Omega-3 fatty acids supplementation effects on paraoxonase-1 enzymatic activity

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Summary

Paraoxonase-1 activity (PON1) and expression modulation using different pharmacological, nutritional and life-style approaches are current scientific foci. We have assessed the influence of ω-3 fatty acids from different dietary sources on PON1 activity and total oxidative status (TOS) in middle-aged dyslipidemic subjects. The study group consisted of 35 subjects, assigned to use commercial fish oil capsules or 150 g of smoked salmon two times per week during 8 weeks with 6 months of wash-out period. After the wash-out period, the same participants were invited again for the second part of the study, and the study was repeated with reversed interventions. PON1 activity and TOS were measured four times, before and after each supplementation period. Consumption of salmon and fish oil capsules both increased PON1 activity ($p < 0.001$). Pro-oxidative effect (TOS) during the first supplementation period was decreased at the end of supplementation ($p < 0.001$). The present study showed that ω-3 fatty acids, regardless of the dietary source, increased the activity of PON1 in patients with dyslipidaemia. This is possibly a consequence of an enzymatic response to the initially pro-oxidant effect of ω-3 fatty acids, which led to a reduction in oxidative stress after complete supplementation.

Keywords

omega-3 fatty acids; oxidative stress; paraoxonase activity; high-density lipoprotein

Serum paraoxonase 1 (PON1), as a component of high-density lipoprotein (HDL), has important antioxidant and anti-inflammatory roles. This glycosylated protein is synthesized in the liver, then secreted into plasma where it is bound to HDL. PON1 protects low-density lipoprotein (LDL), HDL and cellular membranes from oxidative modification and modulates lipid metabolism in adipose tissue. These protective functions of PON1 lead to reduced risk for atherosclerosis. It also takes part in detoxification of homocysteine, a well-known pro-oxidant and atherosclerosis risk factor [1].

PON1 possessed peroxidase and lactonase activities. It hydrolyses many different non-physiological substrates, but normal physiological substrate for paraoxonase is still unknown. Association of PON1 with apolipoproteins Apo A-I and Apo J (clusterin) is necessary for stability and activity of the enzyme. It is also known that the presence of calcium is required for enzyme activity and that many amino acid residues are important for organophosphatase and arylesterase activities [1]. Besides structural factors that influence the activity of the enzyme, there are many external factors that can change the environment in which the enzyme acts and contribute to its activity. Dietary lipids and lipid

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peroxidation products could decrease PON1 activity and gene expression. On the contrary, consumption of pomegranate juice, which is rich in polyphenols and several antioxidants, results in higher PON1 activity [2, 3]. In addition, other antioxidants such as flavonoids, quercetin and glabridin protect PON1 from oxidation, which leads to its increased activity [4]. Pharmacological agents, in particular lipid-lowering drugs, can also modulate PON1 activity. An increase in activity was found in patients treated with statins and fibrates, though this influence was dose-dependent [5]. Life style, namely, smoking [6], alcohol consumption [7], exercise [8] and environmental toxins [9] may also affect PON1 activity.

In addition to their roles as structural components, polyunsaturated fatty acids modulate signal transduction and gene expression in many tissues [10]. Omega-3 (ω-3) fatty acids, docosahexaenoic (DHA), eicosapentaenoic (EPA) and α-linolenic acid (ALA) are important nutrients that are involved in many diverse physiological processes in humans. Most biological effects of ω-3 fatty acids can be explained by their influence on the metabolism of eicosanoid components, i.e. prostaglandins, leukotrienes and thromboxane [11]. Essential fatty acids contained in membrane phospholipids are particularly important for their overall structure and function, because they form and maintain the integrity and the functionality of biological membranes. ω-3 fatty acids are highly concentrated in the brain, especially in myelin and white matter, and appear to be very important for cognitive and behavioural functions [12]. The anti-inflammatory properties of ω-3 fatty acids are especially beneficial in prevention of serious degenerative illness like heart disease, rheumatoid arthritis and psychiatric disease [13–15]. Several studies have shown increased PON1 activity after ω-3 fatty acid supplementation in many different diseases [16–17]. Increased intake of ω-3 fatty acids is a part of accepted dietary recommendations, but while it can restore the desirable cellular structure and have various positive health effects, it can also increase susceptibility of the membrane to lipid peroxidation and cause increased oxidative stress in the organism [18].

