EPA-Mediated Up-regulation of PON2 Gene in The Patients with Type 2 Diabetes Mellitus

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Abstract: Background: Paraoxonase 2 acts as an antioxidant enzyme at the cellular level, and because of its antioxidant and lactonase activities have the protective role against the development of atherosclerosis. EPA has the antioxidant, antiinflammatory, antithrombogenic, and antiarteriosclerotic properties. Therefore, we investigated the influence of the supplementation of EPA on the gene expression of PON2 in the PBMC of type 2 diabetic patients. **Methods:** This study was designed as a randomized, double-blind, and placebo-controlled clinical trial. Thirty six patients with type 2 diabetes were given written; informed consent, randomly were classified into 2 groups. They were supplemented with 2 g/day of the softgels of EPA or placebo. At the start and the end of the intervention, were given blood sample for measurement of the genes expression of PON2 and β-actin, and the serum levels of lipids, FBS and HbA1c. **Results:** Patients supplemented with EPA showed a significant increase in the gene expression of PON2 than β-actin gene. There were no significant differences between the two groups regarding any demographic, clinical or biochemical data, total energy intake, and macronutrient intake at the baseline, and during the intervention, except for a significant increase of protein intake and the levels of HbA1c in the placebo group, and a significant increase of HDL-c, as well as a slight reduce of total cholesterol, LDL-c, TG and FBS in the supplement group. **Conclusions:** We conclude that EPA is atheroprotective via the upregulation of PON2 at the gene level.
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Introduction

Type 2 diabetes is as a serious chronic metabolic disease, and one of the most common endocrine disorders resulting from defect in the insulin secretion, the insulin resistance, or both [1]. In the twenty-first century, type 2 diabetes is recognized as a major public health problem all over the world [2], and its prevalence has reached epidemic proportions worldwide [3]. The World Health Organization (WHO) was estimated at approximately 171 million adults (>20 years old) affected by diabetes worldwide in 2000 [4], and the International Diabetes Federation (IDF) has predicted that the number of people with diabetes will increase from 240 million in 2007 to 380 million for the year 2025 [5], with 80% of individuals with diabetes in the low and middle-income countries [6].

This disease and its chronic complications impose a substantial economic burden on individuals, families, society, and the healthcare system of the country and make it as a public health challenge [7]. It is anticipated that in the year 2025, the healthcare expenditures of diabetes will be between 7% and 13% of the healthcare budget of worldwide [5].

The paraoxonase (PON) multigene family consists of three different members (PON1, PON2, PON3) [8], with genes adjacent to each other on chromosome 7 q21-q22 [9]. PONs are calcium-dependent hydrolases, and can hydrolyze organophosphate (OP) compounds, platelet activating factor (PAF), L-homocysteine thiolactone (L-HcyT); as a risk factor for atherosclerotic vascular disease; decrease lipoprotein peroxides, impede macrophage cholesterol biosynthesis, excite cholesterol efflux from macrophages, and postpone atherosclerosis [10].

It is thought that PON2 is the first member of the PON family proteins which discovered [11]. PON2 is nearly expressed in all human tissues, including the liver, heart, kidney, small intestine, stomach, lung, spleen, testis, and placenta [12]. Moreover, PON2 mRNA in humans (hPON2 mRNA), unlike hPON1and hPON3, is also found in the cells of the artery wall; including smooth muscle cells, endothelial cells, and macrophages [13]; white blood cells, and skeletal muscle [14]. PON2 protein is mostly located in the cell membrane and intracellularly. Thus, its mechanism of action is likely related to its function in the cell [15].

Although there is still little information about its specific functions, characteristics and regulation of PON2, Paraoxonase 2 acts as an antioxidant at the cellular and not humoral level. PON1, PON2 and PON3 have a protective role against the development of atherosclerosis (antiatherogenic) [16], and has also demonstrated that this role of PON2 can be related to its antioxidative properties [14]. In addition, PON2 indicates the lower antioxidant properties than PON1, but has the uttermost lactonase activity [10]. Thus, the PON2 preventing role of the development of atherosclerosis can also be dependent on its lactonase activity. PON2 is able to reduce the intracellular oxidative stress, prevent the cell-mediated oxidation of LDL, alter the properties of LDL and HDL via the interactions of these lipoproteins with a number of cell types including macrophages [14], protect from triglyceride accumulation in the macrophages [17], and the most efficient paraoxonase protein at hydrolysis of the Pseudomonas quorum sensing factors [10].

Eicosapentaenoic acid (EPA) is one of the n-3 polyunsaturated fatty acids (PUFA n-3) which are found at the great amounts in the fish oil [18]. Findings of studies showed that EPA has the antioxidant [19], antiinflammatory [20], antithrombogenic [21], and antiarteriosclerotic [22] properties.

The aim of this study was to determine the effect of the supplementation of EPA on the gene expression of PON2 in PBMC of the patients with type 2 diabetes mellitus.

Material and Methods

1. Patients and Study design

1. 1. Patients

The study subjects were 36 patients with type 2 diabetes mellitus who were selected from Iran Diabetes Association (Tehran, Iran). Only patients with a previous clinical diagnosis of type 2 diabetes mellitus according to the criteria for the diagnosis of diabetes as recommended by American Diabetes Association [23]

1.1.1. Inclusion/exclusion criteria:

Inclusion criteria for the participation in the study were, willingness to collaborate in the study, aged 35-50 years, having a history of at least 1 year of the diagnosis of type 2 diabetes mellitus before the participation in the study based on FBS ≥126 mg/dl or 2hPG ≥200 mg/dl (2-hour plasma glucose), 25≤BMI<30 kg/m^2, identified and maintaining of the antidiabetic's drug (s) dose from 3 months ago.

Participants were excluded from the study if they had, unwillingness to continue the cooperation in the study, need to take insulin, change in the dose (s) and type of medication to the treatment of diabetes, change in the levels of physical activity, do not use (noncompliance) supplements (<10%), affected to the acute inflammatory diseases; according to the consultant physician endocrinologist.

