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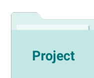

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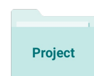

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DHA-enriched fish oil upregulates cyclin-dependent kinase inhibitor 2A ($P16^{INK}$) expression and downregulates telomerase activity without modulating effects of PPARγ Pro12Ala polymorphism in type 2 diabetic patients: A randomized, double-blind, placebo-controlled clinical trial

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DHA-enriched fish oil upregulates cyclin-dependent kinase inhibitor 2A (P16^{INK}) expression and downregulates telomerase activity without modulating effects of PPARγ Pro12Ala polymorphism in type 2 diabetic patients: A randomized, double-blind, placebo-controlled clinical trial

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65 **Abstract**

66 **Objective:** The present study investigated the effects of docosahexaenoic acid (DHA)-
67 enriched fish oil supplement on telomerase activity, mRNA expression of $P16^{INK}$, IL-6,
68 and TNF-α considering Pro12Ala polymorphism in the PPARγ gene.

69 **Methods/Design**: In this double-blind randomized controlled trial, 72 PPARγ Pro12Ala
70 polymorphism genotyped type 2 diabetic patients aged 30-70 years were randomly
71 assigned to receive 2.4 gr of DHA-enriched fish oil or a placebo for 8 weeks.
72 Genotyping of the Pro12Ala polymorphism in the PPARγ gene was assessed using
73 polymerase chain reaction-restriction length polymorphism (PCR-RFLP), telomerase
74 activity in the peripheral blood mononuclear cell (PBMC) was measured using PCR-
75 ELISA based on the telomeric repeat amplification protocol (TRAP), and changes in the
76 mRNA expression of P16, IL-6, and TNF-α were measured using real-time quantitative
77 reverse transcription–polymerase chain reaction (RT-PCR).

78 **Results:** In the DHA group, telomerase activity was decreased (p=0.001) during the
79 intervention. In addition, between-group comparisons showed significant differences in
80 the changes in telomerase activity (p=0.003) and P16 mRNA expression (p=0.028) and
81 non-significant differences in TNF-α and IL-6 mRNA expression. The gene*DHA
82 interaction could not affect changes in P16, IL-6, or TNF-α mRNA expression or in
83 telomerase activity in PBMC.

84 **Discussion:** Short-time DHA-enriched fish oil supplementation caused increased levels
85 of P16 expression and a decline in telomerase activity compared with the control group
86 without modulating the effects of Pro12Ala polymorphism on the PPARγ gene. Because

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87 of the positive correlation between P16 activity and cellular senescence, the possibility
88 of senescence stimulation by DHA is proposed.

89

90

91

92 **Keywords:** Type 2 diabetes, Docosahexaenoic acid, Telomerase activity, Cyclin-
93 dependent kinase inhibitor p16, PPAR gamma, Interleukin-6

94

95

96

97

98 Introduction

99 Proliferator-activated receptors (PPARs) are a superfamily of nuclear receptors that act
100 as transcriptional factors and regulate gene expression (1). The role of PPARα
101 activation in the proliferation of intima media smooth muscle cells by inhibiting the cell-
102 cycle progression in vascular smooth muscle cell (VSMC) has been proposed and
103 attributed to the induced levels of the tumor suppressor gene $p16^{INK}$ (2, 3).
104 P16 (also known as cyclin-dependent kinase inhibitor 2A and multiple tumor suppressor
105 1) plays important roles in cell-cycle control and cell senescence by reducing cyclin-
106 dependent kinase 4 (CDK4) and CDK6 as well as their phosphorylation properties and
107 providing cell proliferation regulation by inhibiting the activity of cyclin–CDK complexes
108 (3).

