium-derived microparticles and microparticle tissue factor antigen were not significantly different between the two groups. However, the number of platelet-derived microparticles [from a median of 431 (126-1796, range) $\times 10^6$/L to a median of 226 (87-677, range)] $\times 10^6$/L and monocyte-derived microparticles [from a median of 388 (9-1681, range) $\times 10^6$/L to a median of 265 (7-984, range) $\times 10^6$/L] in plasma were significantly ($p<0.05$) decreased by n-3 fatty acids, while they were unchanged in the placebo group. Total microparticle tissue factor-procoagulant activity was also reduced in the n-3 fatty acid group compared to that in the placebo group.

Conclusions

Treatment with n-3 fatty acids after myocardial infarction exerts favorable effects on levels of platelet- and monocyte-derived microparticles, thus possibly explaining some of the anti-inflammatory and anti-thrombotic properties of these natural compounds.

Key words: tissue factor, microparticles, platelets, endothelium, thrombosis, coronary heart disease.

Citation: Del Turco S, Basta G, Lazzerini G, Evangelista M, Rainaldi G, Tanganelli P, Hagstrup JC, Schmidt EB, and De Caterina R. Effect of the administration of n-3 polyunsaturated fatty acids on circulating levels of microparticles in patients with a previous myocardial infarction. Haematologica 2008 June; 93(6):892-899. doi: 10.3324/haematol.11845

Manuscript received June 14, 2007. Revised version arrived on January 28, 2008. Manuscript accepted January 29, 2008.

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Introduction

Microparticles (MP) are small membrane vesicles, released from circulating cells or endothelial cells upon cell activation or during apoptosis.[1] Levels of circulating MP have been reported to be raised in various thrombotic disorders and, specifically, in coronary heart disease, in which they may play an important role in promoting coagulation, inflammation, and vascular dysfunction.[2,3] MP support coagulation through both the externalization and shedding of the procoagulant anionic phospholipid phosphatidylserine upon membrane remodeling occurring with cell stimulation, and through the possible presence of tissue factor (TF), the main trigger of coagulation *in vivo*.[4] In addition to their direct effect of promoting and amplifying coagulation, circulating MP may also act in inflammatory processes,[3,5] and may, therefore, be responsible for the dissemination of thrombosis and inflammation to sites remote from the microenvironment where their formation occurs, with effects that depend on their membrane and cytoplasmic composition.

The long-chain n-3 polyunsaturated fatty acids eicosapentaenoate (EPA) and docosahexaenoate (DHA), mainly derived from fish, have been epidemiologically linked to protection from coronary heart disease both in primary and secondary cardiovascular prevention studies.[6-8] In fact, numerous investigations have shown that diets rich in n-3 fatty acids are associated with a reduction of cardiovascular mortality, myocardial infarction, and sudden death.[9-11] Many of the health effects of these compounds occur through their incorporation into membrane phospholipids, in particular into anionic phospholipids,[12] which have important functions in membrane and cell physiology.

We, therefore, investigated the effects of an *in vivo* treatment with n-3 fatty acids in patients after an acute myocardial infarction evaluating circulating levels of MP and their *in vitro* thrombogenic potential in activating TF-dependent and -independent coagulation.

Design and Methods

Patients and healthy control subjects

Forty-two patients discharged from the Department of Cardiology at Aalborg Hospital after an acute myocardial infarction and with a ventricular ejection fraction <0.40% were considered eligible for a study investigating the effect of dietary supplementation with n-3 fatty acids on heart rate variability.[13] Blood samples collected in that study served for the here-reported investigations. Patients over 75 years of age, with pacemakers or permanent tachyarrhythmias, as well as those with diabetes or other diseases potentially affecting heart rate variability were not included because of the aims of the original study.[13] Patients were studied out of the acute phase, 6 or more months after discharge. They were randomly allocated to receive either 5.2 g of total n-3 fatty acids daily (corresponding to 4.3 g of EPA and DHA, given as 8 capsules/day of Pikasol, a re-esterified triglyceride preparation; EPAX 5500, Pronova Biocare A/S, Norway), or olive oil, for 12 weeks. Treatment was started immediately at the time of randomization. Diet and drugs were both kept constant during the trial. Serum lipid parameters were measured with standard techniques.