1.2. Study design

The study protocol was designed as a randomized, double-blind, and placebo-controlled clinical trial. At the first, the study protocol was approved by the ethics committee of Tehran University of Medical Sciences (ID: 84153), and all participants gave written, informed consent before the participation in the study. This trial was registered in the Clinical Trial. gov-register (NCT03258840).

The patients were randomly classified into 2 groups to the supplementation with 2 g/day of the softgels of EPA or placebo (supplied as 1-g softgels), the two groups were randomly allocated to the supplement and placebo groups by balanced permuted block on the sex. The softgels containing Eicosapentaenoic acid ethyl ester (75%) [EPA, Mino Pharmaceutical Co. Iran], or edible paraffin were provided by Mino Pharmaceutical Co., Iran. They were strictly advised to maintain their usual diets and nutritional habits, level of physical activity, and not to change their medication dose (s) during the study, as well as were asked to record and report any side effect of taking capsules gave to them.

Compliance with the supplementation was assessed by counting the number of softgels had used and the number of softgels returned to the study center at the time of specified visits. The patients were followed up by telephone each week.

1.2.1. Nutritional assessment

At the beginning and at the end of the intervention, nutrients intakes were estimated using a 24-hour diet recall questionnaire for 3 days.

1.2.2. Questionnaires, anthropometric and biometric measurements

At the start and the end of the study, each participant was evaluated with the physical examination and a general questionnaire containing questions regarding demographic variables (age, sex), anthropometric data (weight, height, waist and hip circumference, heart rate, and measurements of systolic, diastolic and mean blood pressure (SBP, DBP and MBP), and pulse pressure (PP)), family history of diseases (diabetes, hyperlipidemia and hypertension, cardiovascular, etc), age at the diagnosis of type 2 diabetes, type of the treatment and medication used, and lifestyle habits (including the history of smoking, alcohol consumption). The average of type and duration of all physical activities were measured using the International Physical Activity Questionnaire (IPAQ), at the beginning and at end of the intervention.

Anthropometric measurements, including weight, height, as well as waist and hip circumference, and blood pressure were measured at the start and at end of the study according to standard protocols. Weight, changes in the level of physical activity, and any disease were recorded at the baseline and during weeks 2, 4, 6, and 8 of the intervention.

Subjects were weighed without shoes; in light indoor clothes by a Seca scale with an accuracy of ±100 g. Standing height was measured without shoes to the nearest 0.5 cm using a commercial stadiometer. Body mass index (BMI) was calculated as weight/height² (kg/m²). According to the recommendation of International Diabetes Federation, hypertension was defined as blood pressure ≥130/85 mmHg [24].

Each participant gave a blood sample in the early morning after an overnight fast for 10–12 hours and before taking any oral hypoglycemic agent (s) at the beginning and at the end of intervention (8th week). Samples were drawn from the antecubital vein, and were collected into blood tubes containing EDTA or heparin, and tubes without the aforementioned substances. After at least 30 minutes, plasma and serum were separated by centrifugation at 3000 ×g for 10 minutes at 4 °C. Serum and plasma aliquots of each sample stored at −80 °C, for analysis of biochemical parameters [FBS (fasting blood sugar), HbA1c, the serum total cholesterol (TC), triglyceride (TG), LDL-c and HDL-c]. The blood samples were collected only for this study.

1.2.3. Molecular Analysis

Isolation of PBMCs: At the first, published guidelines were followed to guard against bacterial and nucleic acid contamination [25]. Subsequently, PBMCs (peripheral blood mononuclear cells) were isolated. Briefly, the remaining blood was returned to its original volume with adding PBS, and then blood gently added to tubes containing Ficoll, followed by the centrifugation at 2500 rpm (1100 ×g) for 20 minutes at room temperature (25 °C), PBMC-containing band (buffy coat) was aspirated, washed with adding PBS and centrifuge at 1600 rpm (450 ×g) for 15 minutes at room temperature (25 °C). Finally, cells analysed microscopically.

RNA isolation and cDNA synthesis: After PBMCs were separated, total RNA was isolated from cells using RNeasy Mini Kit (Qiagen, Hilden, Germany), and cDNA synthesis was done by QuantiTect Reverse Transcription (Qiagen, Hilden, Germany) according to the manufacturer's instructions.

Primers design: Using information contained public database in GeneBank of the National Center for Biotechnology Information (NCBI), the sequences of primers were designed by Primer 3 online software (http://www.basic.nwu.edu/biotools/Primer3.html or http://www.cgivo.2 [26].), and theirs specificity were verified in BLAST domain (http://www.ncbi.nlm.nih.gov/BLAST). Primers for the PON2 and β-actin genes were synthesized by Bioneer Co. Ltd. The sequences of primers used for real time PCR reactions are shown in Table 1.

Real-time PCR: Analysis and measurement of the selected genes expression were performed by real-time PCR using ABI Step One (Applied Biosystems, Foster City, CA, USA). PCR amplification was performed in 40 cycles of 15 seconds at 95 °C, followed by 40 seconds at 60 °C.

The β-actin gene was used as a housekeeping and endogenous control. Thus, the mRNA expression of PON2 gene was normalized by β-actin.

The real time PCR results were imported into Microsoft Excel, and the ratios of the expression levels of PON2 and β-actin genes were calculated (using the Pfaffl method [27]) by subtracting the threshold cycle number (Ct) of target gene (PON2) from the Ct of β-actin and raising 2 to the power of this difference. The Ct values are defined as the number of PCR cycles at which the fluorescent signal while the PCR reaches a constant threshold. Target gene expressions are expressed relative to β-actin expression, and are shown as mean ± SD.