The primary aim of the present study was to estimate PON1 activity in middle-aged Serbian adults with dyslipidemia. This study also attempted to determine whether different dietary sources of EPA and DHA (dietary supplement and fatty marine fish (salmon)) would affect differently the circulating levels of a total oxidative stress marker (TOS) and, in particular, to follow possible PON1 changes.

METHODS AND MATERIALS

Subjects

Of the 130 subjects who were screened, a total of 35 participants met the inclusion criteria and were enrolled into the intervention study. All baseline measurements were completed within 6 months. The baseline data collected at the time of the first clinical visit are shown in Tab. 1.

The study was designed as a randomized, controlled, crossover trial of the effect of ω-3 fatty acids from smoked salmon fish and fish oil supplements on markers related to cardiovascular risk. The 35 healthy volunteers (18 males and 17 females) with estimated dyslipidemia, according to Adult Treatment Panel III (ATP III), aged between 44 and 64 years, were enrolled into the study. Volunteers were invited to participate by advertisement and their eligibility was screened using a health and lifestyle questionnaire.

Exclusion criteria included weight changes (± 3 kg) within 3 months before the beginning of the study, use of any regular medication or dietary supplements containing ω-3 fatty acids, calcium or vitamin D during the previous three months. The participants had no evidence of any chronic illness including diabetes mellitus or other endocrine disorder, hepatic, renal or cardiac dysfunction. They were not heavy smokers, as they were smoking only 0–9 cigarettes per day, nor were they taking medications known to affect plasma lipid levels or non-steroidal anti-inflammatory drugs such as aspirin. Excluded were volunteers who habitually consumed more than one fish meal per week or who drank more than three standard alcoholic drinks per day (a standard drink equals 10 g of pure alcohol).

The study was approved by the Clinical Research Ethics Committee of the Faculty of Pharmacy, Belgrade, Serbia. Informed consent was obtained from all subjects before starting experimental procedures and the study followed the Helsinki guidelines.

Tab. 1. Characteristics of the studied population at baseline.

Characteristic	Value
Gender	17 females, 18 males
Age [years]	55 (44–64)
Number of smokers	9 (26%)
Body weight [kg]	79.5 ± 14.0*
Body mass index [kg·m^{-2}]	26.1 ± 3.4*

Whole group was $n = 35$.
* Data are expressed as mean ± standard deviation.

Study design

Participants were randomly assigned to one of two groups: Group 1 (20 subjects) consumed 150 g of cold smoked skinned filet of Norwegian atlantic farmed salmon providing 274 mg EPA + 671 mg DHA per day (by Squadra, Belgrade, Serbia); Group 2 (15 subjects) consumed fish oil capsules of commercially available dietary supplement providing 396 mg EPA + 250 mg DHA per day (Pharmanova, Belgrade, Serbia). This amount is frequently recommended by producers. Fish oil capsules were consumed with meals two times per week. Each diet phase lasted 8 weeks with 6 months of a subsequent wash-out period. After the wash-out period, the same participants were invited again for the second part of the study. The study was repeated with reversed interventions and lasted 8 weeks. Compliance with the study protocol was confirmed by demonstrating changes in relevant fatty acids in plasma phospholipids [19–20]. Group 1 was called the Salmon – Fish oil supplement (S-FOS) and Group 2 the Fish oil supplement – Salmon (FOS-S) according to the intervention schedule. The whole trial included four clinical visits, one before and one after each intervention period. Participants were instructed not to consume any oily fish during the 8-week study period, but were otherwise encouraged to follow their normal dietary habits.