Ten healthy volunteers without a history or clinical evidence of coronary heart disease were recruited through advertisement, and served as control subjects.

Blood sampling

Citrate-anticoagulated blood samples were centrifuged at 1,500 x *g* for 20 min at room temperature to remove cells. Platelet-free plasma was prepared by centrifugation of the above plasma at 12,000 x *g* for 1 min at room temperature to remove all residual platelets or cell fragments of similar size. The supernatant plasma from both patients and healthy individuals was divided into 250-μL aliquots, snap-frozen in liquid nitrogen to preserve MP structure, and stored, for an identical time interval, at –80° C until further analyses.

Isolation of microparticles

MP were isolated from plasma samples as described previously.[14,15] Briefly, a 250 μL plasma sample was centrifuged at 17,570 x g at 20°C for 30 min to pellet MP. After centrifugation, 225 μL of the supernatant were removed, and the MP pellet, in a volume of 25 μL, was resuspended into a further 225 μL of filtered buffer (10 mM HEPES/NaOH, 140 mM NaCl, 2.5 mM $CaCl_2$, here referred to as *buffer*), and again centrifuged in the same conditions. The washed MP pellet (25 μL) was finally resuspended in 75 μL of filtered buffer, of which 5 μL and 10 μL were used for flow cytometry and coagulation assays, respectively.

Flow cytometry

Five μL of washed MP suspension were diluted into 35 μL of buffer, gently vortexed, and then with 5 μL of 1:500 buffer-diluted normal mouse serum added. After incubation for 15 min at room temperature, MP were labeled with 5 μL of fluorescein isothiocyanate (FITC)-conjugated annexin-V (annexin-V FITC, final dilution 1:50, Bender MedSystems, Vienna, Austria) or the specific antibody, for 20 min in the dark. To identify annexin-V-positive MP, a threshold was set based on a MP sample prepared without the addition of Ca^{++}, essential for annexin-V binding (autofluorescence). Cell-specific mono-clonal antibodies used to determine the cellular origins of MP and TF-exposing MP were: anti-human tissue factor 4508CJ (final dilution 1:25, American Diagnostica Inc., Greenwich, CN, USA), anti-human CD-61 (against the platelet-specific antigen glycoprotein IIIa (β_3 integrin), final dilution 1:50, from Cymbus Biotechnology, Eagle Close, UK), anti-human CD14 (against the monocyte-specific antigen lipopolysaccharide receptor; final dilution 1:10, from Cymbus), anti-human CD-62E (against the endothelium-specific inducible antigen E-selectin, final dilution 1:100, also from Cymbus). The cell specificity of these antibodies in our MP flow cytometry experiments was verified in preliminary tests using platelets and

endothelial cells (*data not shown*). All antibodies were titrated in preliminary experiments to determine the optimal labeling concentrations for each. In order to define the background noise of the flow-cytometry analysis, we also treated plasma samples with the corresponding isotype-matched non-relevant antibodies as controls. After labeling, 200 μL of buffer were added, and the suspensions centrifuged as above to reduce the background signal. Finally, 200 μL of (MP-free) buffer were removed and the MP pellet (in 55 μL) resuspended in a further 300 μL of buffer. All samples were analyzed for 1 min. Light scatter and fluorescence channels were set at logarithmic gain. The region corresponding to shed MP was defined in forward light scatter *vs.* side-angle light scatter intensity dot-plot representations, using 0.05 μm, 0.8 μm and 3 μm latex beads as references (Sigma Aldrich, Milan, Italy). Freezing MP did not affect MP forward scatter and side scatter distribution (*data not shown*). MP were defined as elements smaller than 1 μm that were positively labeled with annexin-V. The number of total MP and cell-specific MP per liter of plasma was calculated using the number of fluorescence events (n) of annexin-V and cell-specific MP-binding antibody at flow cytometry, after correction for control immunoglobulin (Ig) G binding, as follows:

$$Number/L = n \times [100/5] \times [355/60] \times [10^6/250]$$

where 100 (μL) is the total volume of washed MP suspension, 5 (μL) is the volume of analyzed washed MP suspension, 355 (μL) is the total volume in the tube before analysis, 60 (μL) is the sample volume analyzed, 10^6 is the number of μL/L, and 250 is the original volume of plasma.[16]