1.2.4. Other laboratory analyses

Serum was used for the determination of lipids and glucose. Glucose and HbA1c were measured by enzymatic methods. Serum lipid (serum total cholesterol, HDL-cholesterol, triglyceride and LDL-cholesterol) analyses were performed by spectrophotometric method (Pars azmoon, Iran).

1.2.5. Statistical analyses

The data were analyzed using SPSS software (version 16.0 for Windows; SPSS Inc., Chicago, IL, USA), and the results are expressed as mean ± SD. The Independent t-test was used for the comparison of variables between two groups. The Paired t-test and Levene's test were also used for data analysis. 24-hour diet recalls analysed using Food processor II software [28], and the comparison of means in different intervals was performed using Independent t-test. Values of $p < 0.05$ were considered statistically significant.

Results

Patient characteristics

The baseline and after characteristics of the two groups of patients are shown in Table 2. There were no significant differences in age, sex, duration of diabetes, weight, height, body mass index (BMI), waist circumference, hip circumference, waist/hip ratio, measurements of systolic, diastolic and mean blood pressure (SBP, DBP and MBP), pulse pressure,

heart rate and biochemical data between the two groups at the baseline.

Dietary intake and lifestyle

There were no significant differences in total energy intake, macronutrient intake, and body weight between the two groups of patients at the baseline (Table 2), and no significant changes observed during the intervention, except for a significant increase of protein intake in the placebo group. Medication dose (s), and the levels of physical activity from both groups had no significant difference at the baseline, and remained constant during the intervention period (data not shown).

Compliance and side effect

All patients were fulfilled the intervention program, and were well tolerated intervention with study capsules for 8 weeks. Also, they were reported no side effects throughout the study.

The gene expression of PON2

The results analysis of real-time PCR showed that the supplementation with EPA resulted in a significant increase in the gene expression of PON2 than β-actin gene (P = 0.041) (Table 3).

The Serum Levels of Lipids:

The serum total cholesterol was 226.27 ± 38.73 mmol/L after receiving placebo and 207.16 ± 39.69 mmol/L after the supplementation with EPA. The serum LDL-cholesterol was 95.73 ± 29.86 mmol/L after receiving placebo and 81.4 ± 32.63 mmol/L after the supplementation with EPA. The serum HDL-cholesterol was 31.38 ± 4.76 mmol/L after receiving placebo and 37.11 ± 5.97 mmol/L after the supplementation with EPA. The serum triglycerides was 162, 8± 158.81 mmol/L after receiving placebo and 176.48 ± 133.75 mmol/L after the supplementation with EPA (Table 4).

Discussion:

A number of studies have established that EPA has several effects including preventing of the insulin resistance [29], increasing the insulin secretion [30], enhancing the size of LDL particle [31], reducing the levels of serum triglyceride, lowering the blood viscosity, increasing the production of NO, having antiinflammatory and antithrombotic properties [32-35], and decreasing the blood pressure [36].

Investigators have suggested that EPA is more effective than DHA in the suppression of inflammatory response [37]. EPA plays as a substrate that decreases the production of inflammatory eicosanoids from arachidonic acid, via competing for the cyclooxygenase-2 and lipooxygenase (COX-2/LOX) enzymes. These alternative eicosanoids have identified as a group of mediators, which are termed E-series resolvins, and exert the antiinflammatory actions. Moreover, both DHA and EPA reduce the release of arachidonic acid via the inhibition of phospholipase-2 [42, 43].

EPA also have an inhibitory role on the endotoxin-induced expression of adhesion molecules upon the endothelial cells of human venous, and results in the reduction in excessive number of monocytes attached to arterial endothelium [44].

Findings of an epidemiological study of Greenland Eskimos suggested that EPA could be has antithrombogenic and antiarteriosclerotic properties [22]. It has been postulated that the mechanisms of these actions are including the suppression of platelet aggregation and the improvement of blood rheologic properties [21].

It has also been reported that EPA has beneficial effects on the levels of serum lipids, which is suggesting that EPA may be useful as a supplement for the prevention and treatment of arteriosclerotic disease [47]. These results suggest that the administration of EPA to patients with Type 2 diabetes may prevent of the development of cardiovascular complications caused by some different risk factors. It seems that a combination of these actions and mechanisms explained above are responsible for the antiinflammatory, antiatherosclerotic, and antithrombotic effects caused by EPA. This study demonstrated that EPA can significantly increase the serum levels of HDL-c.

Three different members of the PON family (PON1, PON2, PON3) can prevent from the development of atherosclerosis and control oxidative stress in the blood circulation. Thus, in recent years because of their antioxidant and antiinflammatory role, PONs have received special attention in the field of atherosclerosis [50].

Authors demonstrated that although the gene expression of PON2 was already detected in several human tissues, but it does not exist in lipoproteins. Thus, they concluded that this describes ubiquitous intracellular distribution of this gene. These authors also reported that cells transfected with hPON2 gene compared with those cells that were not transfected, had greater antioxidant capacity and were less effective in the LDL oxidation [14]. PON2 is as a protective protein against the cellular oxidative stress i.e. as a potent cellular antioxidant [13, 14], and can protect macrophages from triglyceride accumulation [17]. Moreover, PON2, like PON1, may prevent of the lipid peroxidation via the hydrolysis of cellular oxidized lipids [51].

The PON2 gene has two common polymorphisms, G/A148 and C/ S311[55], which involve in variations in the levels of plasma lipoproteins, fasting glucose, apolipoprotein A-I in a variety of human disorders, such as type 2 diabetes [56], cardiovascular diseases [56, 57], and early

microvascular complications in type 1 diabetes [58]. Therefore, this may express that PON2 gene can have an important metabolic role in vascular disease.

Indeed, as mentioned above PON2 has a role antioxidant, and increase in the oxidative stress by a lot of agents can lead to enhancement in the gene expression of PON2 and its lactonase activity [13, 14]. It is reported that indeed, a moderate reduction in activity occurs before the acute increase in the activity of PON2 gene associated with enhancement of oxidative stress [59], and increment of the levels of human PON2 can effectively protect against the development of atherosclerosis [60].