Biochemical analyses

Lipid status parameters, total cholesterol (TC), LDL cholesterol (LDLC), HDL cholesterol (HDLC) and triglycerides (TG), were measured in serum, using an ILAB 300 plus analyzer (Instrumentation Laboratory, Milan, Italy), employing commercial kits.

Atherogenic index of plasma (AIP) was calculated as the TG/HDLC ratio.

Oxidative stress and antioxidative defence parameters

Rates of PON1 activity toward paraoxon (POase, 1.2 $mmol \cdot l^{-1}$) and diazoxon (DZOase, 1 $mmol \cdot l^{-1}$) were measured spectrophotometrically, POase activity with an ILAB 300 plus analyzer and DZOase activity with a UV-1800 Spectrophotometer Shimazu (Shimazu, Kyoto, Japan) in serum according to the method described by Richter and Furlong [21].

Total oxidative status (TOS) was determined according to Erel's method [22]. This assay is based on the oxidation of ferrous ion to ferric ion in the presence of various oxidant species in serum. Concentration of ferric ion is measured using xylenol orange. The assay was calibrated with hydrogen peroxide and was incorporated into the ILAB 300 plus analyzer. The intra-assay and inter-assay coefficients of variance were 5.6% and 9.5%, respectively. The results wre expressed as micromoles of hydrogen peroxide equivalent per litre.

Fatty acid analysis

Fatty acid composition of lipids from fish (after Bligh-Dyer extraction) and from dietary supplement was determined using gas chromatography [23]. Fatty acid methyl esters (FAMEs) from lipid extracts were trans-esterified with HCl in methanol according to the method described by Ichihara and Fukubayashi [24]. FAMEs were quantified using an Agilent Technologies 7890A Gas Chromatograph with a flame ionization detector (Agilent Technologies, Santa Clara, California, USA). A 112-88A7, HP-88 capillary column 100 m × 0,25 mm × 0, 2 μm (Agilent Technologies) was used with He as a carrier gas at a flow rate of 105 $ml \cdot min^{-1}$. Samples were injected at a starting oven temperature of 175 °C, injector temperature was 250 °C and detector temperature was 280 °C. The oven temperature was programmed to increase from 175 °C to 220 °C at 5 $°C \cdot min^{-1}$. FAMEs were identified on the basis of their retention times with reference fatty acid standards (Supelco FAME Mix; (Sigma-Aldrich, Saint Louis, Missouri, USA).

Statistical analysis

The results were expressed as means and standard deviations. Differences between variables before and after dietary intervention were calculated using two-way mixed model ANOVA with repeated measurement with Bonferoni post-hoc analysis for pairwise comparisons. To test the effect of other oxidative status parameters on POase activity before and after both parts of intervention trials, multiple linear regression analysis with backward selection was used. The initial model consisted of lipid, inflammation and oxidative status parameters: TC, HDLC, TG, sum of EPA + DHA, TOS. All statistical analyses were performed using the Statistical Package for the Social Science (SPSS) statistical software (PASW Statistic 18, IBM, Armonk, New York, USA).

RESULTS

The baseline data collected at the beginning of the study are shown in Tab. 1. Thirty-three participants completed two phases of the intervention; only two participants withdrew from study group, the reason being poor compliance within the study

group. Groups of subjects were uniform regarding age and sex (Group S-FOS 48.4 ± 10.8 years, 8 males, 11 females; Group FOS-S 46.1 ± 9.5 years, 9 males, 5 females). No significant changes in TC, LDLC and triglycerides were present in either group after complete supplementation, whereas the concentration of HDLC decreased remarkably just in the S-FOS group, after the first supplementation ($p < 0.05$). Concentrations returned to baseline values after the second supplementation period (Tab. 2). Comparison of AIP regarding the two dietary interventions, before and after supplementation, showed no significant changes (Tab. 2).