Fibrin generation test

A fibrin generation test was used to assess the *in vitro* procoagulant activity of MP, as described.[17] Fibrin formation was monitored by kinetic measurements of the optical density at a wavelength of 405 nm. The procoagulant potential of MP in such conditions is inversely proportional to the clotting time. We used factor VII- and factor XII-deficient plasma (Dade Behring, Milan, Italy) to determine the contribution of TF-dependent and independent coagulation pathways, respectively, to MP-induced fibrin generation. In control experiments, MP were also pre-incubated with a TF-antibody (#4509, American Diagnostica, used at 1 μg/mL) to evaluate the contribution of TF-dependent procoagulant activity. To test whether fibrin formation was dependent on MP, we also performed assays with saline alone±MP added to plasma and buffer at increasing concentrations. Fibrin generation tests were also performed on fresh MP isolated from healthy individuals to identify possible differences in procoagulant activity between thawed and fresh MP samples. Fresh MP samples showed a time to fibrin generation similar to that of thawed samples (582 [567-643] s *vs.* 556 [538-623] s (*p=NS*). Moreover, changes of fibrin generation times by fresh and thawed MP reconstituted in factor VII- and XII-deficient plasma were similar, suggesting that the relative contributions of pathways initiating fibrin generation are unaffected by the single freeze/thawing cycle used in our plasma processing.

Measurement of coagulation markers

Plasma levels of thrombin-antithrombin (TAT) complexes and the prothrombin fragment 1+2 (F_{1+2}) were determined as such, without the addition of MP, by commercially available enzyme immunoassays (Enzygnost TAT microkit and Enzygnost F_{1+2} kit, Dade Behring, Marburg, Germany), according to the manufacturer's instructions. The measurement ranges of TAT and F_{1+2} assays were 2-60 μg/L and 20-1200 pmol/L, respectively.

Extraction and methylation of lipids from platelets

Platelets isolated from whole blood anticoagulated with EDTA were washed and resuspended in 250 μL of 0.9% NaCl. Lipids were extracted in CH_2Cl_2/MeOH according to a previously described method,[18] dissolved in hexane, and transesterified as described elsewhere.[19] The fatty acid composition was assessed by gas chromatography on a Chrompack CP-9002 apparatus (Varian, Palo Alto, CA, USA), quantifying fatty acid methyl esters with 12 to 24 carbon atoms.[20]

Statistical analysis

Since the numbers of MP in patients were not normally distributed, results are expressed as median and range comprised between the 5th and the 95th percentile. Comparisons of differences in various parameters measured before and after treatments were tested by the paired Student's *t* test and Wilcoxon's test for variables distributed normally and non-normally, respectively. Differences between the n-3 fatty acid and the olive oil groups were analyzed with the unpaired Student's *t* test and the Mann-Whitney *U* test for variables distributed normally and non-normally, respectively. The $\chi 2$ test was used to compare frequency values in baseline characteristics of the study groups. Sperman's ρ correlation test was used to investigate the relationship between non-parametric variables.

Results

Characteristics of the populations studied

The two groups of patients did not differ to any significant extent with regards to sex, age, risk factor profile, infarct size, infarct location and medications used (Table 1). The ten control subjects were sex- (7 male and 3 female) and age- (58±9 years) matched with the original study population. Three of the healthy subjects were smokers. As the result of the matching, there were also no significant differences between patients and the ten healthy control subjects with regards to age (p=0.09), gender (p=0.345) and smoking habits (p=0.625).

Compliance to treatment was monitored by measuring the levels of incorporation of EPA and DHA in total platelet lipids, before and after treatment (Table 2). The intake of n-3 fatty acids increased n-3 fatty acid incor-

poration in patients randomized to n-3 fatty acids compared with the olive oil-treated controls (Table 2). To assess the effects of n-3 fatty acids and olive oil on patients' serum lipid profile, the levels of total cholesterol, triglycerides, LDL- and HDL-cholesterol were measured. As shown in Table 3, the baseline levels of these lipid parameters did not differ between the two groups before treatment. Treatment with n-3 fatty acids decreased triglyceride levels significantly, further demonstrating satisfactory compliance, while total cholesterol, LDL-cholesterol and HDL-cholesterol were unaffected. Treatment with olive oil reduced total cholesterol and LDL cholesterol (Table 3).