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109 Telomeres are stabilized by telomerase to serve as a protective capping and prevent
110 cellular senescence; telomerase activation affects cell proliferation by maintaining
111 telomere length (4). Saito et al. found that $p16^{INK}$ inhibits telomerase activity through the
112 suppression of human telomerase reverse transcriptase (hTERT) expression at
113 transcriptional levels (5), and Gizard et al. showed that the effects of $p16^{INK}$ on cell-cycle
114 arrest are mediated through the inhibition of telomerase activity (3).
115 Although DNA replication is precisely regulated, some important body of evidence has
116 expressed that telomerase deficiency reduces atherosclerosis and neointima formation
117 because of telomere shortening and the inhibition of mitotic proliferation (3). In contrast,
118 higher telomerase activity is linked to smooth muscle cell proliferation. In support,
119 Narducci et al. found higher telomerase activity in neutrophils driven from unstable
120 coronary plaques (6).
121 PPARγ, another member of PPAR family, is involved in adipocyte function and
122 differentiation, lipid storage by adipocytes, and glucose responsiveness (7). Some
123 experimental studies have demonstrated that PPARγ activation by thiazolidinedione
124 (TZD) inhibits the proliferation and migration of VSMCs (8). Furthermore, a study
125 conducted by Gan et al. defined an important role for PPARγ in accelerating cellular
126 senescence through the overexpression of $p16^{INK}$ in human diploid fibroblasts (9). Some
127 point mutations and single nucleotide polymorphisms (SNPs) are defined for this
128 nuclear receptor. Pro12Ala missense mutation in PPARγ2 specific B exon and a
129 CCA→GCA base exchange is a highly prevalent polymorphism which causes the
130 replacement of alanine amino acid instead of proline. The possibility of an association
131 between this SNP by lower blood levels of total and LDL cholesterol and improved lipid

132 profile (10); reduced risk of type 2 diabetes mellitus (T2DM) and coronary heart disease
133 (CHD) (11); diabetic nephropathy; metabolic syndrome; homeostasis model
134 assessment (HOMA-IR); hepatic growth factor (HGF); and nerve growth factor (NGF)
135 has been proposed by previous studies (12). Investigating the effects of Pro12Ala SNP
136 on BMI and insulin sensitivity has had some conflicting results (10, 13, 14). Because
137 alanine substitution at codon 12 of exon B is related to the ligand-independent activation
138 domain in the PPARγ gene, the possibility of this SNP altering transcriptional activity
139 has been proposed. Recently, Lapice et al. reported that the effects of changes to the
140 energy content and composition of the diet on anthropometric and body composition
141 measures are more intense in ◌Ala carriers than in wild types, and the Ala allele could
142 result in weight loss in response to a healthy lifestyle (15).
143 Docosahexaenoic acid (DHA, 22:6 n-3), a long-chain polyunsaturated fatty acid (PUFA),
144 is a nutritional ligand for PPARα and PPARγ (16, 17), and the anti-inflammatory effects
145 of this ligand through the inhibition of activated NF-κB in a PPARα-dependent pathway
146 have been proved. PPARα activation by DHA can inhibit the gene expression of acute-
147 phase and inflammatory cytokines, such as interleukin-6 (IL-6) and tumor necrosis
148 factor alpha (TNFα), which implies an anti-inflammatory role for PPARα (18). Some
149 evidence shows that omega-3 fatty acid levels are correlated with telomere aging in
150 PBMC (19). T lymphocytes and other immune cells release TNFα and IL-6 during
151 chronic inflammatory responses that are inflammatory cytokines and have an important
152 role in plaque formation (18). Since serum levels of TNFα and IL-6 are related to intima
153 media thickness, preventing inflammation by inhibiting cytokine expression can be a
154 critical issue in modulating atherosclerotic cascade responses.

155 The risk of macro- and micro-vascular disorders is greater in T2DM patients than in
156 non-diabetic subjects, and this contributes to altered lipid and lipoprotein metabolism,
157 low-grade inflammation, endothelial dysfunction, etc. Hence, the present study was
158 conducted to identify the effects of DHA as a natural ligand for PPARs on telomerase
159 activity, P16, TNFα, and IL-6 regarding the effects of the highly prevalent polymorphism
160 of the PPARγ gene. To the best of the authors' knowledge, the present study is a
161 pioneer work that attempts to combine in vivo nutrigenetics and nutrigenomics in the
162 format of a randomized, controlled trial to investigate the alteration in cell signaling
163 pathways.