We wanted to test whether the specific ω-3 therapy (i.e. by salmon or commercial fish-oil capsules) caused any difference in the pattern of PON1 changes). Two-way repeated measures ANOVA showed that the increase in PON1 enzymatic activities at both ω-3 fatty acids supplementations was significant ($p < 0.0001$ for POase and for DZOase). There were no interaction effects between supplementation periods and the order of administering ω-3 fatty acids supplement. Changes of PON1 enzymatic activities are presented in Fig. 1A.

Repeated measures ANOVA with order of supplementation as a between-subjects factor was used to test differences between supplementations. Both groups of subjects had the same pattern of POase changes (Fig. 1). The enzyme activities increased after the wash-out period as well as at the end of complete supplementation compared with the period before the first supplementation ($p < 0.001$). We also found a significantly increased activity of PON1 after the wash-out period and after the end of complete supplementation compared with the period after the first supplementation ($p < 0.001$ respectively), so the activity was significantly higher at the end of the study compared to beginning. However, we noticed slight decrease in PON1 activity at the end of the second supplementation compared with the wash-out period ($p < 0.05$). Subjects from the Group S-FOS had higher POase activities, but this difference was not significant (Fig. 1B). The same trend was also seen in changes in DZOase activities in supplement order subgroups. After the first period, the group using salmon as ω-3 fatty acids supplement had a significantly higher DZOase activity compared with the fish-oil capsule consumers ($p < 0.05$, Fig. 1B).

To study the joint action of lipids and oxidative stress on POase activity, we implemented multiple linear regression analysis with backward selection.The initial model included TC, HDLC, LDLC, TG, and sum EPA + DHA for the different

Tab. 2. Effect of salmon and fish oil intervention on plasma lipids.

Parameter	S-FOS					FOS-S				
	SP 1	SP 2	SP 3	SP 4	*p*	SP 1	SP 2	SP 3	SP 4	*p*
Total cholesterol [mmol·l^{-1}]	6.6 ± 1.0	6.2 ± 0.27	6.5 ± 0.27	6.4 ± 0.91	0.345	6.4 ± 0.84	6.6 ± 0.24	6.4 ± 0.25	6.7 ± 1.08	0.361
LDL cholesterol [mmol·l^{-1}]	4.3 ± 0.88	4.1 ± 0.22	4.3 ± 0.23	4.2 ± 0.85	0.754	4.2 ± 0.80	4.4 ± 0.20	4.2 ± 0.21	4.5 ± 0.86	0.870
HDL cholesterol [mmol·l^{-1}]	1.4 ± 0.08	1.3 ± 0.09*	1.3 ± 0.10	1.4 ± 0.09	< 0.05	1.4 ± 0.30	1.3 ± 0.08	1.3 ± 0.09	1.4 ± 0.36	0.906
Triglycerides [mmol l^{-1}]	2.0 ± 1.17	1.6 ± 0.21	1.7 ± 0.33	1.7 ± 0.83	0.380	1.8 ± 0.86	1.9 ± 0.19	2.3 ± 0.29	1.8 ± 0.70	0.528
Atherogenic index of plasma	1.6 ± 1.20	1.4 ± 0.22	1.5 ± 0.33	1.4 ± 0.97	0.686	1.4 ± 0.94	1.5 ± 0.19	1.9 ± 0.29	1.3 ± 0.71	0.483

Intervention modality: S-FOS – salmon followed by fish oil supplements; FOS-S – fish oil supplement followed by salmon.
Supplementation periods: SP 1 – before the first supplementation (baseline), SP 2 – after the first supplementation, SP 3 – after wash-out and before the second supplementation, SP 4 – after the second supplementation.
The results are expressed as mean ± standard deviation. *p* values indicate statistical differences between all supplementation periods among the same intervention group, *p* values < 0.05 were considered significant.
* – $p < 0.05$ difference between baseline and the first supplementation in S-FOS group.

