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THE EFFECT OF N-3 FATTY ACIDS ON PLASMA MYELOPEROXIDASE LEVELS IN HEALTHY ADULTS

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Abstract – Objective: To examine the effect of marine n-3 polyunsaturated fatty acids (PUFA) supplementation in a low to moderate and a high dose on plasma levels of myeloperoxidase (MPO) in healthy individuals. Background: Atherosclerosis is a chronic inflammatory disease and MPO, which is secreted primarily from activated neutrophils and monocytes, has pro-inflammatory properties and has been linked with both initiation and propagation of atherosclerosis. Marine n-3 PUFA have anti-inflammatory properties, but whether n-3 PUFA affect plasma levels of MPO is largely unknown. Methods: Sixthy healthy adults were randomized to three groups receiving either 6.6 g PUFA/day, 2.0 g PUFA/day or a control oil (olive oil) for 12 weeks. Blood samples were drawn at baseline and after exposure. Plasma levels of MPO were measured using a MPO ELISA-kit (from Mercodia, Uppsala, Sweden) with specific mouse monoclonal antibodies. Results: Plasma MPO concentrations (µg/L) at baseline were 36.9 ± 9.4; 36.2 ± 7.1 and 35.4 ± 11.3 (for high dose-, low dose- and control-group, respectively). After 12 weeks of supplementation we found no significant changes in plasma MPO in any of the groups nor between groups, with values after intervention of 36.1 ± 8.6; 37.0 ± 8.2 and 34.4 ± 11.1, respectively. Conclusion: Supplementation with n-3 PUFA has no effect on plasma levels of MPO in healthy adults with low baseline levels of MPO.

Key words: Myeloperoxidase, MPO, atherosclerosis, CAD, Coronary artery disease, CHD, coronary heart disease, n-3 PUFA, n-3 polyunsaturated fatty acids.

INTRODUCTION

Despite the multifactorial nature of atherosclerosis it is increasingly recognized as a chronic inflammatory process. Many of its underlying mechanisms, however, remain to be explored.

Myeloperoxidase (MPO), an enzyme realeased from cytoplasmic granules of activated neutrophil granulocytes and monocytes, catalyzes the formation of powerful oxidants (e.g. HOCL) involved in the destruction of invading microbes. However, MPO can be released to the outside of the cell where its oxidant products can cause damage to adjacent tissue contributing to disease (12). In fact, MPO has been reported to be a potential marker of atherosclerotic cardiovascular disease (18) with elevated levels predicting increased risk of coronary artery disease (CAD) in healthy individuals (15), more severe outcomes in CAD patients(2), and risk of myocardial infarction in patients with chest pain (3). MPO has been linked with both initiation and propagation of atherosclerosis through oxidation of LDL (10,20), creation of dysfunctional HDL, limitation of NO availability causing endothelial dysfunction (1,8,29), activation of metalloproteinases affecting plaque stability (9), and through induction of endothelial cell apoptosis triggering plague erosion and thrombosis (26).

There is substantial evidence indicating beneficial effects of long chain n-3 polyunsaturated fatty acids (PUFA). These fatty

Abbreviations: BMI, body mass index; **CAD,** coronary artery disease; **CV,** coefficient of variation; **DHA,** docosahexaenoic acid; **ELISA,** enzyme linked immunosorbent assay; **EPA,** eicosapentaenoic acid; **HDL,** high density lipoprotein; **hs-CRP,** high sensitivity C-reactive protein; **LDL,** low density lipoprotein; **LVF,** left ventricular function; **MPO,** myeloperoxidase; **PAD,** peripheral arterial disease; **PUFA,** polyunsaturated fatty acids; **ROS,** reactive oxygen species; **STEMI,** ST-elevation myocardial infarction.

acids, especially eicosapentaenoic acid (20:5 n-3; EPA) and docosahexaenoic acids (22:6 n-3; DHA), are found in high proportions in fish oils and fatty fish. The protective mechanism of n-3 PUFA seem to be multifactorial including antiatherogenic, antiarrhythmic, antithrombotic and anti-inflammatory effects. Animal studies have shown that n-3 PUFA supplementation prior to an induction of inflammation, gives lower levels of MPO in tissues compared to control groups (5,19,31) proposing a possible mechanism for one of the beneficial effects of n-3 PUFA on CVD. In this study we aimed to evaluate the effect of supplementation with n-3 PUFA on levels of MPO in healthy adults, and to reveal a possible dose response relationship we compared two different doses of n-3 PUFA with a control oil.

