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CLINICAL TRIAL

N-3 fatty acid supplementation to routine statin treatment inhibits platelet function, decreases patients' daytime blood pressure, and improves inflammatory status

Keren Doenyas-Barak · Sylvia Berman · Ramzia Abu-Hamad · Ahuva Golik · Naomi Rahimi-Levene · Shai Efrati

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Abstract
Objectives N-3 fatty acids reduce the risks of cardiovascular morbidity and mortality. Administration of N-3 fatty acids to patients treated with statins may potentiate the treatment effects. We examined the operating mechanisms underlying such a combination.
Methods Thirty-two hypercholesterolemic patients aged 30–70 years with hypercholesterolemia controlled by statins, received sequential treatments with placebo followed by 1.9 g/day of N-3 fatty acids for 23 weeks. Scheduled clinical visits included physical examination, 24-h blood pressure measurement, endothelial function evaluated by pulse wave analysis, analyses for platelet function, inflammation markers [interleukin (IL)-6, plasminogen activator inhibitor-1 (PAI-1)] and oxidative stress parameters (STAT-8-Isoprostane) were undertaken at baseline, after placebo treatment, and after 6 and 20 weeks of N-3 fatty acid intake.
Results Platelets functions were significantly inhibited, whereas endothelial function parameters were unaltered. IL-6 significantly decreased whereas PAI-1and STAT-8-Isoprostane levels remained unaffected. Daytime blood pressure significantly decreased; however, nighttime pressure and heart rate remained unchanged. No evidence of lipid-profile improvement was observed following combined treatment with statins and N-3 fatty acids.
Conclusions In hypercholesterolemic patients, combination of statins and N-3 fatty acid inhibits platelet aggregation, alters inflammatory status, and positively affects daytime blood pressure. Close long-term follow-up might reveal additional beneficial effects of N-3 fatty acids in this patient population.

Keywords N-3 fatty acid · Statins · Platelets function · Inflammation · Blood pressure

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Introduction

Long-chain N-3 fatty acids, obtained either from dietary sources or from pharmacological supplements, have been shown to exert significant cardiovascular (CV) protective effects [1–3] . As such, they have been recommended by various health associations for primary and secondary prevention of CV events [4] . N-3 fatty acids significantly improve lipid profiles in dyslipidemic patients, mainly via

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reduction of blood triglyceride concentrations. Recent studies demonstrated reduced susceptibility to ventricular arrhythmias [5] , improved antithrombogenic effects [6] , endothelial relaxation [7], and nitrous oxide (NO) availability [8, 9], attenuated systemic inflammation [10], and decreased intima-media thickness [11] in patients treated by N-3 fatty acids. Routine maintenance on statins is the cornerstone for treating patients at high CV risk due to lipid profile improvement [12] and antiatherosclerotic pleiotropic effects [13, 14] . Administration of N-3 fatty acids to patients already treated with statins was shown to further reduce their CV morbidity risk [15] . From the lipid profile point of view, combination of N-3 fatty acids and statin coadministration seems to exert additive effects: it reduces triglycerides and non-high-density-lipoprotein (HDL) cholesterol and elevates HDL cholesterol compared with treatment with statins alone [16] . However, regarding other pleiotropic effects, the picture is less clear; both statins and N-3 fatty acids have anti-inflammatory effects, both improve aggregation and endothelial function, and both share an antiarrhythmic effect. A combination of these drugs may have additive effects but may have negligible effects compared with statins alone.

In this study, we investigated the effects exerted by combined administration of statins and N-3 fatty acids on platelets function of hypercholesterolemic patients. Blood pressure (BP) and heart rate (HR), endothelial function, lipid profile, interleukin (IL)-6 and oxidative stress (STAT-8-Isoprostane) were concomitantly assessed.

