Effect of DHA-Rich Fish Oil on *PPAR* γ Target Genes Related To Lipid Metabolism in Type 2 Diabetes: A Randomized, Double-Blind, Placebo-Controlled Clinical Trial

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Introduction

PPAR γ is a nuclear transcription factor, which regulates genes involved in lipid metabolism, fat storage, adipocyte function and insulin action, including adipocyte fatty acid binding protein (ap2), phosphoenolpyruvate carboxykinase (PEPCK), lipoprotein lipase (LPL), the uncoupling protein (UCP1), liver x receptor- α (LXR α) and fatty acid translocase/CD36 (CD36) ¹. Polyunsaturated fatty acids (PUFAs) are considered as natural ligands of PPAR γ ²⁻³. It has been suggested that decosahexaenoic acid (DHA) might be a more potent activator of PPAR γ than eicosapentaenoic acid (EPA) ⁴. Studies in cell cultures and experimental models ⁵⁻⁷ as well as human studies ⁸⁻⁹ suggest that n-3 PUFAs improve lipid metabolism and anti-inflammatory/antioxidant capacity through the regulation of genes related to PPAR γ signaling. However, the influence of n-3 PUFAs on a number of PPAR γ -related genes involved in lipid metabolism remain to be discovered.

Patients with type 2 diabetes mellitus (T2DM) are frequently affected by coronary heart diseases. Hypertriglyceridemia is the most common lipoprotein alteration in diabetic patients, often accompanied by a decrease in high density lipoprotein cholesterol (HDL-c). Therefore, impaired reverse cholesterol transport (RCT) has been implicated ¹⁰. In RCT process, excess cholesterol is removed from peripheral cells, and returned to the liver for clearance into the bile and ultimately the feces ¹¹. The membrane-associated ATP-binding cassette A1 (ABCA1) has pivotal role in RCT ¹¹. ABCA1 protein mediates cholesterol efflux from cells to apolipoprotein A-1, and promotes the formation of HDL particles ¹¹. Functional mutation in the *ABCA1* gene lead to very low level of circulating HDL in both Tangier disease and familial HDL deficiency ¹². *ABCA1* expression in peripheral blood

leukocyte was correlated negatively with circulating HbA1c and glucose level, and also reduced in T2DM compared with control group ¹³. *ABCA1* expression in several tissues is upregulated by LXRa ¹⁴. Human *LXRa* gene promoter contains functional peroxisome proliferator response element (PPRE), and *LXRa* transcription is stimulated by PPAR γ agonist ¹⁴. Some anti-lipid drugs, PPAR γ agonist, and curcumin promote lipid metabolism via PPAR γ -LXRa-ABCA1 pathway ¹⁵⁻¹⁹.

CD36 is a lipid scavenger receptor with the broad cell type expression ²⁰. The ability of this receptor to recognize and internalize modified forms of LDL, including oxLDL, is well documented ²¹. Upregulation of this receptor leads to increased uptake of oxLDL ²². The role of CD36 in the intestinal lipid absorption, chylomicron formation and secretion is also reported in many studies ²³⁻²⁴. In the proximal intestine, lipid binding to CD36 leads to chylomicron secretion ²⁵. Moreover, CD36 is involved in the downstream signaling, which promote the production of large triglyceride-rich lipoprotein that are rapidly cleared in the blood ²⁶. Thus, CD36 may be targeted for reducing the postprandial hypertriglyceridemia ²⁶⁻²⁷. In addition, *CD36* expression is upregulated by PPAR γ ²⁸.

A comprehensive meta-analysis reported administration of 4g/day of n-3 PUFAs leads to decreased TG level by 25-30%, while producing an increase in low density lipoprotein-cholesterol (LDL-c) and HDL-c levels of 10% and 1-3%, respectively ²⁹. However, the gene expression modulations by which n-3 PUFAs affect lipid metabolism are not well documented. Therefore, the aim of the present study was to investigate whether DHA-rich fish oil supplementation mediate the modulation of some PPAR γ responsive genes related to lipid metabolism.

Materials and methods

Participants and study design. This parallel randomized, double-blind, placebo-controlled clinical trial was conducted on 72 T2DM patients in Tehran, Iran, during January to December 2014. The inclusion criteria were age 30-70 y, and a fasting blood glucose concentration ≥ 126 mg/dl. The exclusion criteria included 1) unwillingness or inability to participate, 2) insulin or thiazolidinediones therapy, 3) pregnancy, lactation or hormone replacement therapy, 4) clinical disease that could be related to n-3 metabolism (e.g. renal, hepatic, thyroid and coagulation disorders and malignancies), 5) the history of allergic reaction to fish or fish oil, 6) intake of n-3 supplement within last 3 months, 7) intake of vitamin D, A and B6 supplement that could influence PPAR activity ³⁰⁻³², 8) any change in the type or dosage of medications during the study.