In vitro, the PON2 gene is mainly expressed in monocytes, and influences the cellular oxidation and lipoprotein properties [14]. It is previously shown that only PON2 expresses in the human macrophages [13], and unlike PON1 and PON3, which inactivate by the oxidative stress, the expression and enzymatic activities of PON2 increase under the cellular oxidative stress [13, 59], and in parallel with it from cholesterol-loaded macrophages decreases [61]. Increase in the oxidative stress leads to the differentiation of monocytes into macrophages; an inflammatory phenomenon that play a role in the initiation of atherosclerosis [13, 62]; and during the differentiation of monocytes into macrophages, the production of superoxide anions via the cellular activation of nicotinamide adenine dinucleotide phosphate (NADPH) oxidase (this process is partly regulated by activating the transcription factor AP-1 [63], and STAT1 [64]) will increase, as well as in parallel with it the mRNA expression and activities of PON2 increases [13, 62]. Thus, it is possible that increase in the mRNA expression of PON2 is as a mechanism to compensate against the enhancement in oxidative stress, which occurs during the differentiation of monocytes into macrophages and thereby protects from the development of atherogenesis. It is demonstrated that PON2 indirectly decreases the release of superoxide anions from the inner membrane of mitochondrion [65].

As yet, interaction between EPA and the gene expression of PON2 in vitro and in vivo was not studied, and this is the first time that has been demonstrated EPA can upregulate the gene expression of PON2 in vivo. Our present study clearly shows that the supplementation of EPA for 8 weeks in patients with type 2 diabetes mellitus significantly upregulates the gene expression of PON2 (Table 2). Thus, it is significant to point out that our data provides evidence compatible with the hypothesis that EPA influences the expression of PON2.

Meanwhile, several studies have shown that the ω-3 PUFAs have various effects on the lipid profile in type 2 diabetic patients, including enhancing the size of LDL-c particle [69], reducing the serum levels of TG [70], increasing the plasma levels of HDL-c and HDL2-c [70, 71], and decreasing the plasma levels of HDL3-c [70]. This study demonstrated that EPA can significantly increase the serum levels of HDL-c which is compatible with the results in the other studies with ω-3 PUFAs [70, 71], but did not significantly affect the other serum levels of lipids.

The study limitations: There were several limitations for our study. First, a relatively small sample size of patients, therefore, it should point out that our study result is preliminary and need to be confirmed in a larger sample size of patients. Second, the mechanism by which EPA influences the gene expression of PON2 has not been clarified and further work is necessary to delineate the molecular mechanism of action of EPA on the regulation of PON2. For these reasons, additional studies will be necessary to determine the general applicability of our study result.

Conclusions:

The manipulation of the expression and activity of PONs by nutritional or pharmacological agents and products can be has an important role on the oxidative stress, and many of the inflammation and oxidative stress-related diseases. Unlike PON1, manipulation of PON2 has been less studied. Since PON2 gene has shown a significant antioxidative and lactonase potential, and these may be have the capacity to help the prevention of the development of atherosclerosis and other oxidative stress-related diseases. Thus, it has major clinical importance and is necessary that more studies be increasingly performed in the future in order to develop specific nutritional and pharmacological agents targeting PON2.

To conclude, the present study provides evidence for the upregulation of PON2 at the gene level by EPA in the patients of type 2 diabetes mellitus supplemented with EPA. Since the level of the gene expression of PON2 has a significant impact on serum lactonase and paraoxonase activities. Thus, it is very beneficial in the reduction of oxidative stress, and as a key regulator in the pathogenesis of atherosclerosis leading to several cardiovascular diseases. Thereby, the clinical efficiency of EPA in this application has worth evaluating.

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Table 1. The sequences of primers used for real time PCR reactions.

Primer Sequence (5′→ 3′)
Forward TGTAGACCTTCCACACTGCCACCT PON2 Reverse TGGTGCAAAGCTGTGGAGTCCTG
β -actin Forward CCTGGCACCCAGCACAATGAAG Reverse CTAAGTCATAGTCCGCCTAGAAGC

Table 2. The baseline and after characteristics of the two groups of patients

Variable / Group	Placebo [n (Female/Male)=18]		P-value	EPA [n (Female/Male)=18]		P-value
	Baseline	After		Baseline	After	
Age (years)	44.72 ± 4.69			44.44 ± 3.79		> 0.05
Duration of DM (years)	6.61 ± 3.68			6.44 ± 2.83		> 0.05
Weight (kg)	78.30 ± 12.34	78.24 ± 13.39	> 0.05	78.03 ± 12.68	77.15 ± 12.68	> 0.05
Height (cm)	165.11 ± 8.85			165.39 ± 8.12		> 0.05
Body mass index (kg/m²)	28.92 ± 5.39	28.87 ± 5.61	> 0.05	28.49 ± 3.95	28.17 ± 3.94	> 0.05
Waist circumference (cm)	97.47 ± 10.93	97.08 ± 11.73	> 0.05	97.55 ± 9.65	96.44 ± 10.16	> 0.05
Hip circumference (cm)	106.00 ± 11.82	105.61 ± 12.32	> 0.05	105.33 ± 6.70	104.61 ± 7.59	> 0.05
Waist/hip (ratio)	0.92 ± 0.08	0.92 ± 0.07	> 0.05	0.92 ± 0.05	0.92 ± 0.06	> 0.05
Systolic blood pressure (SBP) (mmHg)	124.11 ± 15.32	124.89 ± 18.08	> 0.05	124.00 ± 16.25	123.06 ± 18.78	> 0.05
Diastolic blood pressure (DBP) (mmHg)	80.00 ± 6.69	80.00 ± 7.22	> 0.05	79.78 ± 13.40	79.44 ± 11.83	> 0.05
Mean blood pressure (MBP) (mmHg)	94.70 ± 7.87	94.96 ± 8.98	> 0.05	94.52 ± 13.69	93.98 ± 13.41	> 0.05
Pulse Pressure (PP) (mmHg)	44.11 ± 14.42	44.89 ± 16.83	> 0.05	44.22 ± 9.59	43.62 ± 11.84	> 0.05
Heart rate (HR) (beat/minute)	89.44 ± 12.49	89.33 ± 11.73	> 0.05	89.67 ± 10.50	89.33 ± 10.91	> 0.05
FBS (mg/dL)	138.06 ± 49.13	142.06 ± 52.34	> 0.05	143.72 ± 53.53	137.94 ± 23.566	> 0.05
HbA1C (%)	7.47 ± 1.67	7.77 ± 1.42	0.022	7.89 ± 1.75	7.86 ± 1.58	> 0.05
Total energy intake (kcal)	1953.94 ± 297.12	1961.56 ± 232.21	> 0.05	1955.94 ± 279.49	274.36 ± 1973.61	> 0.05
Carbohydrates intake (g/d)	260.32 ± 35.44	37.22 ± 265.08	> 0.05	260.85 ± 41.78	42.89 ± 260.82	> 0.05
Proteins intake (g/d)	63.19 ± 14.78	11.97 ± 70.09	0.041	14.34 ± 63.83	63.92 ± 14.06	> 0.05
Lipids intake (g/d)	22.68 ± 76.11	76.39± 16.56	> 0.05	16.78 ± 73.82	20.13 ± 76.86	> 0.05
Fibers intake (g/d)	14.75 ± 4.64	2.28 ± 14.64	> 0.05	16.66 ± 4.99	16.84 ± 3.82	> 0.05

