Expert Opinion

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Effects of long chain ω-3 fatty acids on metalloproteinases and their inhibitors in combined dyslipidemia patients

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We evaluate the effect of a standardized dietary supplementation with ω-3 polyunsaturated fatty acids (n-3 PUFAs) on the level of some markers of vascular remodeling in patients with combined dyslipidemia. Three hundred and thirty-three patients received placebo or n-3 PUFAs for 6 months. We evaluated body mass index, glycemic profile, blood pressure, lipid profile, lipoprotein(a), plasminogen activator inhibitor-1, homocysteine, fibrinogen, high-sensitivity C reactive protein, ADP, MMP-2 and MMP-9, and tissue inhibitors of metalloproteinase-1 and -2. A significant increase of high-density lipoprotein-cholesterol, and a significant decrease of triglycerides were present after 3 and 6 months with n-3 PUFAs intake. A significant plasminogen activator inhibitor-1, fibrinogen and high-sensitivity C reactive protein decrease was obtained after 3 and 6 months and a significant ADP increase was observed after 3 and 6 months of n-3 PUFAs. A significant MMP-2, MMP-9, tissue inhibitors of metalloproteinase-1 and tissue inhibitors of metalloproteinase-2 decrease was obtained after 6 months compared to the baseline value with n-3 PUFAs intake. n-3 PUFAs give a better lipid profile and a better improvement of coagulation, fibrinolytic and inflammatory parameters than placebo. Furthermore, lowers levels of MMP-2, MMP-9 and their tissue inhibitors are obtained with n-3 PUFAs compared to placebo.

Keywords: combined dyslipidemia, inflammatory parameters, matrix metalloproteinases, tissue inhibitors of metalloproteinases, ω-3 polyunsaturated fatty acids

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1. Introduction

The inverse association between dietary intake of ω-3 polyunsaturated fatty acids (n-3 PUFAs) and cardiovascular disease morbidity/mortality was primarily established following the observation that the Greenland Inuits had low mortality from coronary heart disease despite a fat-rich diet. In the 1970s, some Danish investigators proposed that this could be because of the n-3 PUFAs high content in the Inuit diet, which consisted largely of fish, seal and whale [1]. Plasma n-3 PUFAs concentrations are highly correlated with dietary n-3 PUFAs [2]. The inverse association between n-3 PUFAs intake and stroke incidence observed in different longitudinal epidemiological studies [3,4], even reflected in a slower age-related cognitive decline [5], supports the hypothesis that n-3 PUFAs could play a significant role in modulating also the systemic arterial tree. However, other authors suggest that the observed inverse relationship between n-3 PUFAs consumption and stroke incidence is the consequence of the atrial fibrillation

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preventive action of n-3 PUFAs [6]. This preventive action is effective and safe in a base population of patients with heart failure [7]. Nevertheless, it could be also possible that a small but significant blood pressure decreasing effect of n-3 PUFAs contribute to their cardiovascular disease preventive effect [8].

Beyond their well-known antiarhythmic effects and triglycerides lowering action [6], the systemic vascular disease protection induced by n-3 PUFAs could be also related to a direct modulation of arterial wall matrix metabolism, and in particular by stabilization of atherosclerotic plaque, as demonstrated by preclinical and clinical evidences [9-11]. In this context, the aim of our study is to evaluate the effect of a standardized dietary supplementation with n-3 PUFAs on the level of some markers of vascular remodeling, such as MMP-2 and -9 and tissue inhibitors of metalloproteinase (TIMP-1 and TIMP-2), in patients affected by combined dyslipidemia.

2. Materials and methods

2.1 Study design

This multi-center, case-control, placebo, randomized trial was conducted at the Department of Internal Medicine and Therapeutics, University of Pavia (Pavia, Italy); and the "G. Descovich" Atherosclerosis Study Center, Department of Internal Medicine, Aging and Kidney diseases, University of Bologna, (Bologna, Italy).

Patients received placebo or n-3 PUFAs (ethylic esters, EPA and DHA in the proportion of 0.9 – 1.5), 3 g/day (three times a day, during the meals), for 6 months.