Cellular origin of microparticles in plasma samples

The total number of MP isolated from all patients was significantly higher than that of MP isolated from plasma of healthy control subjects (1054 [352-7486]$\times 10^6$/L; *vs.* 599 [451-720]$\times 10^6$/L; p=0.021). The largest proportion of circulating MP originated from platelets in both groups of patients (Table 1) (median percentage of total MP: 44% in the group receiving n-3 fatty acids; 38% in the group receiving olive oil), while total endothelial cell-derived MP always constituted a minor fraction (median percentage of total MP: 18% in the group receiving n-3 fatty acids; 20% in the group receiving olive oil). Platelet-derived MP were also the highest proportion in samples from healthy subjects (469 [152-566]$\times 10^6$; median % of total MP: 78), with endothelial-derived MP constituting a minor fraction (36 [10-63]$\times 10^6$; median % of total MP: 6) (*data not shown*). We observed a decrease of both platelet- and monocyte-derived MP after treatment with n-3 fatty acids (49% reduction, p=0.016, and 34% reduction, p=0.010, respectively), while no significant decrease was observed in the olive oil group (5% reduction, p=0.353, and 6% reduction, p=0.770, respectively) (Table 4). There were no differences in endothelial-derived MP and TF-exposing MP in either group after treatment with n-3 fatty acids or olive oil. The total number of TF-exposing MP tended to be lower in patients after treatment with n-3 fatty acids (Table 4), but the difference was not statistically significant (p=0.249).

Figure 1 shows representative examples of dot plots

Table 1. Baseline characteristics of the population studied.

	n-3 fatty acid group (n=21)	*Olive oil group (n=21)*	*p value*	*Healthy subjects (n=10)*	*p value*
Age (years)	65±5	62±8	0.065	58±9	0.091
Sex (male, n)	18	18	1.000	7	0.345
Ejection fraction (%)	33±5	33±6	0.876	66±6*	<0.001
Q-wave infarction (n)	21	17	0.432	–	–
Infarct location					
Anterior (n)	16	17	0.685	–	–
Inferior (n)	7	6	0.531	–	–
Other (n)	3	3	1.000	–	–
Smoking	8	9	0.481	3	0.625
Hypertension	4	2	0.435	–	–
Platelet count, x10^3/µL	234±69	217.6±45.4	0.683	210±11	0.230
Leukocyte count, x 10^3/µL	6.34±2.4	6.3±1.7	0.927	7.3±1.6	0.420
Medications					
Aspirin	20	18	0.294	–	–
Diuretics	11	10	0.736	–	–
ACE-inhibitors	5	9	0.195	–	–
Digoxin	4	3	0.680	–	–
β-blockers	6	2	0.635	–	–
Calcium channel blockers	6	6	1.000	–	–

*Data are mean ± SD. All p values between the two post-infarction study groups (n-3 fatty acids vs. olive oil) and between healthy subjects and patients, apart from the ejection fraction in this latter comparison, are not statistically significant. *Estimated by transthoracic echocardiography.*

Table 2. Platelet composition of the main fatty acids (expressed as percentage of total fatty acids) before and after treatment with n-3 fatty acids or olive oil.

	Before treatment	*After treatment*	*Before vs. after p value*	*n-3 fatty acids vs. olive oil, post-treatment p value*
Stearic acid (18:0)				
n-3 fatty acid group	18.0±1.7	17.2±1.9	0.057	0.234
olive oil group	17.3±0.9	17.9±2.0	0.184	
Oleic acid (18:1, n-9)				
n-3 fatty acid group	20.3±2	21.0±1.7	0.056	0.474
olive oil group	21.1±1.5	20.6±1.7	0.339	
Linoleic acid (18:2, n-6)				
n-3 fatty acid group	7.1±1.7	6.8±1.6	0.195	0.49
olive oil group	6.9±0.7	6.7±1.1	0.183	
α-linolenic acid (18:3, n-3)				
n-3 fatty acid group	0.3±0.2	0.3±0.1	0.116	0.627
olive oil group	0.3±0.1	0.4±0.3	0.258	
EPA (20:5, n-3)				
n-3 fatty acid group	1.4±0.6	4.2±1.3	<0.001	<0.001
olive oil group	1.6±0.5	1.3±0.8	0.202	
DPA (22:5, n-3)				
n-3 fatty acid group	1.4±0.4	2.0±0.4	<0.001	<0.001
olive oil group	1.3±0.3	1.3±0.5	0.469	
DHA (22:6, n-3)				
n-3 fatty acid group	2.6±0.6	3.6±0.5	<0.001	<0.001
olive oil group	2.8±0.7	2.7±0.7	0.433	

EPA : eicosapentanoic acid; DPA: docosapentanoic acid; DHA docosahexaenoic acid. Values are expressed as mean ± SD.