All 72 participants stratified based on sex (male or female) and age (<50 or ≥ 50 year), using random number table to allocate into DHA-rich fish oil supplement or placebo group. The odorless DHA-rich fish oil softgels (containing 600 mg fish oil; DHA: 362.5mg and EPA: 100mg; vitamin E (mixed tocopherols): 1IU in each softgel) known as DHA Ultimate product were provided by Pure Encapsulation Co. (Boston, USA). Placebo softgels containing paraffin oil with the same shape, size and color as the supplements were provided by Zahravi Pharm Co. (Tabriz, Iran). The participants were instructed to consume four soft gels per day (containing 2400 fish oil; DHA: 1450mg and EPA: 400mg or 2400 mg paraffin oil), two soft gels after both breakfast and dinner for eight weeks.

On the first visit, the study protocol was completely described to the participants and the written informed consent form was obtained from them. After that, a general questionnaire including demographic data, diseases history and medication use was completed.

On the first day of the intervention, patients received one bottle of supplement (containing 120 softgels) which they needed for about four weeks consumption. They were asked to bring the first bottle at the end of four weeks and receive the second one. The patients obligated to note the number of taken softgels for each day during 8 weeks. Compliance was assessed by checking the consumption notes, counting the eaten softgels, and making bi-weekly phone call. The study was conducted according to the guidelines of the Declaration of Helsinki. The ethics committee of Tehran University of Medical Sciences approved the study and the trial was registered at the Iranian registry of clinical trial website (www.irct.ir) as IRCT2013071914013N1.

Assessment of dietary intake, physical activity and anthropometric parameters. To verify whether the participants maintained dietary intake at a constant level during the intervention, dietary intake of participants was assessed by means of 3-d food diaries (including a weekend day) completed in the first and the last weeks of the intervention period. All participants were given written and verbal instruction with some examples by a dietitian to complete the food records. Dietary energy values and nutrient contents from 3-d food diaries were calculated using NUTRITIONIST IV software, modified for Iranian (First Data Bank, San Bruno, CA, USA) foods. Participants were instructed to maintain a constant level of physical activity restricted to walking, and they were advised not to engage in any form of strenuous physical activity throughout the study, and Follow-up of lifestyle was done by bi-weekly phone call. The classified physical activity questionnaire according to Metabolic equivalent tasks (METs) was used to assess the amounts of daily physical activity at the baseline and last day of study (11). Anthropometric measurements were performed by the same person, after overnight fasting with a light cloth and without shoes. Body

weight was measured to the nearest of 0.1 kg by the use of digital scale (Seca). Height was measured in relaxed position and freely hanged arms using a stadiometer to the nearest of 0.1 cm (Seca). BMI was calculated as body weight (kg) divided by the square of the height (m).

Laboratory assessment. After fasting for 12 hours, the participants' blood samples were collected into tubes either with or without an anticoagulant (ethylene diamine tetraacetate, EDTA). TG, TC, LDL-cholesterol (LDL-c), HDL-c and fasting blood sugar were measured on sera from clot samples by enzymatic colorimetric assays, using commercially available kits (Pars Azmoon, Iran) and an auto-analyzer (Biolis 24i Premium, Japan). Glycated hemoglobin (HbA1c) was measured by using high performance liquid chromatography (HPLC) method (Toso, Japan). Serum insulin was measured by using an ELISA kit (Monobind, USA). Homeostasis model assessment-estimated of insulin resistance (HOMA-IR) calculated based on suggested formula ³³.

Isolation of RNA and RT-PCR assays. Peripheral Blood Mononuclear Cells (PBMCs) were isolated from anticoagulant-treated blood by Ficoll standard density gradient centrifugation. RNA was extracted and purified using Hybrid-R™ Blood RNA kit (GeneAll, Seoul, Korea) according to the company's protocol. The extracted RNA was checked for quality and purity by spectrophotometer (Nanodrop, Thermo Scientific, USA). RNA from all subjects was reverse transcribed with the use of a cDNA synthesis kit (Thermo Scientific, USA). Standard quantitative real-time PCR (RT-PCR) was carried out in StepOne system (Applied Biosystems, Foster City, USA) with SYBR Green method (Takara Bio Inc., Japan). The primer sequences used for the RT-PCR are listed in **Table 1**. GAPDH was used as

housekeeping gene. LinRegPCR software was used to determine PCR efficiency ³⁴. The fold change of gene expression was computed using Pfaffl method ³⁵.