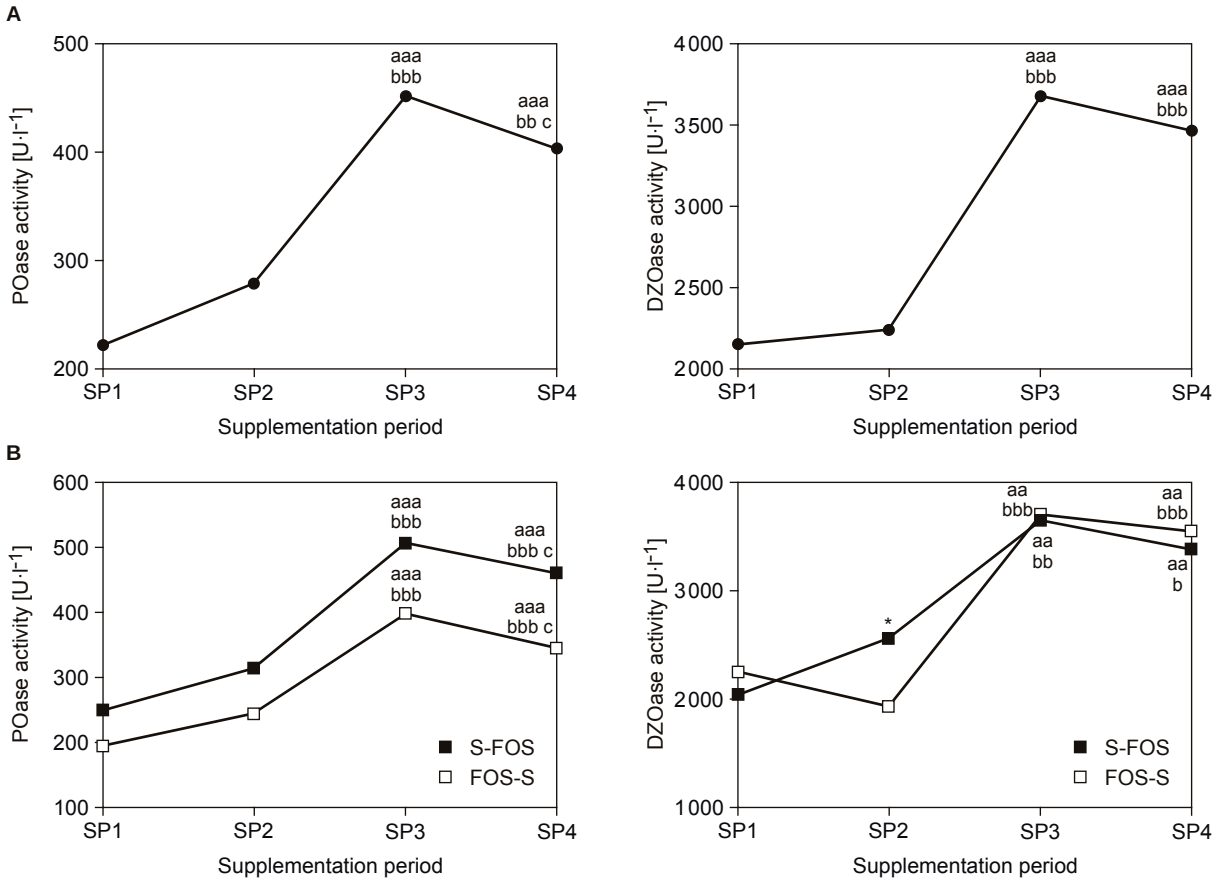

Fig. 1. Paraoxonase-1 activity.