Randomization was done using a drawing of envelopes containing randomization codes prepared by a statistician. A copy of the code was provided only to the responsible person performing the statistical analysis. The code was only broken after a database lock, but could have been broken for individual subjects in case of an emergency. Medication compliance was assessed by counting the number of pills returned at the time of specified clinic visits. At baseline, we weighed participants and gave them a bottle containing a supply of study medication for at least 100 days. Throughout the study, we instructed patients to take their first dose of new medication on the day after they were given the study medication. A bottle containing placebo (sucrose, mannitol and mineral salts) or the new study medication (kindly given to the authors by the pharmaceutical companies) for the next treatment period was given to participants every 3 months. At the same time, all unused medication was retrieved for inventory. All medications were provided free of charge.

Standard diet advice was given by a dietitian and/or specialist doctor. Dietitian and/or specialist doctor periodically provided instruction on dietary intake recording procedures as part of a behavior modification program and then later used the subject's food diaries for counseling. Individuals were also encouraged to increase their physical activity by walking briskly for 20 – 30 min 3 – 5 times a week or by cyclette. The recommended changes in physical activity throughout the study were not assessed.

The study protocol was approved at each site by institutional review boards and was conducted in accordance with the Declaration of Helsinki and its amendments.

2.2 Study population

Caucasian patients aged ≥ 18 years of either sex were eligible for inclusion in the study if they had combined dyslipidemia (defined by the International Lipid Information Bureau) [12], identified by total cholesterol (TC) > 200 mg/dl and triglycerides (Tg) > 200 mg/dl, and who had never previously taken lipid-lowering medications.

They were 333 patients (164 males and 169 females), identified from review of case notes and/or computerized clinic registers, who were contacted by the investigators in person or by telephone.

Patients were excluded if they had a genetic condition affecting lipid metabolism (e.g., familial hypercholesterolemia, type III hyperlipidemia, LPL deficiency, etc.); a history of microalbuminuria or nephrotic syndrome; an impaired hepatic function (defined as plasma aminotransferase and/or γ-glutamyltransferase level higher than the upper limit of normal for age and sex); an impaired renal function (defined as serum creatinine level higher than the upper limit of normal for age and sex); thyroid diseases; endocrine or metabolic disease; a history of alcohol or drug abuse; a neoplastic, infectious or autoimmune disease; poor mental condition or if they were taking any other drug that was able to influence lipid metabolism. Patients with serious cardiovascular disease (e.g., New York Heart Association class I–IV congestive heart failure or a history of myocardial infarction or stroke) or cerebrovascular conditions in 6 months before study enrollment were also excluded. All patients provided written informed consent to participate.

2.3 Assessments

Before starting the study, all patients underwent an initial screening assessment that included a medical history, physical examination, vital signs, a 12-lead electrocardiogram, measurements of fasting plasma glucose (FPG), fasting plasma insulin (FPI), homeostasis model assessment (HOMA index), blood pressure, lipid profile, coagulation, fibrinolytic and inflammation parameters, MMP-2, MMP-9, TIMP-1 and TIMP-2.

All parameters were determined in a fasting state, after a 12-h overnight fast, in the plasma. Venous blood samples were taken for all patients between 08.00 and 09.00 and were drawn from an antecubital vein with a 19-gauge needle without venous stasis.

We used plasma obtained by addition of Na_2-EDTA, 1 mg/ml, and centrifuged at 3000 g for 15 min at 4°C. Immediately after centrifugation, the plasma samples were frozen and stored at -80°C for not > 3 months. All measurements were performed in a central laboratory.

Table 1. General characteristics of the dyslipidemic patients at baseline.

	Placebo	n-3 PUFAs
N	165	168
Sex (M/F)	82/83	82/86
Age (years)	50.7 ± 6.8	51.3 ± 7.2
Current smoking (n)	41	37
Comb. dysl. dur. (years)	7.1 ± 2.0	7.4 ± 2.3
Height (m)	1.68 ± 0.05	1.69 ± 0.06
Weight (Kg)	73.4 ± 9.1	74.8 ± 9.4
BMI (Kg/m^2)	26.0 ± 1.1	26.2 ± 1.3
Waist circumference (cm)	97.8 ± 2.2	98.6 ± 2.5
Hip circumference (cm)	105.9 ± 3.0	107.4 ± 3.2
W:H ratio	0.92 ± 0.1	0.92 ± 0.1
Abdominal circumference (cm)	95.4 ± 7.1	96.8 ± 7.3

Data are means ± s.d.
BMI: Body mass index; Comb. dysl. dur.: Combined dyslipidemia duration; n-3 PUFA: ω-3 Polyunsaturated fatty acid; W:H: Weight:height.