Table 3. Serum lipid profile of patients before and after treatment with n-3 fatty acids or olive oil.

Parameter (mmol/L)	*Before n-3 fatty acids*	*After n-3 fatty acids*	*Before olive oil*	*After olive oil*
Total cholesterol (mmol/L)	6.1±1.2	5.9±1.0	6.4±1.5	6.0±1.5**
LDL cholesterol (mmol/L)	4.2±0.9	4.3±0.9	4.5±1.3	4.2±1.3**
Triglycerides (mmol/L)	2.0±1.7	1.4 ±1.1*	1.6±1.1	1.6±1.0
HDL (mmol/L)	1.0±0.2	1.0±0.3	1.1±0.3	1.0±0.4

*All values are expressed as mean ± SD. The n-3 fatty acid and olive oil groups did not differ significantly at baseline. *p<0.05 between groups of patients before and after n-3 fatty acid treatment. **p<0.05 between patients before and after olive oil treatment.*

(left panels) and the corresponding histogram (right panel) for platelet-derived MP isolated from a patient before and after treatment with n-3 fatty acids. We also found that the number of platelet-derived MP after treatment with n-3 fatty acids had a significant inverse relationship with the level of incorporation of n-3 fatty acids in platelets (r=-0.55; p=0.031).

Fibrin-generating capacity of microparticles

Overall data for fibrin generation curves obtained from patients before and after treatment with n-3 fatty acids or olive oil are provided in Table 5. MP-free normal plasma clotted after a lag time >600 s (Table 5). Incubation of normal plasma with MP isolated from patients receiving n-3 fatty acids or olive oil before treatment shortened this lag time to 280 [270-290] s and to 278 [261-292] s, respectively (p<0.05), indicating the procoagulant potential of MP from patients after myocardial infarction.

MP isolated after treatment with n-3 fatty acids caused a significant increase in the fibrin generation time compared with pre-treatment values, while those isolated after olive oil treatment caused only a minor and non-significant increase in fibrin generation compared with pre-treatment values, suggesting that only n-3 fatty acid supplementation overall reduces the MP thrombogenic potential. MP (before and after treatment) were then reconstituted in factor VII- and factor XII-deficient plasma samples to determine whether the observed generation of fibrin in myocardial infarction patients before and after treatment was due to stimulation of the TF-dependent or the TF-independent coagulation pathway. For this purpose, the original plasma volume containing MP (10 μL) was diluted 1:400 before assaying MP procoagulant activity in factor VII- and factor XII-deficient plasma samples. The result of such dilution is that the contribution to fibrin generation of coagulation factors in the original sample volume is negligible (*data not shown*). The fibrin generation time in the presence of MP isolated from patients before treatment with n-3 fatty acids (as well as before treatment with olive oil), and incubated with factor VII-deficient plasma, was prolonged. However, preincubation of MP with factor XII-deficient plasma prolonged the time to clotting much more than with VII-deficient plasma, indicating overall that the intrinsic pathway of coagulation contributes more than the TF-dependent pathway to the shortening of coagulation times occurring after myocardial infarction.

When MP isolated after treatment with n-3 fatty acids were incubated with factor VII-deficient plasma, the time to fibrin generation was prolonged compared with the value before treatment (by about 13%), suggesting that n-3 fatty acids decrease TF-dependent procoagulant activity. Further evidence for a role of the TF-dependent pathway was provided by the similar findings obtained when MP from the n-3 fatty acid group were added to normal plasma in the presence of an antibody against TF (before treatment: 295 [215-313] s; after treatment: 374 [350-398] s; n=10, p=0.0013). However, preincubation of MP with factor XII-deficient plasma also prolonged the time to clotting after treatment (by about 11%). These results suggest that

Table 4. Cellular origin and TF-exposing of microparticles in post-myocardial infarction patients before and after treatment with n-3 fatty acids or olive oil.