164

165 ## Subjects and Methods

166 ### Study participants

167 Based on the 5.94% frequency of Pro12Ala in the PPARγ gene among the Iranian
168 population, 465 type 2 diabetic patients were subjected to polymorphism screening. To
169 obtain additional information on the treatment, a 2:1 allocation rate (two wild types
170 versus one polymorphic subject) was used (20). Seventy-two T2DM males and females
171 were selected based on this particular SNP. Forty-eight non-Ala carriers (Pro/Pro) and
172 24 Ala carriers (22 Pro/Ala and two Ala/Ala genotypes) were enrolled in this RCT. The
173 stratification was based on sex (male or female) and age (<50 or ≥50 year), and a
174 random number table was applied to randomly allocate the participants to DHA-
175 enriched fish oil supplementation or control groups as mentioned previously. The final
176 gene*DHA intervention groups were as follows: Ala+*DHA supplementation group,
177 Ala+*control group, Ala-*DHA supplementation group, and Ala-*control group. Pregnant

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178 and/or lactating women; patients with a history of severe reactions to fish or fish oil, a
179 history of liver, kidney, coagulation, or thyroid disorders, insulin therapy,
180 thiazolidinedione therapy, intake of anticoagulant and non-steroidal anti-inflammatory
181 agents, daily consumption of fish or omega-3, vitamins A or D, or B12 supplements
182 within the last 3 months, or any changes in medications during the study; and patients
183 with an intake of less than 90% of total soft gels were excluded.
184 For 8 weeks, all patients took four 600 mg soft gels orally per day (two after breakfast
185 and 2 after dinner). Each DHA-enriched fish oil soft gel contained 600 mg omega-3 fatty
186 acids; more specifically, 362.5 mg DHA plus 100 mg EPA according to the
187 manufacturer's information (DHA Ultimate, Pure Encapsulations, Boston, USA). Other
188 ingredients were gelatin capsule, fish oil (tilapia), 1 IU natural mixed tocopherols, and
189 rosemary extract (leaf). The control group received soft gels that were identical in
190 appearance to the DHA-enriched fish oil soft gels but contained 600 mg paraffin
191 (Zahravi, Tabriz, Iran).
192 Patients who met the inclusion criteria were fully informed about the study's protocol
193 which was approved by the Medical Ethics Committee of Tehran University of Medical
194 Sciences and was in accordance with the Declaration of Helsinki. Informed consent was
195 obtained from each subject, and the study was registered in Iranian Registry of Clinical
196 Trials (www.irct.ir) as IRCT2013071213964N1.
197

198 Forms and questionnaires

199 Demographic and physical activity questionnaires were completed at the baseline and
200 repeated for physical activity at the endpoint of the study as previously described (21). A

201 3-day food record (2 weekdays and 1 weekend day) in the first week of intervention and
202 another 3-day diet record in the last week of intervention were completed by
203 participants, analyzed by Nutritionist IV (The Hearst Corporation, San Bruno, CA), and
204 compared within and between the studied groups.
205

206 Laboratory methods

207 After 12h overnight fasting, 20 ml samples of venous blood were obtained and
208 subjected to biochemical and genetic assessments. Telomerase activity and mRNA
209 gene expression were assessed at both baseline and endpoint of the intervention.
210

211 Genotyping of Pro12Ala polymorphism in PPARγ

212 Genomic DNA was extracted from samples of whole blood using the salting-out method
213 (22). PCR was performed in 25 μL of the mixture containing 2X Taq polymerase mix
214 (Amplicon co.), 75 ng DNA, and 0.6 mM of each primer (Forward: 5'-
215 CTGATGTCTTGACTCATGGGTGTATTCAC-3'; Reverse: 5'-
216 ACAGTGTATCAGTGAAGGAATCGCTTTCCG -3') in 35 cycles at 94 ℃ for 30 s, 58 ℃
217 for 40 s, and 72 ℃ for 40 s on a peQSTAR thermocycler system (PeQLab, Germany).
218 Genotyping was conducted by the RFLP method using BstU-I at 37 ℃ overnight. The
219 digested PCR products (CC: one 196 bp band; CG: 196, 166, and 30 bp bands; GG:
220 two 166 and 30 bp bands) were analyzed using 8% polyacrylamide gel electrophoresis
221 and subsequent staining with fluorescent red dyes (Biotium Inc.) as previously
222 described.
223