Table 2. Data at baseline and after placebo in dyslipidemic patients.

	Baseline	3 months	6 months
N	165	163	162
Sex (M/F)	82/83	81/82	81/81
BMI (Kg/m^2)	26.0 ± 1.1	26.2 ± 1.2	25.9 ± 1.1
FPG (mg/dl)	88.1 ± 8.7	87.4 ± 8.6	87.0 ± 8.5
FPI (μU/ml)	8.1 ± 1.7	8.2 ± 1.8	8.1 ± 1.7
HOMA index	1.7 ± 0.8	1.7 ± 0.8	1.7 ± 0.8
SBP (mmHg)	129.6 ± 6.8	129.2 ± 6.7	128.1 ± 6.6
DBP (mmHg)	81.4 ± 7.1	81.1 ± 7.0	80.8 ± 6.9
TC (mg/dl)	227.5 ± 16.3	225.2 ± 15.9	220.1 ± 15.5
LDL-C (mg/dl)	149.9 ± 7.5	147.9 ± 7.1	148.1 ± 6.5
HDL-C (mg/dl)	39.7 ± 5.1	40.2 ± 5.3	40.6 ± 5.4
Tg (mg/dl)	189.3 ± 41.8	185.6 ± 40.3	182.1 ± 39.5
Lp(a) (mg/dl)	9.3 ± 14.9	9.2 ± 14.7	9.1 ± 14.5
PAI-1 (ng/ml)	37.2 ± 8.1	36.9 ± 8.0	37.4 ± 8.2
Hct (μmol/l)	12.1 ± 3.0	12.1 ± 3.0	12.5 ± 3.2
Fg (mg/dl)	386.2 ± 47.6	379.8 ± 46.1	375.1 ± 44.7
Hs-CRP (mg/dl)	1.6 ± 0.8	1.5 ± 0.7	1.5 ± 0.7
ADP (μg/ml)	5.5 ± 0.8	5.6 ± 0.9	5.5 ± 0.8

Data are means ± s.d.
BMI: Body mass index; DBP: Diastolic blood pressure; Fg: Fibrinogen;
FPG: Fasting plasma glucose; FPI: Fasting plasma insulin; Hct: Homocysteine;
HDL-C: High-density lipoprotein-cholesterol; HOMA index: Homeostasis model assessment index; Hs-CRP: High-sensitivity C reactive protein;
LDL-C: Low-density lipoprotein-cholesterol; Lp(a): Lipoprotein(a); PAI-1: Plasminogen activator inhibitor-1; SBP: Systolic blood pressure;
TC: Total cholesterol; Tg: Triglycerides.

Body mass index (BMI) was calculated by the investigators as weight in kilograms divided by the square of height in meters. The estimate of insulin resistance was calculated by HOMA index with the formula: FPI (μU/ml) × FPG (mmol/l)/22.5, as described by Matthews *et al.* [13].

Blood pressure (BP) measurements were obtained from each patient (using the right arm) in the seated position, using a standard mercury sphygmomanometer (Erkameter 3000, ERKA, Bad Tolz, Germany) (Korotkoff I and V) with a cuff of appropriate size. BP was measured by the same investigator at each visit, in the morning, after the patient had rested for ≥10 min in a quiet room. Three successive BP readings were obtained at 1 min intervals, and the mean of the three readings was calculated.

Plasma glucose was assayed by glucose-oxidase method (GOD/PAP, Roche Diagnostics, Mannheim, Germany) with intra- and inter-assay coefficients of variation (CsV) of < 2% [14]. Plasma insulin was assayed with Phadiaseph Insulin RIA (Pharmacia, Uppsala, Sweden) by using a second antibody to separate the free and antibody-bound ^{125}I-insulin (intra- and inter-assay CsV: 4.6 and 7.3%, respectively) [15].