Marker	*Before n-3 fatty acids*	*After n-3 fatty acids*	*p* value*	*Before olive oil*	*After olive oil*	*p** value*
Platelet (CD61/β_3 integrin)	431 (126-1796)	226 (87-677)	0.016*	386 (75-1565)	361 (111-1222)	0.353
Monocyte (CD14)	388 (9-1681)	265 (7-984)	0.010*	407 (22-1558)	385 (13-1068)	0.770
Endothelial (CD62E/ E-selectin)	97 (3-650)	88 (1.5-526)	0.159	109 (2-569)	93 (3-365)	0.305
Tissue factor (CD142)	76 (6-494)	66 (9-453)	0.249	95 (10-498)	98 (11-482)	0.602

*Values are shown as median number x 10^6/L plasma with range. Non-significant differences were found between two groups of patients before treatments (Mann-Whitney U-test). Differences before and after n-3 fatty acid and olive oil treatments were analyzed by the Wilcoxon test. *Differences before and after n-3 fatty acid treatment. **Differences before and after olive oil treatment.*

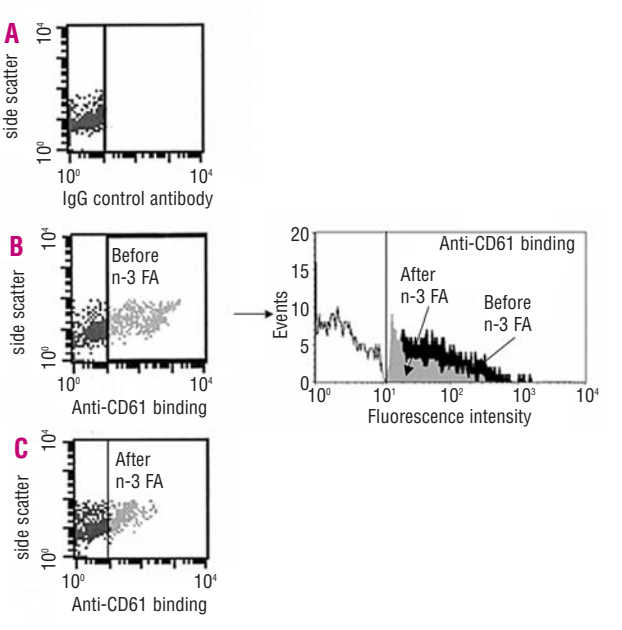

Figure 1. Dot plots (left panels) and histograms (right panel) of platelet-derived microparticles (MP) from one representative patient before and after treatment with n-3 fatty acids. The dot plots are obtained as plots of side scatter (on the ordinate) as a function of fluorescence intensity (logarithmic scale, on the abscissa). Fluorescence thresholds (vertical lines) were set using a sample of MP labeled with a control non-specific antibody (A). MP isolated from the plasma of a patient before (B) and after treatment with n-3 fatty acids (C) were stained with anti-CD61 (characterizing platelet-derived MP). The two superimposed histograms on the right panel (fluorescence intensity on the abscissa *vs.* event number on the ordinate) show CD-61-positive MP before (black area) and after (gray area) treatment with n-3 fatty acids. The empty area to the left shows the background fluorescence, detected as described above.

also the TF-independent coagulation pathway accounts for the anticoagulant effect of n-3 fatty acids. On the other hand, MP isolated after treatment with olive oil and preincubated with factor VII-deficient plasma and factor XII-deficient plasma did not prolong the times to fibrin generation, demonstrating that olive oil treatment did not reduce the procoagulant potential of MP. Fibrin generation in plasma from healthy subjects was not enhanced by MP, and not inhibited by preincubation with either factor VII-deficient plasma or factor XII-deficient plasma (Table 5), indicating that these MP have virtually no procoagulant activity unlike those from post-myocardial infarction patients.