Patients and methods

Study population

Forty-six ambulatory hypercholesterolemic patients aged 30–70 years routinely treated by statins for at least 3 months were recruited. The study was approved by local Helsinki committee. All patients signed an informed consent before enrollment and were in good health based on their medical history, physical examination, electrocardiogram (ECG), and laboratory evaluation. In all patients, hypercholesterolemia was stable and under control according to their National Cholesterol Education Program Adult Treatment Panel III (NCEP ATP III) goals, and fasting triglyceride level was ≤200 mg/dl.

Exclusion criteria were thrombocytopenia; significant bleeding history (incidence of blood transfusion during the last year); inadequately controlled hypertension (systolic BP ≥160 mmHg or diastolic BP ≥100 mmHg); poorly controlled diabetes mellitus [hemoglobin (Hb)A1c ≥7.5%); acute CV/cerebrovascular event during the last 3 months; N-3 fatty acid treatment during the last 6 months; and known inflammatory or infectious condition.

Study protocol

The study was designed as a sequential self-controlled trial. All patients received placebo (Capsugel®, France, filled with 1ml of soya oil) for 3 weeks, followed by study medication of two pills of Omega 950®, Solgar, NJ, USA. Each pill contained 542 mg eicosapentaenoic acid (EPA), 408 mg docosahexanoic acid (DHA), and 2.75 IU vitamin E. Patient visits were scheduled prior to starting the study. Visit 1: screening and placebo treatment initiation; visit 2: baseline after 3 weeks of placebo treatment, initiation of study medication; visit 3: after 6 weeks of treatment with N-3 fatty acids; visit 4: after 20 weeks of treatment with N-3 fatty acids (Fig. 1). Each visit included medical interview and physical examination. Fasting blood samples were withdrawn at each visit for lipid profile assessment, complete blood count, platelet function parameters, IL-6, STAT-8-Isoprostane, and plasminogen-activator inhibitor (PAI). At each visit, pulse wave augmentation index (Aix) was assessed and calculated as detailed later. Twenty-four hour BP was monitored and recorded at visits 2, 3, and 4. BP monitoring was not applied before placebo treatment because of the expectantly low compliance of patients with a study protocol whenever the latter involves BP monitoring performed too frequently. Patients were requested to bring the remaining pills every visit during the study for drug accountability.

Cone and platelet analysis (CPA)

The Impact cone and plate(let) analyzer (CPA) test for platelet adhesion and aggregation under high shear conditions, which represent arterial flow, were applied in this study. In brief, 130 μl citrated whole blood were placed into polystyrene wells and subjected to circulation at a high shear rate (1,875 s^{-1}) for 2 min with a rotating Teflon cone. The

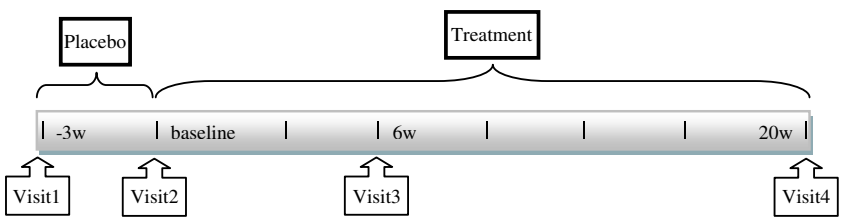

Fig. 1 Study design

wells were then rinsed with water, stained by May-Grünwald dye, and analyzed by IMPACT (Diamed Istael) under inverted light microscope (magnification x100) connected to an image analysis system. The results were expressed as percentages of the well surface covered by platelets [surface coverage (SC)] and as the average size (AS) of the stained objects. SC reflects interaction between the surface of the well and platelets. Hence, it correlates with platelet adhesion, whereas AS reflects platelet aggregation. Platelet functioning was analyzed in duplicate. Every measurement was performed twice. The interassay variability was 7–11% (Fig. 2).

Blood Pressure monitoring

Ambulatory 24-h BP was monitored using the Spacelabs™ 90207 apparatus (ABPM, USA). The recordings were registered every 20 min between 6:00 a.m. and 10:00 p.m. and every 40 min between 10 p.m. and 6:00 a.m. The daytime mean BP was calculated as the average of the measurements between 6 a.m. and 10 p.m, whereas the nighttime mean BP was calculated as the average of the values between 10 p.m and 6 a.m. BP load was calculated as percentage of values >140/90 during the day and120/80 at night. Patients were instructed to keep their regular activity and to report every special event during the 24 h period.