MATERIAL AND METHODS

Subjects and study design

This report is a substudy of a dose-response study regarding the effect of n-3 PUFA on heart rate variability (6). Briefly, 60 healthy volunteers (35 men and 25 women with no known diseases and none taking any medication) participated in a double-blinded, controlled trial. The regional ethics committee approved the study, and all subjects consented to participate.

Dietary supplements

The subjects were randomized to 3 groups, and for a period of 12 weeks they were daily given either

1) a high dose of n-3 PUFA (6.6 g/ day containing 3.0 g EPA and 2.9 g DHA)

2) a low/moderate dose of n-3 PUFA (2.0 g/ day containing 0.9 g EPA and 0.8 g DHA) or

3) a control oil (olive oil, 0.7 g/capsule)

The participants were all provided with 3 capsules in the morning (n-3 PUFA for group 1 and 2, and control oil for group 3) and 7 capsules in the evenings (n-3 PUFA for group 1 and control oil for group 2 and 3) to maintain the blinding.

Laboratory methods

Blood samples were drawn after an overnight fast before and after the 12 weeks of intervention. Blood samples for MPO analysis were collected into tubes containing EDTA as anticoagulant and were kept on ice until centrifugation (within half an hour after collection). Plasma was isolated and stored at -80°C until the analyses were made. Plasma MPO levels were measured using a MPO ELISA-kit from Mercodia (Uppsala, Sweden) - based on the direct sandwich ELISA method with two mouse monoclonal antibodies directed against separate antigenic determinants on the MPO molecule. The test-kit recommended dilution of samples was 1 + 10, however, with this dilution some of the MPO-concentration were lower than the detection limit set by the lowest calibrator. We therefore used a stronger dilution of our samples (1+ 4) after consulting the manufacturer. Plasma concentrations of MPO were determined in double and the results are given as means. The coefficient of variation was 5.1% (at a mean concentration of 36.2 µg/ L).

Serum samples were collected to determine serum lipids using standard methods. High-sensitive C-reactive protein (hs-CRP) was measured with a BNII nephelometer (Dade Behring Marburg GmbH, Marburg, Germany) as described previously (14).

To separate granulocytes for analysis of fatty acid composition whole-blood collected in EDTA of granulocytes was layered on top of a solution of Hypaque Ficoll (Nycomed, Oslo, Norway) and after discontinues gradient centrifugation the granulocytes were harvested into RPMI. The red blood cells were haemolysed and the granulocytes were washed in isotonic phosphate buffer and resuspended in 0.5 ml Sodium-chloride. The vials were then filled with nitrogen and stored at -80°C (28).

Lipids from granulocytes were extracted and fatty acids were methylated and analysed using a CP-9002 Gas chromatograph equipped with CP-Wax 58 CB capillary column (Varian, Middleburg, Nederland) using temperature programming from 40-195 °C and constant flow. Results are expressed as a percentage of total fatty acids content. Further details have previously been described (24).

Statistical analysis

When required for analyses assumptions of normality were checked by visual inspection of histograms and Q-Q plots. Variance homogeneity was checked by Bartlett's test. Paired t-tests were used to assess the possible change in MPO-levels within groups after 12 weeks of supplementation. Differences between the three intervention groups in baseline characteristics and in MPO changes during the period of intervention were tested with one-way ANOVA in case of variance homogeneity and normality – otherwise Kruskal Wallis' test was used.