Effects of microparticles on in vivo *tests of coagulation activation*

We also investigated *in vivo* coagulation markers in patients, measuring plasma concentrations of TAT complexes and F_{1+2}. The plasma levels of TAT complexes and F_{1+2} in patients in control conditions (TAT: 2.5 [1.9-4.8] µg/L, median [5-95 percentiles]; F_{1+2}: 218 [77-510] pmol/L) did not differ from those measured in healthy controls (TAT: 2.3 [2-2.9] µg/L; F_{1+2}.: 191 [185-220] pmol/L). There were no statistically significant differences in either parameter in the comparisons before and after n-3 fatty acid treatment (TAT: 2.5 [1.8-9.4] µg/L before treatment *vs.* 2.4 [1.7-4.9] µg/L after treatment, p=0.23; F_{1+2}: 247 [61-614] pmol/L before treatment *vs.* 263 [79-640] pmol/L after treatment, p=0.78), or in comparisons before and after olive oil treatment (TAT: 2.3 [2.0-3.9] µg/L before treatment *vs.* 2.4 [2.2-2.8] µg/L after treatment, p=0.58; F1+2: 203 [173-230] pmol/L before treatment *vs.* 202 [175-235] pmol/L after treatment, p=0.91). We found no significant correlations between MP parameters and TAT and F_{1+2} levels (*data not shown*). On the other hand, the number of total and platelet-derived MP correlated inversely with the time of fibrin generation in all patients before treatment with n-3 fatty acids or olive oil (r=-0.69; p=0.036 and r=-0.80; p=0.027, respectively). Moreover, the number of platelet-derived MP correlated inversely with the time of fibrin generation after treatment with n-3 fatty acids (r=-0.87; p=0.04).

Discussion

This study demonstrates that the administration of n-3 fatty acids, in parallel with their increased incorporation in total cell lipids, reduces platelet- and monocyte-derived MP number and their procoagulant activity in patients after an acute myocardial infarction.

Patients studied had elevated levels of MP compared with healthy subjects. An increased number of circulating procoagulant MP after myocardial infarction, with their potential of being both markers of vascular cell activation and effectors of inflammation and thrombosis,[3] is in line with previous observations in patients with acute coronary syndromes[21] and of persistent intracoronary thrombi 24 h to 30 days after the ischemic episode,[22] but is here demonstrated an average of more than 6 months after the qualifying episode. This suggests that elevated levels of circulating MP may be useful as markers of the persistence or recurrence of thrombosis or of the patient's thrombotic risk, and highlights the possibility that they could be tested as markers for the recurrence of ischemic events.

We found that the majority of *in vivo* circulating MP derive from platelets, both in patients and in healthy subjects, in agreement with previous findings,[15,21] and that endothelium-derived MP constitute the smallest proportion.[15] The quantification of circulating MP from different cell sources varies substantially in the literature published to date, probably because of the absence of appropriate standardization of detection methods,[23] since factors such as assay protocols, as well as clones, sources, and dilutions of the antibodies used can have dramatic quantitative and qualitative effects on the results of MP analysis.[23]

We found that numbers of MP from specific cellular sources are influenced by n-3 fatty acid treatment. The administration of n-3 fatty acids resulted in lower numbers of platelet- and monocyte-derived MP compared with those following olive oil treatment in our patients. Endothelium-derived MP, on the other hand, were not influenced by treatment. This is the first demonstration of a selective effect of a treatment of any kind on select-

Table 5. ***In vitro*** **fibrin generation test of microparticles isolated from plasma of patients before and after treatment and of healthy subjects, reconstituted in normal human plasma and in factor VII-and factor XII-deficient plasma.**

	Patients				*Healthy subjects*
	Before n-3 fatty acids	*After n-3 fatty acids*	*Before olive oil*	*After olive oil*	
MP-depleted plasma	>600	>600	>600	>600	>600
MP-depleted plasma + MP	280 (270-290)	382* (330-410)	278 (261-292)	300 (292-325)	556 (538-623)
MP-depleted factor VII-deficient plasma + MP	405 (398-410)	461* (400-470)	430 (390-461)	447 (410-450)	564 (555-610)
MP-depleted factor XII-deficient plasma + MP	515 (510-548)	570* (545-580)	538 (510-540)	555 (548-570)	588 (570-567)

*MP: microparticles. Fibrin generation is expressed as time (in seconds) to clotting. Values are shown as median (5^{th}-95^{th} percentile range). *$p<0.05$ between patients before and after n-3 fatty acid treatment.*

ed MP populations in humans.