A – Summed changes in paraoxonase-1 activity during ω-3 supplementation periods, B – Changes in paraoxonase-1 during different dietary intervention types.
POase – paraoxonase-1 activity toward paraoxon, DZOase – paraoxonase-1 activity toward diazoxon.
Intervention types: S-FOS – salmon followed by fish oil supplements; FOS-S – fish oil supplement followed by salmon.
Supplementation periods: SP 1 – before the first supplementation (baseline), SP 2 – after the first supplementation, SP 3 – after wash-out and before the second supplementation, SP 4 – after the second supplementation.
Different letters mean significant differences between supplementation periods: a – difference between wash-out period vs pre-supplementation point (a – $p < 0.05$, aa – $p < 0.01$, aaa – $p < 0.001$); b – difference between wash-out period vs first supplementation period (b – $p < 0.05$; bb – $p < 0.01$; bbb – $p < 0.001$); c – difference between wash-out period vs second supplementation period (c – $p < 0.05$; cc – $p < 0.01$; ccc – $p < 0.001$); * – $p < 0.05$ first supplementation period salmon vs fish-oil supplement.

period of supplementation. Backward selection enabled us to get the best model, which consisted of several of the most influential selected parameters (Tab. 3). Multiple linear regression analysis showed that the best model of parameters determining POase activity after the complete ω-3 fatty acids intervention period consisted of the sum of TC, HDLC and EPA + DHA, and this model could explain changes (increase) of nearly 23% of POase activity at the end of the study (Tab. 3). TOS as an oxidative stress marker was also included in the model, though having no significant effect. It was not included in the best model.

During the wash-out period, pro-oxidative effects (TOS) were suppressed and those decreases of TOS were significantly different from the period after the first supplementation ($p < 0.001$). Analysing all the changes of oxidative stress parameter (TOS) during the study period, we found a significant decrease in oxidative stress, after the supplementation was completed ($p < 0.001$). This parameter changed in parallel for the two subgroups according to the order of ω-3 fatty acids supplementation (Fig. 2).

DISCUSSION

Oxidative stress is involved in many diseases. It is believed that free radicals can cause many complications, which are the basis for development of serious chronic disorders including diabetes melli-

Tab. 3. Multiple linear regression analysis with backward selection for the best model.

Parameter	R^2	Adjusted R^2	The best model	β coefficient (standardized)	*p*	Equation
POase 1	0.240	0.174	TC			POase = 474 – 38·TC
POase 2	0.023	0.019	HDLC	0.153	0.465	POase = 319 + 126·HDLC
POase 3	0.158	0.126	LDLC	–0.368	0.036	POase = 1 004 – 133·LDLC
POase 4	0.322	0.229	TC	–0.473	0.030	POase = 647 – 160·TC + 544·HDLC + 56·(EPA + DHA)
			HDLC	0.513	0.013	
			EPA + DHA	0.417	0.045	

Serum paraoxonase 1 activity toward paraoxon: POase 1 – before supplementation (baseline), POase 2 – after the first period of supplementation (S-FOS, FOS-S), POase 3 – after the wash-out period, POase 4 – after the second period of supplementation (S-FOS, FOS-S).
TC – total cholesterol, LDLC – low-density lipoprotein cholesterol, HDLC – high-density lipoprotein cholesterol, EPA – eicosapentaenoic acid, DHA – docosahexaenoic acid.
Equation produced by multiple linear regression best model: Dependent variable = Constant (intercept) ± $\beta_1 x_1$ ± $\beta_2 x_2$ ± ... $\beta_i x_i$

tus, atherosclerosis and underlying diseases or psychiatric disease. On the other hand, positive effects of ω-3 fatty acids were demonstrated in the pathogenesis of many disease processes, including some in which oxidative stress plays a crucial role [25, 26].

The usual recommendations for increasing ω-3 fatty acid intake in everyday life are the consumption of two portions of fatty fish per week or using 1–2 capsules of fish oil supplements daily [27]. As fish lipids and fish oil supplements usually differ in their fatty acid composition (particularly in EPA and DHA contents), it is of interest to investigate how these different dietary sources of ω-3 fatty acids in recommended amounts influence the oxidative status and PON1 activity in an average consumer. The average middle-aged Serbian population has been characterized with one or more cardiovascular risk factors [28, 29]. In the present study, participants had increased levels of TG, TC or LDLC as a lipid-related risk factor.