TC and Tg levels were determined using fully enzymatic techniques [16,17] on a clinical chemistry analyzer (HITACHI 737; Hitachi, Tokyo, Japan); intra- and inter-assay CsV were 1.0 and 2.1 for TC measurement, and 0.9 and 2.4 for Tg measurement, respectively. High-density lipoprotein-cholesterol (HDL-C) level was measured after precipitation of plasma apo B-containing lipoproteins with phosphotungstic acid [18]; intra- and inter-assay CsV were 1.0 and 1.9, respectively; and low-density lipoprotein-cholesterol level was calculated by the Friedewald formula [19].

Plasminogen activator inhibitor-1 (PAI-1) was assayed with a commercial two-stage indirect enzymatic assay (Spectrolyse, Biopool AB, Umea, Sweden); intra- and inter-assay CsV were 5.9% [20]. Fibrinogen (Fg) was determined according to Clauss. The intra-assay CV for the Fg method was < 5% [21].

Homocysteine was measured by a modified procedure of Araki and Sako [22] with high-pressure liquid chromatography and fluorescence detection. The intra-assay CV of the method was 2.5%.

High-sensitivity C-reactive protein (Hs-CRP) was measured with the use of latex-enhanced immunonephelometric assays on a BN II analyzer (Dade Behring, Newark, DE, USA). The intra- and inter-assay CsV were 5.7 and 1.3%, respectively [23].

Lipoprotein (a) [Lp(a)] was measured by a sandwich ELISA method, which is insensitive to the presence of plasminogen, using the commercial kit Macra-Lp(a) (SDI, Newark, DE, USA) [24,25]; the intra- and inter-assay CsV of this method were 5 and 9%, respectively.

Adiponectin level was determined using ELISA kits (B-Bridge International, Inc., Sunnyvale, CA, USA). The intra-assay CsV were 3.6% for low and 3.3% for high control samples, whereas the inter-assay CsV were 3.2% for low and 7.3% for high control samples [26].

Table 3. Data at baseline and after n-3 PUFAs therapy in dyslipidemic patients.

	Baseline	3 months	6 months
N	168	166	164
Sex (M/F)	82/86	81/85	80/84
BMI (Kg/m^2)	26.2 ± 1.3	25.9 ± 1.1	26.1 ± 1.2
FPG (mg/dl)	87.8 ± 8.5	86.3 ± 8.4	86.1 ± 8.3
FPI (μU/ml)	8.3 ± 1.9	8.5 ± 2.1	8.4 ± 2.0
HOMA index	1.8 ± 0.9	1.8 ± 0.9	1.8 ± 0.9
SBP (mmHg)	128.4 ± 6.5	126.1 ± 6.2	126.9 ± 6.3
DBP (mmHg)	80.6 ± 6.8	79.8 ± 6.6	80.2 ± 6.7
TC (mg/dl)	223.4 ± 15.7	217.6 ± 14.3	213.8 ± 14.0
LDL-C (mg/dl)	148.5 ± 7.2	145.4 ± 6.9	147.4 ± 7.0
HDL-C (mg/dl)	38.4 ± 4.2	41.7 ± 5.8*	43.2 ± 6.3‡§
Tg (mg/dl)	182.6 ± 39.7	152.6 ± 29.4*§	116.2 ± 24.6‡¶
Lp(a) (mg/dl)	9.1 ± 14.3	9.0 ± 14.1	8.8 ± 13.8
PAI-1 (ng/ml)	36.1 ± 7.8	31.4 ± 7.1*	26.5 ± 6.4‡§
Hct (μmol/l)	12.3 ± 3.1	11.2 ± 2.9	11.6 ± 3.0
Fg (mg/dl)	394.8 ± 49.1	328.6 ± 45.3*	303.8 ± 42.6‡§
Hs-CRP (mg/dl)	1.4 ± 0.6	1.0 ± 0.5*§	0.8 ± 0.4‡¶
ADP (μg/ml)	5.7 ± 1.0	6.9 ± 1.8*§	7.6 ± 2.0‡¶

Data are means ± s.d.
*p < 0.05 versus baseline.
‡p < 0.01 versus baseline.
§p < 0.05 versus placebo.
¶p < 0.01 versus placebo.
BMI: Body mass index; DBP: Diastolic blood pressure; Fg: Fibrinogen; FPG: Fasting plasma glucose; FPI: Fasting plasma insulin; Hct: Homocysteine; HDL-C: High-density lipoprotein-cholesterol; HOMA index: Homeostasis model assessment index; Hs-CRP: High-sensitivity C reactive protein; LDL-C: Low-density lipoprotein-cholesterol; Lp(a): Lipoprotein(a); n-3 PUFA: ω-3 Polyunsaturated fatty acid; PAI-1: Plasminogen activator inhibitor-1; SBP: Systolic blood pressure; TC: Total cholesterol; Tg: Triglycerides.