Possible associations at baseline between plasma MPO and gender, age, BMI, smoking status, hs-CRP, high-density lipoprotein-cholesterol (HDL)-cholesterol, low-density lipoprotein-cholesterol (LDL), and the granulocyte fatty acid content were investigated by multivariate linear regression.

All results of continuous variables are expressed as mean ± SD. $P<0.05$ was considered statistically significant. STATA/SE 10.1 software was used for the analyses.

RESULTS

Before supplementation

The three intervention groups were comparable with no significant differences at baseline regarding gender, age (P= 0.87), smoking status, BMI (P= 0.55), MPO (P= 0.86), hs-CRP (P= 0.12) total cholesterol (P= 0.16), LDL cholesterol (P= 0.51), HDL cholesterol (P= 0.11) and triacylglycerides (P= 0.24) (Table 1) and also in granulocyte content of EPA (P= 0.30) and DHA (P= 0.71).

The mean plasma MPO concentration was low with a value of 36.2 µg/L (SD 9.3). We found no association between baseline MPO and the content of EPA and DHA in granulocytes (P= 0.75), gender (P = 0.44), age (P= 0.59), smoking status (P= 0.24), BMI (P= 0.71), LDL cholesterol (P= 0.93), HDL cholesterol (P= 0.41) or hs-CRP

(P= 0.30).

After supplementation

The dietary supplements were well tolerated and no subjects dropped out of the study. Subjects receiving n-3 PUFA daily for 12 weeks had a significant increase in cellular concentrations of EPA and DHA with the largest increase occurring in the high dose group suggesting satisfying compliance (Table 2). No statistically significant changes in plasma MPO concentrations were observed subsequent to intervention, with values of -0.8 ± 5.8 (P=0.56), 0.7±6.3 (P=0.60) and -1.0 ± 6.3 (P=0.47) µg/ml in the high dose-, low dose- and control-group, respectively. Nor were there any differences regarding change in MPO levels between the three supplementation groups (P=0.62) (Table 2 and Figure 1).

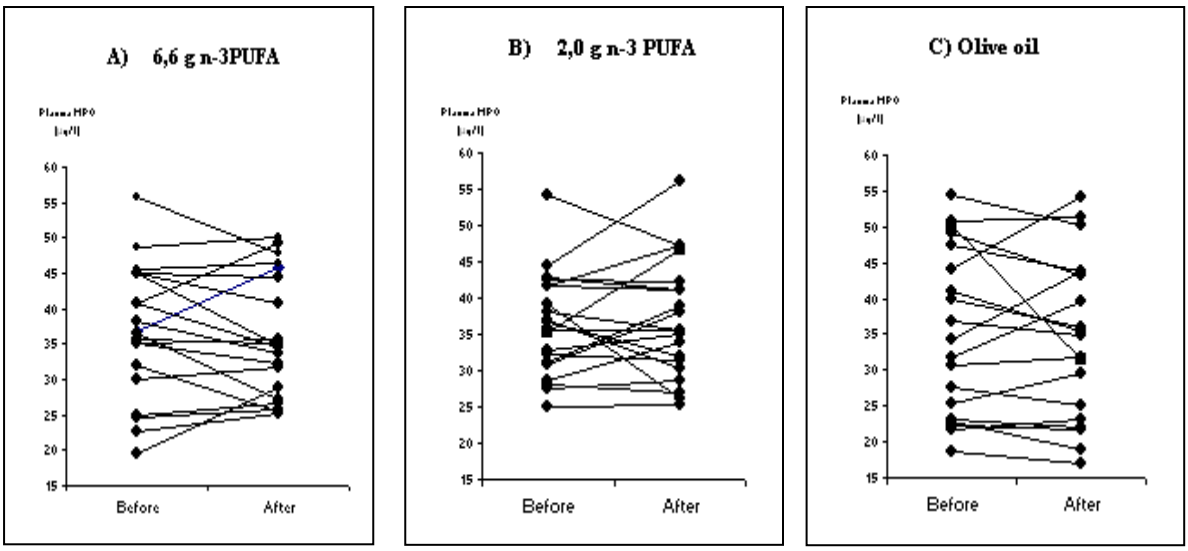

Figure 1. Plasma MPO at baseline and after 12 weeks of supplementation with n-3 PUFA in high dose (A), low dose (B) or with olive oil as control oil (C), with individual results given.