224 **RNA extraction and real-time PCR**

225 Peripheral blood mononuclear cells (PBMCs) were isolated from whole blood using the
226 standard Ficoll method, and RNA was extracted by a Hybrid-R™ Blood RNA Kit
227 (GeneAll, Seoul, Korea). The quality and purity of the extracted RNA were checked by
228 spectrophotometer (NanoDrop, Thermo Scientific, USA).
229 Total RNA was reverse transcribed to cDNA using a cDNA synthesis kit (Thermo
230 Scientific, USA). Standard quantitative real-time PCR (RT-PCR) was carried out in the
231 StepOne system (Applied Biosystems, Foster City, USA) using the SYBR Green
232 method (Takara Bio Inc., Japan). Real-time PCR primers were designed using Primer
233 Blast, Oligocalc, and Generunner 5.0.99; the primer sequences are listed in Table 1.
234 The glyceraldehyde-3-phosphate dehydrogenase (GAPDH) housekeeping gene was
235 included as an endogenous normalization control. PCR efficiency was estimated using
236 LinRegPCR software (23). Changes in mRNA expression were computed using the
237 Pfaffl equation (24).
238

239 **Assessment of telomerase activity in PBMC**

240 Telomerase activity was measured using a commercial telomerase PCR-ELISA (Roche
241 Diagnostics Corp., Indianapolis, IN, USA) based on the telomeric repeat amplification
242 protocol (TRAP). Briefly, 2×10^5 purified PBMCs for each sample were lysed and 2 µl of
243 supernatant was added to 25 µl reaction mixture until the final volume reached 50 µl.
244 Sample-containing tubes were amplified, denaturized, hybridized to a digoxigenin-
245 (DIG)-labeled, immobilized to streptavidin-coated microplate, detected with antibody
246 against digoxigenin, and visualized. Sample absorbance was determined at 450 nm

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247 using the reference wavelength at 690 nm, and the measured optical density was
248 considered to be telomerase activity in PBMC.
249
250
251
252 **Statistical analysis**
253 The one-sample Kolmogorov-Smirnov test was used to establish normal data
254 distribution. Because of the non-parametric distribution of some study parameters, the
255 data was transformed to normal values for statistical analyses and then back-
256 transformed into natural units for exhibition in the related tables or figures. Continuous
257 data was expressed as mean±standard deviation, and categorical variables were
258 presented as number and percentage. Between-group comparisons for nominal
259 variables were made using the chi-square test. The independent sample t-test was used
260 to compare parametric continuous data, and the paired t-test was conducted to evaluate
261 within-group differences (before and after intervention). Factorial ANOVA followed by
262 the least significant difference (LSD) post-hoc test was applied to detect the effects of
263 interaction between the polymorphic phenotype and DHA supplementation, and
264 analysis of covariance (ANCOVA) was conducted by controlling for possible
265 confounders to assess between-group differences. The alpha value was set at 0.05 to
266 indicate the statistical significant level for the comparisons. All data entry and statistical
267 analyses were conducted using Statistical Package for Social Sciences (SPSS Inc.,
268 Chicago, IL, USA), version 18.0.
269

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270 # Results

271 ## Basic information

272 To investigate PPARγ genotyping, 465 participants were enrolled and screened for
273 Pro12Ala polymorphism. The frequencies of Pro/Pro, Pro/Ala, and Ala/Ala genotypes
274 were 90.32%, 8.6%, and about 1%, respectively.

275 The background information of the participants is shown in Table 2. Three patients in
276 the control group were excluded from the study because of traveling (n=1), a diagnosis
277 of cancer during intervention (n=1), and poor compliance (n=1). One patient in the
278 intervention group left the study because of gastrointestinal distress, and the final data
279 for one DHA-enriched fish oil-supplemented patient was excluded because of fibrate
280 usage during the study. At the endpoint, 67 patients (33 males and 34 females)
281 completed the intervention.