MMP-2, MMP-9, TIMP-1 and TIMP-2 levels were determined by a two-site ELISA method using commercial reagents (Amersham Biosciences, Uppsala, Sweden). The intra- and inter-assay CsV for measuring MMP-2 levels were 5.4 and 8.3%, respectively [27]. The intra- and inter-assay CsV to evaluate MMP-9 levels were 4.9 and 8.6%, respectively [28]. The intra- and inter-assay CsV for measuring TIMP-1 levels were 9.3 and 13.1%, respectively [29], whereas those for measuring TIMP-2 levels were 5.4 and 5.9%, respectively [30].

2.4 Statistical analysis

An intention-to-treat analysis was conducted in patients who had received ≥ 1 dose of study medication and had a subsequent efficacy observation. Patients were included in the tolerability analysis if they had received ≥ 1 dose of trial medication and had undergone a subsequent tolerability observation. Considering as clinically significant a difference of at least 10% compared to the baseline and an α error of 0.05, the actual sample size is adequate to obtain a power higher than 0.80 for all measured variables.

Continuous variables were compared by analysis of variance. Intervention effects were adjusted for further potential confounders using analysis of covariance. Analysis of variance was also used to assess the significance in and between the groups. Non-parametric tests were also used in the statistical analysis of the data because some parameters were not normally distributed (Kolmogorov-Smirnov test). Mann-Whitney U test was used to compare two independent groups. Outcome variables with a skewed distribution were transformed to a log scale before statistical testing. The statistical significance of the independent effects of treatments on the other variables was determined using analysis of covariance. A 1-sample t test was used to compare values obtained before and after treatment administration; 2-sample t tests were used for between-group comparisons. The Bonferroni correction for multiple comparison was also carried out [31]. Statistical analysis of data was performed using the Statistical Package for Social Sciences software version 11.0 (SPSS, Inc., Chicago, IL, USA). Data were presented as mean ± s.d. For all statistical analyses, p < 0.05 was considered statistically significant.

3. Results

3.1 Study sample

A total of 333 patients were enrolled in this trial. The characteristics of the patient population at the baseline are shown in Table 1, whereas the characteristics of the patient population after 3 and 6 months of placebo or n-3 PUFAs intake are reported in Tables 2 and 3.

3.2 BMI

No significant BMI change was observed in dyslipidemic patients after 3 and 6 months of n-3 PUFAs intake or placebo compared to the baseline value.

3.3 Glycemic control

No significant FPG, FPI and HOMA index variations were obtained after 3 and 6 months of therapy with n-3 PUFAs or placebo compared to the baseline values.

3.4 BP control

No significant systolic blood pressure or diastolic blood pressure changes were present after 3 and 6 months of n-3 PUFAs or placebo intake.

3.5 Lipid profile and lipoprotein variables

No significant TC or low-density lipoprotein-cholesterol changes were observed after 3 and 6 months of n-3 PUFAs therapy or placebo compared to the baseline values, whereas a significant HDL-C increase was obtained after 3 and

Table 4. MMP-2, MMP-9, TIMP-1 and TIMP-2 levels at baseline and after 6 months of placebo in dyslipidemic patients.