Data are shown as mean ± SD. Statistical analysis was performed using paired t-test and Independent t-test.

Table 3. The mRNA expression of PON2 at baseline and after of the supplementation with EPA or placebo

Group / Variable	Placebo		P-value	EPA		P-value
	Baseline	After		Baseline	After	
PON2 mRNA	**6.09 ± 5.57**	**6.26 ± 4.01**	**0.926**	**5.31 ± 4.90**	**8.43 ± 4.33**	**0.041**

Data are shown as mean ± SD. Statistical analysis was performed using paired t-test.

Table 4. Serum levels of lipids (mmol/L) at baseline and after the supplementation with EPA or placebo

Group / Variable	Placebo		P-value	EPA		P-value
	Baseline	After		Baseline	After	
Total cholesterol (mmol/L)	**204.44 ± 43.91**	**226.27 ± 38.73**	**> 0.05**	**211.22 ± 43.57**	**207.16 ± 39.69**	**> 0.05**
LDL-cholesterol (mmol/L)	**92.61 ± 35.92**	**95.73 ± 29.86**	**> 0.05**	**96.33 ± 38.13**	**81.4 ± 32.63**	**> 0.05**
HDL-cholesterol (mmol/L)	**31.11 ± 4.24**	**31.38 ± 4.76**	**> 0.05**	**29.72 ± 5.31**	**37.11 ± 5.97**	**< 0.05**
Triglycerides (mmol/L)	**221.50 ± 121.49**	**162. 8± 158.81**	**> 0.05**	**218.61 ± 94.52**	**176.48 ± 133.75**	**> 0.05**

Data are shown as mean ± SD. Statistical analysis was performed using paired t-test.