Pulse wave augmentation index (Aix)

Each patient was subjected to 5 min rest. Mean value of two consecutive BP measurements was recorded and registered as the patient's representative brachial BP, and radial artery pressure waveform was sampled. The 10-s samplings were performed on the same arm with a Millar tonometer (SPC-301, Millar Instruments, Houston, TX, USA) and calibrated to the average BP. Waveforms were processed by SphygmoCor, version 7, AtCor software, for calculation of the averaged radial artery waveform and derivation of the corresponding central aortic pressure waveform. Augmentation index was defined as the ratio of the augmentation pressure value to central pulse pressure. It was calculated and expressed as follows:

$$AIx = (\Delta P/PP) \times 100\%,$$

where AIx is the augmentation index, P peak pressure, and PP pulse pressure.

Assessment of STAT-8-Isoprostane, PAI-1, and IL-6

In brief, blood samples were collected in precoated lithium heparin test tubes, centrifuged at 4°C at 3,000 RPM for 15 min, and plasma immediately allocated and stored at −80°C until assessment of STAT-8-Isoprostane or PAI-1. For IL-6 evaluation, sera samples were collected without preservatives and stored at −20°C, as described by the manufacturer. Samples used for STAT-Isoprostane enzyme-linked immunosorbent assay (ELISA) (R&D,USA), were stored in the presence of 0.005% butylated hydroxytoluene (BHT), also according to the manufacturer's instructions.

Blood glucose, liver function (LF), Hb, and lipid profile (LP) analyses

Heparinized plasma samples were processed for biochemical analyses of glucose, liver function (LF), Hb, and lipid profile (LP) in Cobas® autoanalyzer (Hoffmann LaRoch, Switzerland) by enzymatic colorimetric tests.

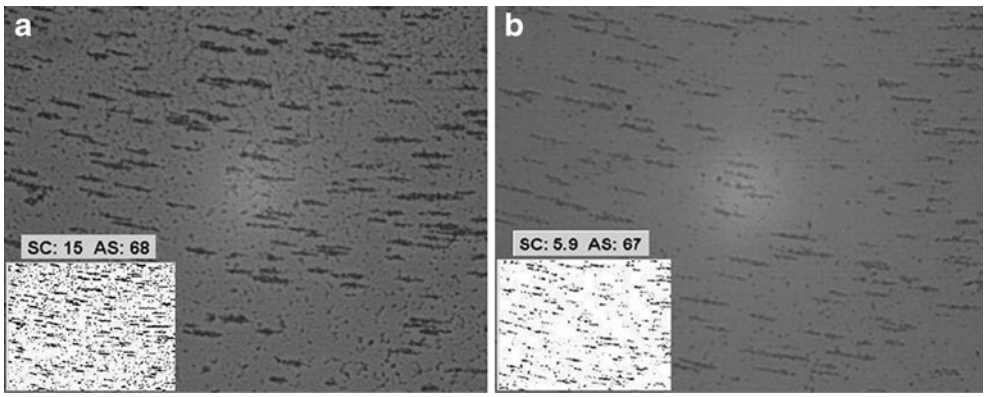

Fig. 2 Representative microphotographs of wells covered with platelet aggregates: **a** before the treatment with N-3 fatty acids; **b** after the treatment with N-3 fatty acids. Wells with strained platelet aggregates were viewed by inverted light microscope (*large images*). IMPACT analyzer software was then applied in order to evaluate the obtained results (*small images*), including calculation of percentages of the well surfaces covered by platelets [surface coverage (SC)] and the average size of the aggregates (AS). For the representative microphotographs exhibited, the calculated percentage of wells covered with platelets decreased from 15% before treatment (*left*) to 5.9% after treatment (*right*), whereas the average size of the aggregates remained unaltered

Statistical analysis

Results are presented as means±standard deviations (SD) and were evaluated using the SPSS-18 software. Repeated-measure analyses of variance methods (ANOVA) were applied to detect treatment trends over time. Differences between variables yielding P values<0.05 were considered significant. Paired Student's t test was used to compare each two time points. Confounding effects were also evaluated by ANOVA.