Table 1. Baseline characteristics of the three intervention groups. Values are means ±SD unless otherwise indicated.

	6.6 g n-3 PUFA (n = 20)	2.0 g n-3 PUFA (n = 20)	Control (n = 20)	P-value
Gender: men (n) / women (n)	11 / 9	12 / 8	12 / 8	
Current Smoking, n	5	6	5	
Age, years	39±10	38±10	37±11	0.87
BMI, kg/m2	24.6 ± 3.7	25.1 ± 2.9	24.0 ± 2.7	0.55
MPO, µg/ml	36.9± 9.4	36.2±7.1	35.4± 11.2	0.86
Hs-CRP, mg/L	1.8 ± 2.0	1.1 ± 1.3	1.3 ± 1.5	0.12
Total cholesterol, mmol/L	5.1 ± 1.2	5.0 ± 0.9	5.0 ± 1.1	0.16
LDL cholesterol, mmol/L	3.2 ± 1.1	3.3 ± 0.9	3.0 ± 1.0	0.51
HDL cholesterol, mmol/L	1.3 ± 0.3	1.3 ± 0.2	1.4 ± 0.4	0.11
Triglycerides, mmol/L	1.3 ± 1.2	1.2 ± 0.6	1.2 ± 0.7	0.24

DISCUSSION

In the present study the effect of n-3 PUFA on plasma MPO levels was assessed by randomizing 60 healthy adults into three groups providing them with either a high or a low dose of n-3 fatty acids or a control oil. We found low baseline MPO levels in general and after the 12 weeks of supplementation changes of MPO in the intervention groups were minor and statistically insignificant.

The low baseline MPO levels in the present study may reflect participants being healthy individuals in whom you might expect low levels of immune cell activation. However, MPO levels vary considerably between studies. Relatively high MPO levels have been observed by Dominguez-Rodriguez el al (7) in STEMI patients complicated with cardiogenic shock (mean plasma MPO 69 µg/L) and by Ndrepepa et al (17) in patients with stable CAD or acute coronary syndrome (mean plasma MPO of 74.5 µg/L). Low plasma MPO levels, however, have also been observed in a number of studies (4,21,22). Rudolph et al (21) found mean MPO levels of 24.5 µg/L and 15.5 µg/L in patients with or without impaired left ventricular function, and Cavusoglu et al (4) found a median plasma MPO concentration of 20.3 µg/L in patients with acute coronary syndrome.

These inconsistent MPO levels may not only reflect different study populations but also a dependence on the type of anticoagulant used when blood samples are drawn, and the preanalytical handling of blood samples (time and storage temperature before centrifugation). After blood is drawn, MPO can leak from the leucocytes to plasma leading to falsely increased plasma levels of MPO - increasing by time before separation of plasma and leucocytes (centrifugation). Shih et al (25) recently reported that blood samples collected in EDTA tubes and kept on ice before centrifugation, as in the present study, give the most consistent measurements with less influence of time before centrifugation. These findings were suggested to be due to an inhibitory effect of EDTA and low temperature on leakage of MPO from leukocytes.

In the present study we included both a high and a low dose group to reveal a possible dose-response relationship. However the changes in plasma MPO were minimal in all intervention groups. These findings indicate that n-3 PUFA have no effect on plasma MPO levels in healthy subjects with low baseline plasma MPO. Regarding the duration of our study, 12 weeks are sufficient to change the cellular content of fatty acids (table 2). Moreover, earlier studies have after only 4 (27) and 6 (13,23) weeks of n-3 PUFA supplementation shown an inhibitory effect on activation of neutrophil granulocytes (with respect to chemotaxis).