PON1, as a calcium-dependent esterase, is associated with HDL and has an important role in protecting not only LDL from oxidative modification, but also HDL and cellular membranes [30]. In addition to genetic polymorphism (Q192R and L55M), PON1 can be modified by factors such as diet, lifestyle and disease [31]. It was found in several studies that paraoxonase activity is decreased in cardiovascular disease [32, 33]. We have also shown that POase and DZOase activities were lower in coronary heart disease patients when compared with the control population [33]. In the present study, we found higher PON1 activity after dietary intervention in both groups (FOS-S and S-FOS) [34].

Increased PON1 activity could be a consequence of slight pro-oxidative effects of ω-3 fatty acids, because the number of double bonds represents a suitable substrate for lipid peroxidation [35]. This increased lipid peroxidation was associated with increased superoxide anion concentrations in our previous study [19]. The TOS parameter, which describes overall oxidative status in the organism, is also in compliance with this, having the same pattern of changes as superoxide anion

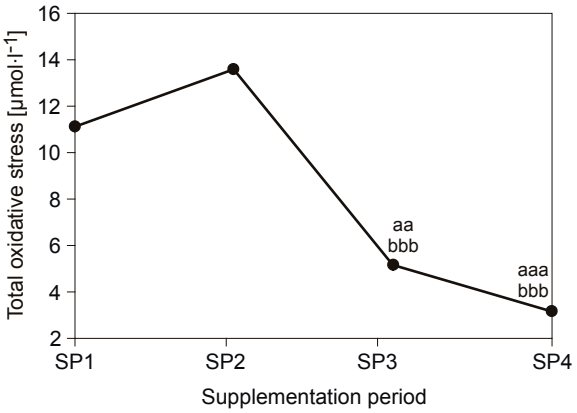

Fig. 2. Summed changes in total oxidative stress during ω-3 supplementation periods.

Supplementation periods: SP 1 – before the first supplementation (baseline), SP 2 – after the first supplementation, SP 3 – after wash-out and before the second supplementation, SP 4 – after the second supplementation.
Different letters mean significant differences between supplementation periods: a – difference between wash-out period vs pre-supplementation point (a – $p < 0.05$, aa – $p < 0.01$, aaa – $p < 0.001$); b – difference between wash-out period vs first supplementation period (b – $p < 0.05$; bb – $p < 0.01$; bbb – $p < 0.001$); c – difference between wash-out period vs second supplementation period (c – $p < 0.05$; cc – $p < 0.01$; ccc – $p < 0.001$).

[19, 36]. Evidence was found that ω-3 fatty acids are potent activators of the peroxisome proliferator-activated receptor α (PPARα) [37], which up-regulates several genes involved in the stimulation of fatty acid oxidation. Oxidative stress induces modification in amino acids of apolipoproteins Apo A-I, which consequently affects binding between HDL and PON1. On the other hand, AVIRAM et al. [37] showed that antioxidants from pomegranate juice (tannins and anthocyanin) directly increased the HDL-PON1 association and increased the enzyme activity. Polyphenols from pomegranate juice have the capability to chelate metal ions and reduce the generation of free radicals [3].

BLOCK et al. [38] showed that decreased blood levels of EPA + DHA associated with risk for acute coronary syndrome. Their results also showed that low EPA + DHA sum can be considered as a possible new cardiovascular marker, particularly for sudden cardiac death. In accordance with this, the present study also showed, through multiple linear regression analysis, a significant positive influence of the EPA + DHA sum on the increase in PON1 activity after the end of complete supplementation (Tab. 3). Thus, we suppose that low EPA + DHA, an established cardiovascular risk factor, probably will contribute to decreasing PON1 activity.

In summary, the current study shows that the initially pro-oxidant effect of ω-3 fatty acids, described with increased levels of TOS, could be the cause of an increased PON1 enzymatic response, which leads to a reduction in oxidative stress at the end of supplementation. A common trend in PON1 activity was seen in both groups regardless of dietary source, which indicate positive effect of ω-3 supplementation.

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