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References

1. Nyenwe, E.A.J., T. W. Umpierrez, G. E. Kitabchi, A. E., *Management of type 2 diabetes: evolving strategies for the treatment of patients with type 2 diabetes.* Metabolism, 2011. 60(1): p. 1-23.
2. Blonde, L., *State of diabetes care in the United States.* Am J Manag Care, 2007. 13 Suppl 2: p. S36-40.
3. Chang, Y.C.C., L. M., *The role of oxidative stress in the pathogenesis of type 2 diabetes: from molecular mechanism to clinical implication.* Am J Transl Res, 2010. 2(3): p. 316-31.
4. Organization, W.H., *Preventing Chronic Diseases: a Vital Investment*. 2005, Geneva: World Health Organization.
5. Federation, I.D., *Diabetes Atlas*. 3rd ed ed. 2006, Brussels, Belgium: International Diabetes Federation.
6. Organisation, W.H., *Stop the Global Epidemic of Chronic Disease*. Quick Diabetes Facts. 2009.
7. Wild, S.R., G. Green, A. Sicree, R. King, H., *Global prevalence of diabetes: estimates for the year 2000 and projections for 2030.* Diabetes Care, 2004. 27(5): p. 1047-53.
8. Primo-Parmo, S.L.S., R. C. Teiber, J. La Du, B. N., *The human serum paraoxonase/arylesterase gene (PON1) is one member of a multigene family*. Genomics, 1996. 33(3): p. 498-507.
9. Bhattacharyya, T.N., S. J. Topol, E. J. Zhang, R. Yang, X. Schmitt, D. Fu, X. Shao, M. Brennan, D. M. Ellis, S. G. Brennan, M. L. Allayee, H. Lusis, A. J. Hazen, S. L., *Relationship of paraoxonase 1 (PON1) gene polymorphisms and functional activity with systemic oxidative stress and cardiovascular risk.* JAMA, 2008. 299(11): p. 1265-76.
10. Draganov, D.I.T., J. F. Speelman, A. Osawa, Y. Sunahara, R. La Du, B. N., *Human paraoxonases (PON1, PON2, and PON3) are lactonases with overlapping and distinct substrate specificities.* J Lipid Res, 2005. 46(6): p. 1239-47.
11. Draganov, D.I.L.D., B. N., *Pharmacogenetics of paraoxonases: a brief review.* Naunyn Schmiedebergs Arch Pharmacol, 2004. 369(1): p. 78-88.
12. Ng, C.J.S., D. M. Hama, S. Y. Villa, N. Navab, M. Reddy, S. T., *The paraoxonase gene family and atherosclerosis.* Free Radic Biol Med, 2005. 38(2): p. 153-63.
13. Rosenblat, M.D., D. Watson, C. E. Bisgaier, C. L. La Du, B. N. Aviram, M., *Mouse macrophage paraoxonase 2 activity is increased whereas cellular paraoxonase 3 activity is decreased under oxidative stress.* Arterioscler Thromb Vasc Biol, 2003. 23(3): p. 468-74.
14. Ng, C.J.W., D. J. Gangopadhyay, A. Hama, S. Grijalva, V. R. Navab, M. Fogelman, A. M. Reddy, S. T., *Paraoxonase-2 is a ubiquitously expressed protein with antioxidant properties and is capable of preventing cell-mediated oxidative modification of low density lipoprotein.* J Biol Chem, 2001. 276(48): p. 44444-9.
15. Levy, E.T., K. Bendayan, M. Seidman, E. Delvin, E. Elchebly, M. Lavoie, J. C. Precourt, L. P. Amre, D. Sinnett, D., *Biological role, protein expression, subcellular localization, and oxidative stress response of paraoxonase 2 in the intestine of humans and rats.* Am J Physiol Gastrointest Liver Physiol, 2007. 293(6): p. G1252-61.
16. Precourt, L.P.A., D. Denis, M. C. Lavoie, J. C. Delvin, E. Seidman, E. Levy, E., *The three-gene paraoxonase family: physiologic roles, actions and regulation.* Atherosclerosis, 2011. 214(1): p. 20-36.
17. Rosenblat, M.C., R. Reddy, S. T. Aviram, M., *Paraoxonase 2 attenuates macrophage triglyceride accumulation via inhibition of diacylglycerol acyltransferase 1.* J Lipid Res, 2009. 50(5): p. 870-9.
18. Hagiwara, S.M., Y. Gu, L. Tanimoto, M. Zhang, M. Nakamura, S. Kaneko, S. Itoh, T. Gohda, T. Horikoshi, S. Tomino, Y., *Eicosapentaenoic acid ameliorates diabetic nephropathy of type 2 diabetic KKAy/Ta mice: involvement of MCP-1 suppression and decreased ERK1/2 and p38 phosphorylation.* Nephrol Dial Transplant, 2006. 21(3): p. 605-15.
19. Kesavulu, M.M.K., B. Apparao, Ch. Kumar, E. G. Harinarayan, C. V., *Effect of omega-3 fatty acids on lipid peroxidation and antioxidant enzyme status in type 2 diabetic patients.* Diabetes Metab, 2002. 28(1): p. 20-6.
20. Figueras, M.O., M. Busquets, S. Lopez-Soriano, F. J. Argiles, J. M., *Effects of eicosapentaenoic acid (EPA) treatment on insulin sensitivity in an animal model of diabetes: improvement of the inflammatory status.* Obesity (Silver Spring), 2011. 19(2): p. 362-9.

21. Terano, T.H., A. Hamazaki, T. Kobayashi, S. Fujita, T. Tamura, Y. Kumagai, A., *Effect of oral administration of highly purified eicosapentaenoic acid on platelet function, blood viscosity and red cell deformability in healthy human subjects.* Atherosclerosis, 1983. 46(3): p. 321-31.
22. Dyerberg, J.B., H. O. Stoffersen, E. Moncada, S. Vane, J. R., *Eicosapentaenoic acid and prevention of thrombosis and atherosclerosis?* Lancet, 1978. 2(8081): p. 117-9.
23. Association, A.D., *Clinical practice recommendations.* Diabetes Care 2010. 33: p. S1-S100.
24. Alberti, K.G.Z., P. Shaw, J., *International Diabetes Federation: a consensus on Type 2 diabetes prevention.* Diabet Med, 2007. 24(5): p. 451-63.
25. Kwok, S.H., R., *Avoiding false positives with PCR.* Nature, 1989. 339(6221): p. 237-8.
26. Rozen S, S.H., *Primer3.* 1998: Code available at http://www-/genome.wi.mit.edu/genome_software/other primer3.
27. Pfaffl, M.W., *A new mathematical model for relative quantification in real-time RT-PCR.* Nucleic Acids Res, 2001. 29(9): p. e45.
28. Stark, K.D.P., E. J. Maines, V. A. Holub, B. J., *Effect of a fish-oil concentrate on serum lipids in postmenopausal women receiving and not receiving hormone replacement therapy in a placebo-controlled, double-blind trial.* Am J Clin Nutr, 2000. 72(2): p. 389-94.
29. Fedor, D.K., D. S., *Prevention of insulin resistance by n-3 polyunsaturated fatty acids.* Curr Opin Clin Nutr Metab Care, 2009. 12(2): p. 138-46.
30. Mustad, V.A.D., S. Huang, Y. S. Mika, A. Lubbers, N. Berthiaume, N. Polakowski, J. Zinker, B., *Differential effects of n-3 polyunsaturated fatty acids on metabolic control and vascular reactivity in the type 2 diabetic ob/ob mouse.* Metabolism, 2006. 55(10): p. 1365-74.
31. Suzukawa, M.A., M. Howe, P. R. Nestel, P. J., *Effects of fish oil fatty acids on low density lipoprotein size, oxidizability, and uptake by macrophages.* J Lipid Res, 1995. 36(3): p. 473-84.
32. Okuda, Y.K., K. Sawada, T. Tsurumaru, K. Asano, M. Suzuki, S. Soma, M. Nakajima, T. Yamashita, K., *Eicosapentaenoic acid enhances nitric oxide production by cultured human endothelial cells.* Biochem Biophys Res Commun, 1997. 232(2): p. 487-91.
33. Okumura, T.F., Y. Morimoto, S. Tsuboi, S. Masai, M. Tsujino, T. Ohyanagi, M. Iwasaki, T., *Eicosapentaenoic acid improves endothelial function in hypertriglyceridemic subjects despite increased lipid oxidizability.* Am J Med Sci, 2002. 324(5): p. 247-53.
34. Tamura, Y.H., A. Terano, T. Takenaga, M. Saitoh, H. Tahara, K. Yoshida, S., *Clinical and epidemiological studies of eicosapentaenoic acid (EPA) in Japan.* Prog Lipid Res, 1986. 25(1-4): p. 461-6.
35. Thies, F.G., J. M. Yaqoob, P. Rerkasem, K. Williams, J. Shearman, C. P. Gallagher, P. J. Calder, P. C. Grimble, R. F., *Association of n-3 polyunsaturated fatty acids with stability of atherosclerotic plaques: a randomised controlled trial.* Lancet, 2003. 361(9356): p. 477-85.
36. Miyajima, T.T., T. Saito, K. Yokoyama, M., *Effects of eicosapentaenoic acid on blood pressure, cell membrane fatty acids, and intracellular sodium concentration in essential hypertension.* Hypertens Res, 2001. 24(5): p. 537-42.
37. Verlengia, R.G., R. Kanunfre, C. C. Bordin, S. Martins De Lima, T. Martins, E. F. Curi, R., *Comparative effects of eicosapentaenoic acid and docosahexaenoic acid on proliferation, cytokine production, and pleiotropic gene expression in Jurkat cells.* J Nutr Biochem, 2004. 15(11): p. 657-65.
38. Jump, D.B., *The biochemistry of n-3 polyunsaturated fatty acids.* J Biol Chem, 2002. 277(11): p. 8755-8.
39. Botolin, D.W., Y. Christian, B. Jump, D. B., *Docosahexaneoic acid (22:6,n-3) regulates rat hepatocyte SREBP-1 nuclear abundance by Erk- and 26S proteasome-dependent pathways.* J Lipid Res, 2006. 47(1): p. 181-92.
40. Berge, R.K.M., L. Vaagenes, H. Tronstad, K. J. Gottlicher, M. Rustan, A. C., *In contrast with docosahexaenoic acid, eicosapentaenoic acid and hypolipidaemic derivatives decrease hepatic synthesis and secretion of triacylglycerol by decreased diacylglycerol acyltransferase activity and stimulation of fatty acid oxidation.* Biochem J, 1999. 343 Pt 1: p. 191-7.
41. Neschen, S.M., K. Dong, J. Wang-Fischer, Y. Cline, G. W. Romanelli, A. J. Rossbacher, J. C. Moore, I. K. Regittnig, W. Munoz, D. S. Kim, J. H. Shulman, G. I., *n-3 Fatty acids preserve insulin sensitivity in vivo in a peroxisome proliferator-activated receptor-alpha-dependent manner.* Diabetes, 2007. 56(4): p. 1034-41.
42. Martin, R.E., *Docosahexaenoic acid decreases phospholipase A2 activity in the neurites/nerve*