Our results differ from a previous study in which 33 healthy women divided in two groups were provided with 2.4 g/day of n-3 PUFA with or without vitamin E for 4 weeks (30). MPO data, however, were given only for 25 of the participants. There was a significant decrease in both groups with baseline plasma MPO levels of 60.4 µg/L in the group with vitamin E and 70.1 µg/L in the group without vitamin E. After 4 weeks of supplementation plasma MPO concentrations were 34.6 µg/L and 34.8 µg/L, respectively which are similar to the levels in our study. Heparin was used as an anticoagulant when blood samples were drawn, making plasma concentrations more dependent on time before centrifugation. The period of time between blood being drawn and centrifugation is not given in the article. If a control group had been included in the study showing no change in plasma MPO, differences in preanalytical handling as a reason for the high baseline levels and observed decrease would be more unlikely.

In another controlled trial Schiano et al (22) measured plasma MPO in 16 patients with peripheral arterial disease (PAD) before and after 3 months' of supplementation with 2 g n-3 PUFA daily in addition to their previous treatment. Another 16 patients continued with their pre-enrolment therapy without any modification serving as control group. After 3 months, plasma MPO remained unchanged in both groups, consistent with our results. Schiano et al also observed low baseline myeloperoxidase levels (20.2 µg/L in invention group and 18.3 µg/L in control group), despite the fact that participants were patients with PAD.

Limitations of the study

Our study included relatively few subjects, which makes it more difficult to detect a possible effect of the intervention, increasing the risk of a statistical type II error. Furthermore, only healthy individuals with low baseline levels of MPO participated in our trial.

Storage of samples at –80 °C for 12 years may have affected the MPO concentrations. However, this would affect all samples – both before and after supplementation, and therefore

Table 2. The plasma MPO concentrations and the content of EPA and DHA in granulocytes in the 3 treatment groups before and after dietary supplementation for 12 weeks with 6.6 g n-3 PUFA daily , 2.0 g n-3 PUFA daily, or control oil. All values are presented as mean ± SD.

	6.6 g n-3 PUFA (n = 20)			2.0 g n-3 PUFA (n = 20)			Control (n = 20)		
	Before	After	Difference[a]	Before	After	Difference[a]	Before	After	Difference[a]
Plasma Myeloperoxidase									
MPO (μg/L)	36.9±9.4	36.1±8.6	-0.8±5.8	36.2±7.1	37.0±8.2	0.7±6.3	35.4±11.3	34.4±11.1	-1.0±6.3
n-3 PUFA in granulocytes (% of total fatty acids)[b]									
EPA	0.58±0.2	4.07±1.0[c]	3.49±1.0[e]	0.56±0.5	1.82±0.8[c]	1.24±0.6[d]	0.51±0.2	0.55±0.3	0.04±0.3
DHA	1.58±0.5	2.14±0.4[c]	0.56±0.4[d]	1.48±0.4	1.85±0.6[c]	0.36±0.4[d]	1.54±0.4	1.54±0.5	0.01±0.4

[a]Defined as after-before.
[b]Values and statistics are from the initial study[13]
[c]Significantly different from before supplementation (paired t test), $P < 0.01$
[d]Significantly different from olive oil, $P < 0.05$.
[e]Significantly different from olive oil and the 2.0-g n-3 PUFA group, $P < 0.05$ (one-way ANOVA).

the size of the MPO changes during intervention should not be affected. In the present study olive oil was used as a control oil. However, large intakes of some phenolic compounds found in olive oil have been associated with lower incidence of CAD (11). This observation may partly be ascribed to an antiinflammatory effect of olive oil (16), but the concentrations, at which an antiinflammatory effect of phenolic compounds has been detected, are unlikely to be achieved in vivo (16).

In conclusion, the present study could not demonstrate any effect of supplementation with a high (6.6 g/day) or a low to moderate (2 g/day) dose of n-3 PUFA on plasma levels of MPO in healthy individuals with low baseline MPO. However, our findings do not rule out an effect of n-3 PUFA on MPO in patients with atherosclerotic diseases or individuals with higher levels of MPO.

Other articles in this theme issue include references (32-44).

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