	Baseline	6 months
MMP-2 levels, means (ng/ml) ± DS, median (ng/ml) (IQR)	1274.3 ± 152.8 1296.5 (1244.2 – 1493.6)	1251.9 ± 148.4 1271.6 (1221.4 – 1408.7)
MMP-9 levels, means (ng/ml) ± DS, median (ng/ml) (IQR)	501.6 ± 53.4 505.8 (442.6 – 539.1)	507.8 ± 59.6 511.6 (456.4 – 548.9)
TIMP-1 levels, means (ng/ml) ± DS, median (ng/ml) (IQR)	505.4 ± 45.8 513.6 (470.9 – 561.6)	494.8 ± 40.7 501.2 (441.6 – 530.4)
TIMP-2 levels, means (ng/ml) ± DS, median (ng/ml) (IQR)	101.2 ± 7.0 103.4 (95.4 – 108.2)	107.1 ± 7.5 109.8 (101.2 – 118.6)

Data are means ± s.d., median and IQR.
IQR: Interquartile range; TIMP-1: Tissue inhibitors of metalloproteinase-1; TIMP-2: Tissue inhibitors of metalloproteinase-2.

Table 5. MMP-2, MMP-9, TIMP-1 and TIMP-2 levels at baseline and after 6 months of n-3 PUFAs therapy in dyslipidemic patients.

	Baseline	6 months
MMP-2 levels§	1242.7 ± 141.4 1272.8 (1225.1 – 1415.8)	935.1 ± 126.8 953.4 (901.5 – 1126.2)*‡
MMP-9 levels§	506.3 ± 58.7 512.6 (463.9 – 552.4)	219.7 ± 31.5 228.9 (201.3 – 264.6)*‡
TIMP-1 levels§	496.2 ± 42.6 505.8 (457.2 – 539.8)	283.1 ± 33.4 299.8 (257.6 – 321.2)*‡
TIMP-2 levels§	104.7 ± 7.3 105.1 (99.4 – 112.5)	89.5 ± 6.1 92.3 (81.8 – 96.7)*‡

*p < 0.001 versus baseline.
‡p < 0.001 versus placebo.
§Data are means (ng/ml) ± s.d., median (ng/ml) and IQR.
IQR: Interquartile range; n-3 PUFA: ω-3 Polyunsaturated fatty acid;
TIMP-1: Tissue inhibitors of metalloproteinase-1; TIMP-2: Tissue inhibitors of metalloproteinase-2.

6 months of therapy ($p < 0.05$ and $p < 0.01$, respectively) with PUFAs but not with placebo. A significant Tg decrease was present after 3 and 6 months ($p < 0.05$ and $p < 0.01$, respectively) of n-3 PUFAs therapy in dyslipidemic patients compared to the baseline, whereas no significant change was observed with placebo. There were no variations in Lp(a) value in both groups. In the group treated with n-3 PUFAs, HDL-C was higher after 6 months ($p < 0.05$), and Tg value was lower after 3 and 6 months ($p < 0.05$ and $p < 0.01$, respectively) compared to the group treated with placebo.

3.6 Coagulation, fibrinolytic and inflammatory parameters

A significant PAI-1 decrease was obtained after 3 and 6 months ($p < 0.05$ and $p < 0.01$, respectively) of n-3 PUFAs therapy but not with placebo compared to the baseline, whereas no significant homocysteine change was observed for both groups. Significant Fg and Hs-CRP decreases were present after 3 and 6 months ($p < 0.05$ and $p < 0.01$, respectively) of n-3 PUFAs therapy, whereas a significant ADP increase was obtained after 3 and 6 months ($p < 0.05$ and $p < 0.01$, respectively) compared to the baseline. No variations of all these parameters were observed in the group treated with placebo. In the group treated with n-3 PUFAs, PAI-1 and Fg values were lower after 6 months (both $p < 0.05$), whereas ADP value was higher after 3 and 6 months ($p < 0.05$ and $p < 0.01$, respectively) compared to the group treated with placebo. Furthermore, Hs-CRP was lower after 3 and 6 months ($p < 0.05$ and $p < 0.01$, respectively) in the group treated with n-3 PUFAs compared to placebo.

3.7 Enzymatic characterization

MMP-2, MMP-9, TIMP-1 and TIMP-2 levels quantified in the dyslipidemic population at the baseline and after 6 months of placebo or n-3 PUFAs intake are reported in **Tables** 4 and 5. A significant MMP-2, MMP-9, TIMP-1 and TIMP-2 decrease ($p < 0.001$) was obtained after 6 months of n-3 PUFAs therapy with respect to the baseline (**Figure** 1), whereas no differences were obtained with placebo. After 6 months, MMP-2, MMP-9, TIMP-1 and TIMP-2 levels were lower ($p < 0.001$) in the group treated with n-3 PUFAs compared to the group treated with placebo.