growth cones of PC12 cells. J Neurosci Res, 1998. 54(6): p. 805-13.
43. Serhan, C.N.C., C. B. Brannon, J. Colgan, S. P. Gronert, K. Chiang, N., *Anti-microinflammatory lipid signals generated from dietary N-3 fatty acids via cyclooxygenase-2 and transcellular processing: a novel mechanism for NSAID and N-3 PUFA therapeutic actions.* J Physiol Pharmacol, 2000. 51(4 Pt 1): p. 643-54.
44. Kim, D.N.S., J. Thomas, W. A., *Dietary fish oil added to a hyperlipidemic diet for swine results in reduction in the excessive number of monocytes attached to arterial endothelium.* Atherosclerosis, 1990. 81(3): p. 209-16.
45. Yazu, K.Y., Y. Niki, E. Miki, K. Ukegawa, K., *Mechanism of lower oxidizability of eicosapentaenoate than linoleate in aqueous micelles. II. Effect of antioxidants.* Lipids, 1998. 33(6): p. 597-600.
46. Wander, R.C.D., S. H., *Oxidation of plasma proteins is not increased after supplementation with eicosapentaenoic and docosahexaenoic acids.* Am J Clin Nutr, 2000. 72(3): p. 731-7.
47. Singer, P.J., W. Wirth, M. Voigt, S. Naumann, E. Zimontkowski, S. Hajdu, I. Goedicke, W., *Lipid and blood pressure-lowering effect of mackerel diet in man.* Atherosclerosis, 1983. 49(1): p. 99-108.
48. Kris-Etherton, P.M.H., W. S. Appel, L. J. American Heart Association. Nutrition, Committee, *Fish consumption, fish oil, omega-3 fatty acids, and cardiovascular disease.* Circulation, 2002. 106(21): p. 2747-57.
49. Wang, C.H., W. S. Chung, M. Lichtenstein, A. H. Balk, E. M. Kupelnick, B. Jordan, H. S. Lau, J., *n-3 Fatty acids from fish or fish-oil supplements, but not alpha-linolenic acid, benefit cardiovascular disease outcomes in primary- and secondary-prevention studies: a systematic review.* Am J Clin Nutr, 2006. 84(1): p. 5-17.
50. Motti, C.D., M. Gnasso, A. Irace, C. Indigeno, P. Angelucci, C. B. Bernardini, S. Fucci, G. Federici, G. Cortese, C., *A multiplex PCR-based DNA assay for the detection of paraoxonase gene cluster polymorphisms.* Atherosclerosis, 2001. 158(1): p. 35-40.
51. Fuhrman, B.V., N. Aviram, M., *Oxidative stress increases the expression of the CD36 scavenger receptor and the cellular uptake of oxidized low-density lipoprotein in macrophages from atherosclerotic mice: protective role of antioxidants and of paraoxonase.* Atherosclerosis, 2002. 161(2): p. 307-16.
52. Ng, C.J.B., N. Grijalva, V. Hama, S. Shih, D. M. Navab, M. Fogelman, A. M. Lusis, A. J. Young, S. Reddy, S. T., *Paraoxonase-2 deficiency aggravates atherosclerosis in mice despite lower apolipoprotein-B-containing lipoproteins: anti-atherogenic role for paraoxonase-2.* J Biol Chem, 2006. 281(40): p. 29491-500.
53. Song, B.S., D. Ron, D. Pennathur, S. Kaufman, R. J., *Chop deletion reduces oxidative stress, improves beta cell function, and promotes cell survival in multiple mouse models of diabetes.* J Clin Invest, 2008. 118(10): p. 3378-89.
54. Horke, S.W., I. Wilgenbus, P. Kruger, M. Strand, D. Forstermann, U., *Paraoxonase-2 reduces oxidative stress in vascular cells and decreases endoplasmic reticulum stress-induced caspase activation.* Circulation, 2007. 115(15): p. 2055-64.
55. Hong, S.H.S., J. Min, W. K. Kim, J. Q., *Genetic variations of the paraoxonase gene in patients with coronary artery disease.* Clin Biochem, 2001. 34(6): p. 475-81.
56. Mackness, B.M., P. Mackness, M. I., *The paraoxonase-2-310 polymorphism is associated with the presence of microvascular complications in diabetes mellitus.* J Intern Med, 2005. 258(4): p. 363-8.
57. Leus, F.R.Z., M. Kastelein, J. J. Voorbij, H. A., *PON2 gene variants are associated with clinical manifestations of cardiovascular disease in familial hypercholesterolemia patients.* Atherosclerosis, 2001. 154(3): p. 641-9.
58. Kao, Y.D., K. C. Chan, A. Bennetts, B. H. Knight, J. Silink, M., *Paraoxonase gene cluster is a genetic marker for early microvascular complications in type 1 diabetes.* Diabet Med, 2002. 19(3): p. 212-5.
59. Shiner, M.F., B. Aviram, M., *A biphasic U-shape effect of cellular oxidative stress on the macrophage anti-oxidant paraoxonase 2 (PON2) enzymatic activity.* Biochem Biophys Res Commun, 2006. 349(3): p. 1094-9.
60. Ng, C.J.H., S. Y. Bourquard, N. Navab, M. Reddy, S. T., *Adenovirus mediated expression of human paraoxonase 2 protects against the development of atherosclerosis in apolipoprotein E-deficient mice.* Mol Genet Metab, 2006. 89(4): p. 368-73.
61. Rosenblat, M.H., T. Hussein, K. Aviram, M., *Decreased macrophage paraoxonase 2 expression in patients with hypercholesterolemia is the result of their increased cellular cholesterol content: effect of atorvastatin therapy.* Arterioscler Thromb Vasc Biol, 2004. 24(1): p. 175-80.
62. Shiner, M.F., B. Aviram, M., *Paraoxonase 2 (PON2) expression is upregulated via a reduced-nicotinamide-adenine-dinucleotide-phosphate*