Results

Patients

Baseline characteristics of 32 patients that completed the study and were included in the final analysis are presented in Table 1. Of the 46 patients initially enrolled in the study, four were excluded due to poorly controlled diabetes, inadequately controlled hypertension, or nonadherence to statins. Two other patients were excluded during the placebo period, as they were diagnosed with transitional cell carcinoma and abdominal aortic aneurism. Eight patients refused to comply because of gastrointestinal disturbances caused by the study drug and did not complete the study protocol.

Analysis of laboratory data

The results of laboratory tests are summarized in Table 2. There were no significant changes in any of the measured biochemical parameters during the placebo treatment. With respect to Hb, total cholesterol, triglycerides, LDL, HDL, or non-HDL cholesterol, no significant changes were observed throughout the study.

Platelet count did not change throughout the entire study period, be it placebo treatment or medication administration. Following the 6-week treatment period, platelet SC was significantly decreased compared with baseline. This decrease progressed further from week 6 to week 20. Average size of platelet aggregates significantly dropped after 6 weeks of N-3 fatty acid treatment but returned to baseline thereafter. As half of our patients were concomitantly receiving aspirin during the study, we tested the possibility that aspirin treatment might have contributed to inhibited platelet aggregation. However, the effect of N-3 fatty acids on platelet aggregation did not differ between patients treated or not treated with aspirin at any time. In other words, no confounding effect was observed (comparison of the effect of N-3 fatty acids in patients receiving vs. patients not receiving aspirin yielded p=0.46). The proinflammatory cytokine IL-6 decreased significantly from 1.68+0.197 pg/ml to 1.50+0.30 pg/ml after 20 weeks of cotreatment (p=0.048). STAT-8-Isoprostane levels remained unchanged throughout the entire study (Table 3).

Clinical and hemodynamic parameters

Figure 3 and Table 3 demonstrate the results of 24-hBP recordings and HR measurements.

Mild, albeit statistically significant, reduction was observed both in systolic and diastolic daytime BP (130.1±14.2/77.9±6.2 mmHg at baseline and 124.2±10.4/74.8±4.6 mmHg after 20 weeks, p=0.02 and p=0.041 for systolic and diastolic BP, respectively). Nighttime BP moderately decreased but did not reach statistical significance (116.1±14.4/67±7.3 mmHg at baseline and 114±59/65±5.7mmHg after 20 weeks; p=0.644 and p=0.319 for systolic and diastolic BP, respectively). BP load decreased significantly, from 31% at baseline to 25% after 6 weeks and 10% after 20 weeks of cotreatment with N-3 fatty acids and statins (p=0.01). Mean HR remained unchanged throughout the entire study. Mean baseline AIx values were 34±10.8%. They were 32±10.7% after placebo treatment and 34±9.2% and 34±8.0% after 6 weeks and 20 weeks, respectively, on N-3 fatty acid combined treatment (p=0.465, Table 3).

Discussion

The results of our study demonstrate that addition of a moderate dose of N-3 fatty acids to to daily maintenance of patients being treated with statins reduces platelet aggregation, decreases IL-6, and improves daytime BP in

Table 1 Baseline patient characteristics

Characteristics	Statistics
Number of participants	N=32
Male	17 (53%)
Age (years)	59±7
Hypertension	15 (47%)
Coronary heart disease	9 (28%)
Diabetes mellitus	4 (12%)
Drug therapy	
β-Blockers	5 (16%)
ACE inhibitors	7 (22%)
Angiotensin receptor blockers	3 (9%)
Diuretics	8 (25%)
Aspirin	17 (53%)
Statin therapy	
Simvastatin	24 (75%)
Atorvastatin	4 (12%)
Pravastatin	2 (6%)
Rozuvastatin	2 (6%)