282 As shown, the DHA-enriched fish oil and control groups did not differ significantly in
283 terms of background and demographics. It should be mentioned that all data analyses
284 based on Pro12Ala polymorphism (regarding Ala and non-Ala carriers) were applied
285 and the same results were obtained (data not shown). No significant difference in
286 background characteristics existed between the studied groups.

287

288 ## Dietary data and anthropometric parameters

289 The results of the baseline and endpoint dietary intakes (including total energy intake
290 and percentage of energy from carbohydrates, protein, total fat, saturated fatty acids,
291 mono-unsaturated fatty acids, and poly-unsaturated fatty acids) were reported in a
292 previous article as well as the data for physical activity (25). Briefly, it was reported that

293 between-group analyses at the baseline and within-group changes at the endpoint of
294 the study did not differ significantly in the studied groups with or without consideration
295 given Pro12Ala polymorphism in PPARγ. It should also be mentioned that the effects of
296 DHA-enriched fish oil on anthropometric and biochemical values were discussed in
297 detail in the authors' previous article (25).

298 **Effects of DHA-enriched fish oil supplementation on telomerase activity**

299 The effects of DHA-enriched fish oil supplementation on telomerase activity are shown
300 in Fig. 1. At the endpoint of the study, telomerase activity was significantly reduced in
301 the DHA group (p=0.001) when compared with the baseline. The same result was not
302 achieved in the control group (p=0.5). The mean changes of telomerase activity in the
303 DHA-enriched fish oil group were significantly higher than those of telomerase activity in
304 the control group, even after adjusting for age and gender (p=0.003) (Table 3).

305

306 **Effects of DHA-enriched fish oil supplementation on mRNA gene**
307 **expression**

308 Figure 2 shows the relative changes of mRNA expression in TNF-α and IL-6 genes in
309 PBMC from the DHA-enriched fish oil and control groups during the intervention. As
310 shown in Table 3, P16 expression was increased 1.86-fold in the DHA-enriched fish oil
311 group. Furthermore, comparisons of the relative changes in mRNA expression between
312 the intervention groups revealed significant differences and proved the overexpression
313 of P16 gene in the DHA-enriched fish oil group (p = 0.021), while between-group
314 analyses of TNF-α or IL-6 mRNA expression levels could not reach significant levels
315 (p=0.4 and p=0.6, respectively). It should be mentioned that, even after adjusting for

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316 confounders, between-group differences in P16 mRNA expression still remained
317 significant (p = 0.028) (Table 3).
318

319 **Effects of gene and DHA interaction on study parameters**

320 In addition to the observed differences in telomerase activity, the polymorphism-based
321 analysis conducted for changes in telomerase activity did not reveal significant gene*-
322 DHA interactions; also, no significant differences were found in changes in telomerase
323 activity levels between the Ala+*DHA and Ala-*DHA groups (See Fig. 3).
324 Telomerase activity reduction levels in the Ala+*DHA group during the study were
325 higher than those of the Ala-*DHA group (-0.64 ± 0.97 ver. -0.43 ± 0.73), but this
326 difference was not statistically significant (p = 0.4). The same analyses were conducted
327 for changes in P16, IL-6, and TNF-α mRNA expressions, and no significant gene*DHA
328 interaction effect was found on the relative changes of P16 mRNA expression (p = 0.7)
329 as well as TNF-α (p = 0.8) and IL-6 (p = 0.3) (Table 4).
330

331 **Discussion**

332 The most important finding of the current study was the significant reduction in
333 telomerase activity and enhanced P16 mRNA expression levels achieved with DHA-
334 enriched fish oil supplementation even after controlling for possible confounders. In
335 addition, a significant inverse correlation between changes in telomerase activity and
336 P16 mRNA expression was observed with no modulation of the effects of Pro12Ala
337 polymorphism in the PPARγ gene.