(NADPH)-oxidase-dependent mechanism during monocytes differentiation into macrophages. Free Radic Biol Med, 2004. 37(12): p. 2052-63.
63. Wisdom, R., *AP-1: one switch for many signals.* Exp Cell Res, 1999. 253(1): p. 180-5.
64. Schieffer, B.L., M. Braun, S. Hilfiker, A. Hilfiker-Kleiner, D. Drexler, H., *Role of NAD (P)H oxidase in angiotensin II-induced JAK/STAT signaling and cytokine induction.* Circ Res, 2000. 87(12): p. 1195-201.
65. Altenhofer, S.W., I. Teiber, J. F. Wilgenbus, P. Pautz, A. Li, H. Daiber, A. Witan, H. Clement, A. M. Forstermann, U. Horke, S., *One enzyme, two functions: PON2 prevents mitochondrial superoxide formation and apoptosis independent from its lactonase activity.* J Biol Chem, 2010. 285(32): p. 24398-403.
66. Rowles, J.S., S. W. Xi, T. Majer, M. Nickle, D. C. Rommens, J. M. Popov, K. M. Harris, R. A. Riebow, N. L. Xia, J. Tsui, L. C. Bogardus, C. Prochazka, M., *Cloning and characterization of PDK4 on 7q21.3 encoding a fourth pyruvate dehydrogenase kinase isoenzyme in human.* J Biol Chem, 1996. 271(37): p. 22376-82.
67. Hegele, R.A., *Paraoxonase genes and disease.* Ann Med, 1999. 31(3): p. 217-24.
68. Rosenblat, M.V., N. Roqueta-Rivera, M. Nakamura, M. T. Aviram, M., *Increased macrophage cholesterol biosynthesis and decreased cellular paraoxonase 2 (PON2) expression in Delta6-desaturase knockout (6-DS KO) mice: beneficial effects of arachidonic acid.* Atherosclerosis, 2010. 210(2): p. 414-21.
69. Patti L, M.A., Iovine C, et al, *Long term effects of fish oil on lipoprotein subfractions and low density lipoprotein size in non-insulin-dependent diabetic patients with hypertriglyceridemia.* Atherosclerosis, 1999. 146: p. 361-367.
70. Woodman, R.J.M., T. A. Burke, V. Puddey, I. B. Watts, G. F. Beilin, L. J., *Effects of purified eicosapentaenoic and docosahexaenoic acids on glycemic control, blood pressure, and serum lipids in type 2 diabetic patients with treated hypertension.* Am J Clin Nutr, 2002. 76(5): p. 1007-15.
71. Luo, J.R., S. W. Vidal, H. Oppert, J. M. Colas, C. Boussairi, A. Guerre-Millo, M. Chapuis, A. S. Chevalier, A. Durand, G. Slama, G., *Moderate intake of n-3 fatty acids for 2 months has no detrimental effect on glucose metabolism and could ameliorate the lipid profile in type 2 diabetic men. Results of a controlled study.* Diabetes Care, 1998. 21(5): p. 717-24.

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