It is well appreciated that n-3 fatty acids may modulate platelet[24,25] and monocyte functions[26] and that changes in the lipid composition of platelet and monocyte membranes reflect the dietary intake of n-3 fatty acids.[27] For this reason, it is likely that the incorporation of n-3 fatty acids in these cell types modifies membrane properties, such as stability and fluidity,[28] as well as cytosolic calcium concentration, protein localization and functionality in caveolae,[29] reducing cell activation and/or apoptosis, with this translating into lower MP release. Differences in the ability of various cell types to incorporate fatty acids or in their ability to respond functionally to such changes may explain the different cellular responses observed in terms of MP release.

Our data indicate that n-3 fatty acids, but not olive oil, decrease total MP procoagulant activity. Moreover, the inverse correlation between platelet-derived MP with time of fibrin generation by MP after treatment with n-3 fatty acids suggests that decreased platelet-derived MP by n-3 fatty acids are associated with lower procoagulant activity. MP isolated from our patients supported coagulation mainly through the intrinsic pathway, as demonstrated by experiments with factor XII- and factor VII-deficient plasma samples, in keeping with a role of the intrinsic pathway in amplifying coagulation events originally triggered by the TF-dependent pathway.[30] However, in such experiments, the reconstitution of MP from patients treated with n-3 fatty acids in both factor XII- and factor VII-deficient plasma samples resulted in prolongation of clotting times, demonstrating that effects on both pathways account for the action of n-3 fatty acids.

We found similar amounts of TF antigen on MP from both groups of patients before and after treatments. These results agree with those of Grundt *et al.*, who reported that TF antigen was unaffected by long-term treatment with high-dose n-3 fatty acids in post-myocardial infarction patients.[31] However this did not exclude the possibility that n-3 fatty acids decrease the activity of TF exposed on MP, through changes in the phospholipid microenvironment necessary for TF-dependent initiation of coagulation,[32,33] or in the levels of its natural inhibitor, tissue factor pathway inhibitor.[34] Indeed we found less TF-dependent procoagulant activity in patients treated with n-3 fatty acids than in those treated with olive oil.

The heightened procoagulant activity of MP isolated from patients (before both treatments) compared with that in healthy controls was not accompanied by detectably higher levels of *in vivo* markers of thrombin generation (TAT and F_{1+2}). Our patients were, however, studied on average more than 6 months after the qualifying myocardial infarction. A marked increase in F_{1+2} and TAT plasma levels has been reported during the acute phase of myocardial infarction, but such levels usually subsequently decline.[35,36] It has also been reported that such coagulation markers cannot differentiate between control subjects and post-myocardial infarction patients without new ischemic events, while levels are elevated in patients with recurrent ischemia.[35] These differences may be interpreted keeping in mind that *in vivo* coagulation markers are measures of the ongoing activity of coagulation, while measurements of MP are an index of the potential to activate coagulation in the presence of a thrombogenic trigger. Thus, the high MP procoagulant activity before treatment in our patients suggests that post-myocardial infarction patients may have a greater propensity to coagulation activation, compared with healthy controls, in the presence of adequate thrombotic triggers. The presence of MP-related procoagulant activity despite normalization of coagulation markers has been previously reported in patients with meningococcal sepsis.[14] The normal values of *in vivo* coagulation markers before treatment and the different background significance of these tests compared with the measurement of MP number and activity may explain the lack of effect of n-3 fatty acid treatment on *in vivo* markers of coagulation in our study.

In conclusion, in this study we observed higher levels of MP in patients at some time (>6 months) after a myocardial infarction than in healthy controls. We also observed that both the number of platelet- and monocyte-derived MP as well as their procoagulant potential are decreased after n-3 fatty acid administration. Such effects may be part of the complex mechanisms by which n-3 fatty acids exert beneficial effects after myocardial infarction in humans.

Authorship and Disclosures

SDT: main author, did most of the experiments and analyzed results; GB helped with flow cytometry analyses; GL: helped with the coagulation assays; ME and GR: helped with the flow cytometry analysis; PT: provided overall supervision on the study; JHC: main author of the original study from which samples were obtained; EBS: corresponding author of the study from which samples were obtained; RDC: conceived this study and wrote the manuscript. The authors reported no potential conflicts of interest.

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