Statistical analyses. The normality of data distribution was tested by using Kolmogorov-Smirnov. The square root transformation was used in the presence of skewness. Student's independent t-test was used to detect differences in participants' characteristics, dietary intakes and gene expression. One-way repeated-measures ANOVA was performed to detect differences in dietary intakes. The effect of treatment on lipid profile was analyzed by analysis of covariance (ANCOVA) adjusted for baseline values. We performed sub-analyses in normo- and hypertriglycerimic subjects (hypertriglyceridemia defined as TG level > 150mg/dL at the baseline of study ³⁶), to evaluate whether the gene expression response to DHA-rich fish oil depends on the baseline of TG levels. Data was analyzed using SPSS (version 18; Chicago, IL), and p value <0.05 was considered significant.

Results

Throughout the study, three participants in the placebo group were excluded for the following reasons: traveling to abroad (n=1), detection of colon cancer (n=1), and poor compliance (n=1). One participant in DHA-rich fish oil group also excluded due to gastrointestinal distress. Eventually, 68 participants completed the study. The baseline characteristics of the study population are shown in **Table 2**. There were no statistical differences in baseline characteristics (**Table 2**). Furthermore, no significant difference was seen between comparable groups in term of dietary intakes of energy, carbohydrate, protein or fat in baseline and throughout the study (**Table 3**). DHA-rich fish oil supplementation resulted in decreased TG level compared with placebo group,

independently of the baseline value of TG (all patients ($p=0.003$), hypertriglyceridemic subjects ($p=0.01$), and normotriglycemic subjects ($p=0.02$)) (**Table 4**). Moreover, a higher reduction in TG level was observed in hypertriglyceridemic subjects, compared to normotriglyceridemic subjects with DHA-rich fish oil supplementation ($p=0.01$). Total cholesterol, LDL-c and HDL-c were not significantly affected by DHA-rich fish oil. The expression of the genes in the PBMCs of DHA-rich fish oil group compared to placebo group is shown in **Figure 1**. In data analysis of all patients, the *PPAR γ* , *LXR α* , *ABCA1* and *CD36* showed a similar level of expression in both groups ($p>0.05$) indicating that DHA-rich fish oil supplementation had no effect on the gene expression of *PPAR γ* , *LXR α* , *ABCA1* and *CD36* (**Figure 1.A**). Moreover, the same results were observed in normo- and hypertriglyceridemic subjects (**Figure 1.B and C**), except for hypertriglyceridemic subjects, in which DHA-rich fish oil supplementation upregulated *CD36* expression, compared to the placebo group ($p=0.01$) (**Figure 1.B**).

Discussion

The results of the current study confirmed that supplementation with a moderate dose of DHA-rich fish oil, independently of baseline value of TG, could reduce TG level in patients with T2DM. Moreover, *CD36* expression was upregulated in hypertriglyceridemic subjects after 8 weeks of supplementation with DHA-rich fish oil. No other significant effects of this supplementation on total cholesterol, LDL-c and HDL-c, and also *PPAR γ* , *LXR α* and *ABCA1* expression were observed.

Supplementation of all subjects as well as normo- and hypertriglyceridemic subjects with DHA-rich fish oil resulted in TG level decrease by 25.5%, 17.3% and 31%, respectively.

These reduction values are comparable to other studies ³⁷. Several biochemical mechanisms may be related to the PUFA effect on TG concentration, such as the suppression of hepatic lipid synthesis and the decrease in very low-density lipoprotein (VLDL) ³⁸ via reducing the nuclear content of sterol regulatory element-binding proteins (SREBPs) ³⁹, or increasing lipoprotein lipase (LPL) gene expression ⁴⁰. However, only a few human studies have investigated molecular mechanisms by which n-3 PUFAs could affect the lipid metabolism through gene expression modification ^{9, 41}. These studies, albeit few in numbers, have shown the regulation of gene expression is a pivotal mechanism of n-3 PUFAs on lipid metabolism ⁴⁰⁻⁴¹.