ACE angiotensin-converting enzymes

Table 2 Biochemical data

		Placebo	Treatment		
		−3 weeks	Baseline	6 weeks	20 weeks
Lipids	Total cholesterol (mg/dl)	157.65±30.09	154.11±28.74	151.91±24.74	154.56±28.63
	LDL (mg/dl)	82±19.6	83.2±21.71	83.1±19.79	82±25.73
	HDL(mg/dl)	47.7±14.71	48.7±15.47	49±14.69	46.7±14.82
	Triglycerides(mg/dl)	122±39.28	120±43.37	110±37.15	127±54.56
Others	Glucose(mg/dl)	100.67±17.46	99.67±15.85	103.7±21.5	100.33±17.9
	Hemoglobin (g/dl)	14.45±1.14	14.46±1.15	14.28±1.21	13.44±2.17
	White blood cells (10^3/μl)	6.64±1.82	6.89±2.08	6.7±1.77	6.74±1.71
	AST (IU/L)	24.58±5.36	22.7±6.24	20.91±4.78	22.74±8.57
	ALT (IU/L)	31.67±12.68	29.15±12.69	24.13±8.13	26.78±13.2
Platelets	Platelets count×10^3/μl	239±53	240±56.7	233±67.9	229±58.56
	Surface coverage (%)*	12±4.53	12±4.26	9.57±3.86**	8.61±3.11**,***
	Average size (μm2)*	57±31.57	56.7±26.74	45±22.48**	68.5±41.7
	PAI (pg/ml)	17.61±7.26	15.36±6.35	14.3±6.24	16.92±6.67
Inflammation and oxidative stress	STAT-8-Isoprostane (pg/ml)	906±81.97	939.21±119	937.41±139.7	913±106
	IL-6(pg/ml)	1.64±0.3	1.68±0.2	1.65±0.43	1.5±0.3**

LDL low-density lipoprotein, *HDL* high-density lipoprotein, *AST* aspartate aminotransferase, *ALT* alanine aminotransferase, *PAI* plasminogen activator inhibitor, *IL6* interleukin 6

*$p<0.05$ significant difference according to the statistical analysis, performed by the methods of repeated-measure analyses of variance (ANOVA) baseline, 6W and 20W

**$p<0.05$ compared with baseline

***$p<0.05$ compared with 6 weeks

hypercholesterolemic patients. No lipid profile improvement was concomitantly observed. Statins, prescribed alone or in combination with other relevant medications, are the most powerful tool for lipid lowering in hypercholesterolemic patients. However, for some patients, statin therapy is not sufficient; hence, a second drug may be required to improve lipid profile and CV risk. The available cotreatments comprise ezetimibe, niacin, bile-acid sequestrants, fibrates, and N-3 fatty acids [17] . Among these options, dietary supplementation of N-3 fatty acids is lately gaining special attention. Essential long-chain N-3 fatty acids are absent from meat and plant-food sources, and sufficient daily sea-fish-oil consumption is characteristic only for a limited number of human populations.

Supplementation with N-3 fatty acids, originating from dietary sources or from pharmacological supplements, exerts protective effects on the CV system, such as antiarrhythmic effects, and decreased rates of sudden cardiac death [1, 3, 5], reduced coronary events [15], stroke [18], and markers of atherosclerosis [6–8]. However, an ideal dose of N-3 fatty acids for patients at high CV risk has not yet been established. Lipid profile amelioration in dyslipidemic patients (mainly via reduction of blood triglyceride concentrations) was achieved only when rather high doses