4. Discussion

The vascular changes associated with the plaques fragility involve both cellular and extracellular components. Among extracellular active substances, MMPs have been postulated to be relevant for atherothrombotic cardiovascular diseases [32]. In fact, circulating MMPs levels are elevated in patients with acute myocardial infarction, unstable angina and also after coronary angioplasty, which is related

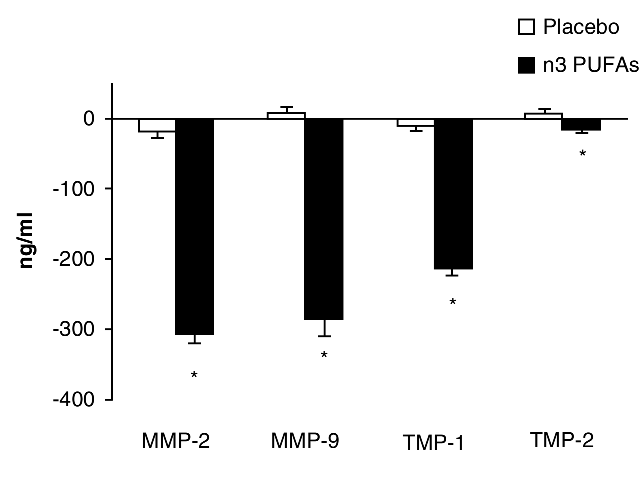

Figure 1. Decrease of MMP-2, MMP-9, TIMP-1 and TIMP-2 levels during placebo and n-3 PUFAs therapy in dyslipidemic patients.
Data are means ± s.d.*p < 0.001 versus placebo.n-3 PUFA: ω-3 Polyunsaturated fatty acid; TIMP-1: Tissue inhibitors of metalloproteinase-1; TIMP-2: Tissue inhibitors of metalloproteinase-2.

to late loss index after the procedure [33]: these observations suggest that MMP expression may be not only related to instability of the plaque, but also to the formation of restenotic lesions.

Previous epidemiological data also showed that both MMP-9 and TIMP-1 plasma levels are markedly increased in coronary artery with unstable atherosclerotic plaque of patients affected by acute coronary syndrome [34]: because TIMP-1 is a potent inhibitor of MMP-9, its increase during the acute phase of acute myocardial infarction may indicate the induced production of MMP-9 in the infarctuated myocardium [35]. We have previously shown that MMP-9 remains significantly elevated in diabetic patients also after 3 months from an acute coronary syndrome [36].

However, even if significant elevation of MMPs and their inhibitors is related to acute coronary events [37], some evidence suggests that they are also slightly, but significantly, increased in some conditions associated with an augmented risk of developing cardiovascular diseases, such as uncomplicated hypertension [38], type 1 diabetes [39], type 2 diabetes [40], obesity [41] as well as dyslipidemia [42].

Beyond doxazosin [43], there is no clear evidence that other preventive treatments are associated with a change in MMP levels. Treatment with n-3 PUFAs have a therapeutic indication for the management of dyslipidemias characterized by a prevalent hypertriglyceridemia [44]. As expected from the available literature [44], in our study we observed that a 6-month treatment with n-3 PUFAs, 3 g/day, significantly improves the plasma level of Tg (-46.6%), HDL-C (+12.5%), PAI-1 (-26.6%), Fg (-23.1%), Hs-CRP (-42.9%) and ADP (+33.3%). However, n-3 PUFAs intake was also associated with a significant reduction in MMP-2 (-24.8%), MMP-9 (-56.5%), TIMP-1 (-43.2%) and TIMP-2 (-14.8%).

These data are in contrast with that recently reported by Furenes *et al.* [45], which did not note any effect of diet or n-3 PUFA supplementation on MMP-9 and TIMP-1 levels. However, in their study, these authors tested a lower daily dose of n-3 PUFAs (2.4 g/day) and they enrolled very different patients, such as smokers and non-smokers, patients with or without history of acute miocardial infarction. In particular, it was already known that MMP-9 level is not influenced by ω-3 supplementation in patients with a history of myocardial infarction [46], maybe because in these patients the baseline MMP-9 level is too high and stabilized by the stimulus of a severe chronic disease, such as coronary heart disease [42].