Since PUFAs are endogenous ligands for PPAR γ activation ⁴², they can regulate the transcription of downstream target genes, such as *LPL* ⁴³ or *SREBP*⁴⁴. Based on previous studies, ligand activation of PPAR γ by PPAR γ agonist in cell culture was resulted in induction of *LXR α* and *ABCA1*, which are associated with RCT by PPAR γ -LXR α -ABCA1 pathway ⁴⁵. Furthermore, previous studies showed synthetic PPAR γ agonist, exercise, fibrates and curcumin promote lipid metabolism by activation of *PPAR γ -LXR α -ABCA1* pathway ^{17, 19}. In the current study, DHA-rich fish oil did not affect this pathway as well as HDL-c level, while reduced TG level was observed. In line with our results, the hypolipidemic action of fish oil diet was not affected by the *PPAR γ* antagonist in mice subjects . Thus, it is suggested that the main mechanisms of this supplementation on lipid metabolism are independent of PPAR γ -LXR α -ABCA1 pathway.

Our results suggest that *CD36* expression is elevated with DHA-rich fish oil supplementation in hypertriglyceridemic subjects, in accordance with the result from cell

culture and animal studies ⁴⁶⁻⁴⁷. In contrast with our result, another study showed that conjugated linoleic acid (as PPAR γ ligand) repressed CD36 gene expression ⁴⁸. In current study, the upregulation of *CD36* expression in hypertriglyceridemic patients is likely to result in a higher reduction in TG level, compared to normotriglyceridemic subjects. . N-3 PUFAs inhibit chylomicron and VLDL assembly and secretion in intestinal epithelial cells, which alleviate hyperlipidemia by reducing absorbed TG from enterocyte ⁴⁹⁻⁵⁰. CD36 is thought to be involved in the intestinal lipid absorption, chylomicron formation and secretion, and may be targeted for reducing the postprandial hypertriglyceridemia ²³⁻²⁷. Additionally, *CD36* is one of PPAR γ target gene ²⁸. Therefore, reduction of TG may contribute to n-3 PUFA impact (as PPAR γ ligand) on PPAR γ activation, which leads to the elevated *CD36* expression ²⁸.

N-3 PUFAs, as ligands of PPAR γ , upregulate *CD36* expression ⁵¹. However, this is due to PPAR γ activation, rather than the upregulation of *PPAR γ* expression ⁵¹. In line with previous findings, in the present study *CD36* upregulation was not accompanied with increased level of *PPAR γ* expression ⁵¹. Furthermore, although we did not assay PPAR γ activation per se, *CD36* upregulation by DHA-rich fish oil might indicate increased PPAR γ activation. However, additional research is required to confirm this result and fully understand the associated mechanisms, and we suggest measuring PPAR γ activity in the future studies. Moreover, inflammatory status may affect CD36 expression ⁵², thus we also suggest proinflammatory cytokines were measured for better interpretation of data.

In conclusion, DHA-rich fish oil supplementation for 8 weeks reduced TG level in all subjects as well as normo- and hypertriglyceridemic subjects. Furthermore, DHA-rich fish

oil supplementation increased *CD36* gene expression in hypertriglyceridemic subjects which might result in a higher reduction in TG level, compared to normotriglyceridemic subjects. However, this finding should be investigated in further studies.

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Figure

Figure 1. Effect of 8 weeks supplementation with DHA-rich fish oil or placebo on expression ratio of target genes in peripheral blood mononuclear cells.

The mRNA expression levels of the target genes were determined by the real-time quantitative reverse transcription-PCR and normalized with GAPDH mRNA levels. LinRegPCR software was used to determine PCR efficiency. The fold change of gene expression was computed using Pfaffl method. p value obtained from Student's independent t-test. Data are means $\pm$ SD.

Table 1. Nucleotide sequences of primers for quantitative real-time polymerase chain reaction

Gene symbol	Sequences (5 ' - > 3 ')
<i>PPARγ</i>	F: GAATTAGATGACAGCGACTTG R: GCTTGTAGCAGGTTGTCTTG
<i>LXRα</i>	F: CGCACTACATCTGCCACAG R: TCAGGCGGATCTGTTCTTCT
<i>ABCA1</i>	F: ATGGCACTGAGGAAGATGCTG R: CTCAGCCATGACCTGCCTTG
<i>CD36</i>	F:GGAAGTGATGATGAACAGCAG R:GAGACTGTGTTGTCCTCAGCG
<i>GAPDH</i>	F: TGGTATCGTGGAAGGACTCATG R: GCTTCACCACCTTCTTGATGTC

Table 2. Clinical characteristics of patients with type 2 diabetes who received either DHA-rich fish oil supplements or placebo