Table 3 Hemodynamic data

		Placebo	Treatment		
		−3 weeks	Baseline	6 weeks	20 weeks
Blood pressure	Mean heart rate (bpm)		70.33±8.36	66±8.53	67.95±7.58
	Day systolic BP (mmHg)*		130.13±14.29	128±13.1	124.22±10.49**
	Day diastolic BP (mmHg)*		77.96±6.23	77±6.63	74.87±4.67**
	Night systolic BP (mmHg)		116±14.45	114±14.65	114±11.59
	Night diastolic BP (mmHg)		67±7.31	66±7.6	65±5.7
	BP load %*		31.1±30.80	25±27.49	10.2±15.53**
	Augmentation index %	34±10.87	32±10.79	34±9.26	34±8.05

BP blood pressure, *BPM* beats per minute

*$p<0.05$ significant difference according to the statistical analysis, performed by the methods of repeated-measure analyses of variance (ANOVA)

**$p<0.05$ compared with baseline

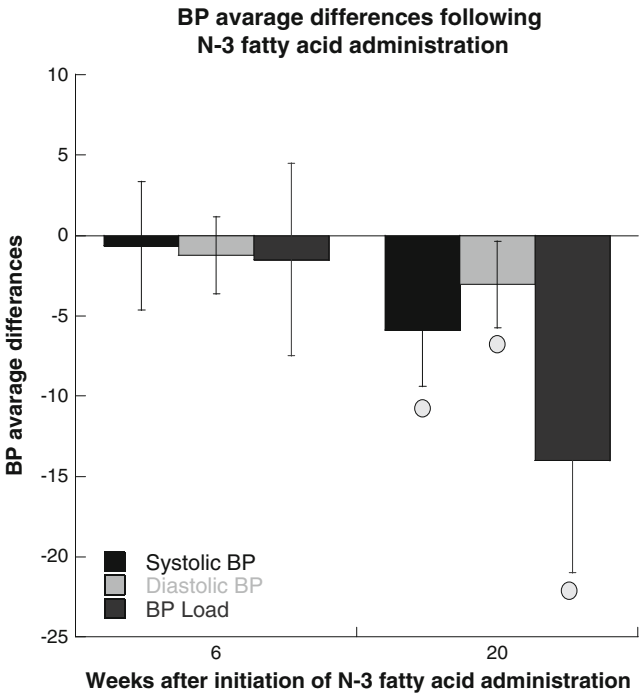

Fig. 3 Six and 20 weeks after initiation of N-3 fatty acid administration

of N-3 fatty acids were applied. Less or no appreciable effects have been demonstrated using low N-3 fatty acid doses (≤1 g/day).

Consumption of high doses of N-3 fatty acids, along with the unequivocal positive impacts, may also bear a number of potential risks. Antithrombogenic effects of N-3 fatty acid consumption may eventually result in prolonged bleeding time [19] and/or increased incidence of hemorrhagic strokes [20] . This is particularly alarming for patients concurrently receiving other anticoagulant or antiplatelet therapies or for those compelled to undergo unplanned surgery. Important negative effects from the patient's point of view, such as gastrointestinal disturbances, annoyingly bad breath, etc, were referred to as unbearable and were the main cause for the lack of patient compliance. With respect to our patients, even with the moderate doses used in our protocol, eight of the 46 patients refused to proceed with the study because of intolerance to the drug under investigation. Beneficial CV effects of very low doses were also controversial [1, 21] . Based on this information, we abstained both from too high (and therefore potentially risky) and from too low (and thus potentially inadequate) N-3 fatty acid dosages when designing the study. We administered 1.9 g/day of N-3 fatty acids to patients already treated with stable daily amounts of statins. These doses were previously used in the Japan EPA Lipid Intervention Study (JELIS) [15] and, similar to our findings, brought about only unappreciable effects with respect to lipid profiles.