Moreover, we observed an increase of ADP concentration after treatment with n-3 PUFAs, despite a substantial stability of BMI during the study. We expected a significant reduction in BMI, because subjects were under strict dietary control. Lack of change in BMI after a 6-month controlled energy-diet may be partially explained by a low adherence to dietary advice and a less strict control on physical activity. In fact, patients were advised to undertake regular physical exercise, but this was not personalized for weight loss. Moreover, some patients may have been previously treated with reduced energy diets, to correct dyslipidemia, so that they were resistant to further weight loss.

As regarding ADP, there are data in literature suggesting an indirect effect of this cytokine on MMPs. Results from experimental studies *in vitro* showed a role for ADP in increasing TIMP-1 expression in human monocyte-derived macrophages through IL-10 induction [47]. In a recent study, data in humans demonstrated a significant inverse relation between ADP and MMP-9/TIMP-1 ratio and a direct relation between ADP and IL-10, thus confirming an interaction between ADP and MMPs through mechanisms that might be independent of significant variations in insulin-sensitivity [48].

A possible role of n-3 PUFAs on insulin-sensitivity has been suggested in different studies [49,50]: n-3 PUFAs reduce adiposity and some atherogenic factors, protecting against high-fat diet induced insulin-resistance and giving a subsequent decrease in intracellular lipid abundance except in patients with type 2 diabetes [51]. Results from experimental studies showed also that n-3 PUFAs from fish oil activate expression and secretion of ADP in adipocytes [52,53].

Dietary n-3 PUFAs activate PPAR-α and -γ increasing lipid oxidation and decreasing insulin resistance, leading in a reduction of hepatic steatosis [54].

Moreover, n-3 PUFAs seem to reduce phosphatidyl inositol 3′ kinase activity and deplete the glucose transporter protein (GLUT4) in the muscle as long as its expression is in adipose tissue.

In our study, we observed an increase of ADP but did not observe any change of HOMA index. At the first glimpse, this could seem contradictory with the literature reported above. We think that the no change in HOMA index despite the increase of ADP is owing to the inclusion criteria we chose: we excluded patients with metabolic diseases or with

a higher cardiovascular risk. Besides, insulin sensitivity decreases in obese patients because of the tissue insulin resistance; our patients were not obese, they were slightly above the normal BMI value, BMI was 26.2 kg/m^2 for the n-3 PUFAs group and 26.0 kg/m^2 for the placebo one. Insulin sensitivity decreases in obese or diabetic patients but we enrolled patients with only combined dyslipidemia, and so they did not have an important insulin resistance and this can explain the lack of improvement with n-3 PUFAs.

Our findings about ADP concentration, MMP-2 and MMP-9 after treatment with n-3 PUFAs in combined dyslipidemia agree with the recent acknowledgements in this field. Also, a longer period of follow-up is necessary to establish the role of n-3 PUFA in precocious modification of inflammatory parameters despite stability of insulin-sensitivity.

Our study has some relevant limitations, such as the relatively limited number of endothelial dysfunction biomarkers studied, the absence of a direct evaluation of endothelial dysfunction and/or vascular integrity. Moreover, we did not perform statistical correlations between MMPs and their inhibitors and the other inflammatory parameters assessed.

However, after an attentive literature search on Med-Line and Embase, we believe that we are the first to describe the change of MMP-2 and MMP-9 plasma levels and their tissue inhibitors after treatment with full-dosed n-3 PUFAs in a large sample of patients with combined dyslipidemia in primary prevention for cardiovascular disease.

In conclusion, we observed that n-3 PUFAs are able to significantly modulate the level of MMP-2, MMP-9, their tissue inhibitors and some inflammatory parameters in patients affected by combined dyslipidemia. The prognostic significance of this observation has to be evaluated in appositely designed prospective studies.

Declaration of interest

The authors certify that they have no affiliation with, or financial involvement in, any organization or entity with a direct financial interest in the subject matter or materials discussed in the manuscript. SPA Societa Prodotti Antibiotici has sponsored the Publication Support Service Charges for this manuscript.

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