	DHA-rich fish oil (n=35)		Placebo (n=33)		p ¹
	Before	After	Before	After	
Age(y)	55.8±7.6		56.0±7.0		0.91
Weight (kg)	81.3±12.9		77.6±6.0		0.37
BMI(kg/m ²)	29.2±2.8		27.4±3.7		0.13
HbA _{1c} (%)	8.7±2.1		8.6±1.8		0.83
Physical activity(MET-h/day)	35.9±4.9	36.5±5.5	37.1±8.1	36.9±6.7	0.48
FBS (mg/dL)	181.8±66.0	181.3±63.4	167.0±50.3	165.4±57.9	0.30
Fasting serum insulin (mU/L)	8.2±5.5	7.2±4.2	8.4±5.1	6.9±3.3	0.92
HOMA-IR	3.5±2.6	3.1±2.0	3.5±2.5	2.8±1.8	0.97
Triglycerides (mg/dL)	193.2±108.1	143.8±63.4	158.4±70.3	174.3±88.0	0.12
Total cholesterol (mg/dL)	185.3±46.8	183.6±46.1	174.2±27.5	168.3±30.8	0.24
LDL-c (mg/dL)	96.4±27.9	99.6±29.5	89.0±17.4	90.5±21.2	0.15
HDL-c (mg/dL)	46.9±9.2	44.3±12.3	47.1±8.9	42.5±8.3	0.91

Data are expressed as means ± SD.

¹Comparison of baseline characteristics between DHA-rich fish oil and placebo groups derived from unpaired t-test.

BMI, body mass index; FBS, fasting blood sugar; HbA_{1c}, glycated hemoglobin; HOMA-IR, Homeostatic model assessment-estimated; LDL-C, LDL- cholesterol; HDL-C, HDL- cholesterol.

Table 3. Dietary intakes of type 2 diabetes who received either DHA-rich fish oil supplements or placebo throughout the study

	DHA-rich fish oil(n=35)		Placebo(n=33)		p ¹	p ²
	Before	After	Before	After		
Energy(kcal/d)	1606±595	1538±613	1620±501	1613±582	0.91	0.56
Carbohydrate(%of energy)	59.3±12.7	62.1±10.9	62.2±11.0	61.0±9.9	0.32	0.20
Protein(% of energy)	15.5±4.7	16.0±5.3	15.2±3.8	15.9±4.7	0.80	0.91
Total fat(% of energy)	25.7±14.6	23.7±10.7	23.6±11.6	23.9±10.7	0.55	0.45
SFA(% of energy)	6.9±4.8	6.3±4.5	5.8±4.0	6.1±3.7	0.34	0.29
MUFA(% of energy)	6.2±3.4	6.0±3.2	5.6±3.7	5.7±3.6	0.52	0.65
PUFA(% of energy)	7.9±9.8	6.3±6.8	5.8±6.1	6.0±5.5	0.34	0.31
Cholesterol(mg/d)	214±261	217.8±253	148±147	221±221	0.23	0.22
Dietary fiber(g/d)	9.6±4.5	9.4±4.6	11.5±5.0	12.0±5.8	0.12	0.47

Data are expressed as means ± SD.
¹Comparision of between-groups difference at the baseline derived from unpaired t-test. ²Comparison of within-groups difference derived from one-way repeated-measures ANOVA.
SFA, saturated fatty acids; MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids

Table 4. Changes of serum lipid concentration in patients with type 2 diabetes throughout the study

	All patients			Hypertriglyceridemic subjects			Normotriglyceridemic subjects		
	DHA-rich fish oil (n=35)	Placebo (n=33)	p ¹	DHA-rich fish oil(n=19)	Placebo (n=16)	p ¹	DHA-rich fish oil(n=16)	Placebo (n=17)	p ¹
Triglycerides	-49.3±87.5	15.9±63.7	0.003	-73.2±109.7	17.1±18.7	0.01	-19.10±28.9	14.7±43.2	0.02
Total cholesterol	-1.6±20.7	-5.9±26.9	0.28	-3.4±17.8	-4.6±30.5	0.34	0.50±24.2	-7.1±23.8	0.56
LDL-c (mg/dL)	3.1±20.0	1.4±16.8	0.48	4.8±22.6	1.1±15.9	0.11	1.10±17.0	1.7±17.5	0.86
HDL-c (mg/dL)	-2.6±9.3	-4.6±6.8	0.30	-2.2±4.5	-4.2±7.5	0.07	-3.0±13.1	-5.0±6.3	0.65

Data are expressed as means ± SD.
LDL-C, LDL- cholesterol; HDL-C, HDL- cholesterol.
¹p values were derived from ANCOVA with baseline values used as a covariate in all analyses.