In our study, 1.9 g/day of N-3 fatty acid supplement on in addition to statin treatment was associated with beneficial effects not related to the lipid-lowering outcomes. Treatment with 1.9 g/day N-3 fatty acids significantly inhibited platelet adhesion. Antithrombotic and antiplatelet aggregation effects of statins have been previously demonstrated [22] . Prolonged treatment with N-3 fatty acids was also shown to exert inhibitory effect on platelet function [23, 24] . N-3 fatty acids can influence platelet function in several ways: They can reduce thromboxane synthesis by altering the ratio between N-3 fatty acids and N-6 (arachidonic acid, for example) in the platelet membrane. They can also reduce platelet aggregation in response to adenosine diphosphate (ADP), or improve platelet/endothelium interaction while improving endothelial functioning. Additionally, with respect to platelet aggregation and thrombogenicity, statins share some common mechanisms of action with N-3 fatty acids via competitively inhibiting synthesis of thromboxane A_2, the factor responsible for platelet aggregation and vascular constriction [25] , as well as through membrane stabilizing [25] . The fact that aspirin did not alter the effect of N-3 fatty acids on platelet function may indicate that some effects might be exerted via a route(s) other than the thromboxane route. One might also conclude that in our study, the observed inhibition of platelet aggregation resulted from the additive effect of statin and N-3 fatty acid treatments.

Another promising finding of this study was the effect of the combined treatments on BP. N-3 fatty acid treatment was shown to have moderate, dose-dependent BP lowering effect in some studies, especially in elderly and hypertensive populations [26, 27] but demonstrated no effect in others [28]. Of note, statins alone have no significant effect on BP [19]. In our study, treatment with 1.9 g/day N-3 fatty acid brought about positive effects not related to lipid-lowering outcomes. Thus, patients' daytime BP, both systolic and diastolic, as monitored by 24-h halter, decreased. The achieved pressure decrements might seem low; however, they are statistically significant and are in keeping with previously reported data. Indeed, according to the Seventh Report of the Joint National Committee on Prevention, Detection, Evaluation, and Treatment of High Blood Pressure (JNC7), every decrement of 2 mmHg in systolic BP yields 5% reduction in coronary heart disease and 8% reduction in stroke [29]. One would conclude that although the amounts of N-3 fatty acids used in our study might have been relatively low, they were sufficient to produce BP-lowering effects.

Baseline inflammatory markers were augmented in our patients compared with the values found in normal individuals in a preliminary investigation (data not shown). N-3 fatty acids have a beneficial effect in this regards. Following 20 weeks of administration, systemic IL-6 was significantly inhibited. According to the study protocol, patients with

clinical signs of acute, severe inflammation were a priori excluded from the study. However, within the cohort of hypercholesterolemic patients finally enrolled, basal levels of inflammatory cytokines were augmented. The observed decline in IL-6 following prolonged treatment with N-3 fatty acids can therefore be interpreted as a sign of amelioration of inflammatory status.

One of the main limitations of this study was the lack of a placebo-control group. The study was intentionally designed as a sequential self-controlled trial, without a placebo-control group, because of the expectantly high interpatient variability. Every patient served as a control for him/herself. Among other advantages, this allowed us to overcome the problem of high baseline interpatient variability of the measured physiological parameters and to also ensure that before the intervention, i.e., the N-3 fatty acid administration, all patients were in a stable condition, as proven by the stability of the results during the placebo period. The respective outcomes at the end of the study were compared with the results obtained prior to the intervention. The main focus of the study was on the physiologic parameters and not on the hard outcomes, such as CV events or death. Yet another limitation was the lack of a proper control for compliance. Our only way to control medication consumption compliance was by counting the drugs left at each patient visit. No dietary monitoring was conducted, and no concurrent biochemical measurements of plasma and/or tissue N-3 levels were completed. Thus, the possible noncompliance of some participants with consumption of the investigated medications might have been missed by us. This, in turn, might have caused us to miss some confounding effects of the dietary N-3 supplementation to patients on regular statin treatment.

In conclusion, combined treatment of hypercholesterolemic patients with statins and moderate doses of N-3 fatty acids reduced platelets function, altered inflammatory status, and effectively decreased daytime BP. These beneficial antiatherosclerotic effects were not associated with lipid profile improvement or oxidative stress amelioration.

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Conflict of interest statement None of the authors had financial or personal relationships with other people, or organizations, that could inappropriately influence (bias) their work.

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