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338 Some cross-sectional studies have reported lower telomerase activity in PBMC for
339 diabetic and hemodialysis patients (26), while some others have reported higher
340 telomerase activity in obese subjects and patients with metabolic syndrome or angina
341 (6). Lower telomerase activity in PBMC contributes to pre-mature senescence and
342 higher inflammatory activity in PBMC (27), while higher telomerase activity in PBMC is
343 explained by the systemic activation of immune blood cells such as macrophages (28,
344 29). Some cellular studies have reported that PPAR activation via synthetic ligands can
345 accelerate PPAR-related cellular senescence in some cell lines that will be discussed
346 further in detail (3, 9). The main objective of the current study was to determine whether
347 DHA-enriched fish oil supplementation as a natural ligand for PPARs (not the synthetic
348 ligand) could induce P16 expression levels and reduce telomerase activity in PBMC,
349 both of which are related to cellular senescence. It was found that DHA
350 supplementation reduced telomerase activity in PBMC of T2DM patients and
351 overexpression of P16 gene. Some mechanisms are described for the control of
352 telomerase activity such as regulation of gene expression, phosphorylation of the
353 enzyme, and interactions by regulatory proteins (30). Regulation of human telomerase
354 reverse transcriptase (hTERT), a catalytic subunit of telomerase which is the most
355 important subunit of the enzyme telomerase, comprises the prominent pathway in
356 regulating telomerase activity in eukaryotic cells. Cyclin-dependent kinase inhibitor p16
357 (p16) is known as a more important cell cycle inhibitor that can inhibit telomerase
358 activity by repressing hTERT subunit of telomerase. In two correlated studies, Gizard et
359 al. showed that PPARα activation via synthetic ligands could modulate telomerase
360 activity through the overexpression of tumor suppressor P16 by an indirect mechanism

361 (2, 3). They showed that PPARα activation via synthetic ligands can lead to
362 RXR/PPARα heterodimer formation and subsequent binding to the DR1 PPAR-
363 response element (PPRE) sequence in the P16 promoter and up-regulation of P16 as a
364 downstream target gene (2). The underlying mechanism for the effects of P16 on
365 telomerase activity is explained by the necessity of P16 to the recruitment of P107 and
366 P130 pocket proteins to the trans-activation site of the TERT promoter, inhibiting E2F-1
367 binding to this promoter and subsequent negative cross-talk and trans-repression (3).
368 Although the pathways mentioned by Gizard et al. can explain the finding of the current
369 study related to P16 upregulation and telomerase activity reduction to a great extent. In
370 another study, Eitsuka et al. suggested that LC-PUFA, specifically EPA or DHA, can
371 reduce telomerase activity by the underexpression of hTERT and myc genes (31) that
372 can be attributed, to some extent, to the inhibition of protein kinase C (32, 33). Very
373 similar results were obtained by Oda et al. for oleic acid (omega-9) (34). One interesting
374 point in the current findings is the lack of differences in PPARγ gene expression in
375 PBMC by DHA-enriched fish oil supplementation during intervention (data not shown).
376 Such result could emphasize different PPARγ regulatory mechanisms in PBMC and
377 propose that a large percentage of the observed DHA effects on P16/telomerase
378 pathway in the present study was related to PPAR binding and PPAR activation instead
379 of PPAR overexpression. Conversely, neither differences in P16 expression levels nor
380 changes in telomerase activity were associated with Pro12Ala polymorphism in the
381 PPARγ gene. Although it is supposed that the alanine substitution in the PPARγ coding
382 gene (instead of proline) leads to structural and functional changes in the PPARγ
383 protein because of the α-helix formation facilitated by alanine amino acid (35), no

384 significant differences between Ala carriers and non-carriers were found in this study,
385 nor was a reduction in transcriptional activity of PPARγ in P16 expression by Ala allele
386 observed. It seems that the phosphorylation status of PPARγ that could be the main
387 determinant of protein activity is not affected by this polymorphism. Besides various
388 underlying pathways that control P16 expression in PBMC, it is possible that the
389 relatively small sample size of polymorphic sub-groups, ethnic differences, or the short
390 interventional period could account for non-significant gene-diet interactions despite the
391 baseline genotype stratification.

392 In the present study, no association was observed between differences in TNF-a or IL-6
393 mRNA expression in PBMC with DHA-enriched fish oil supplementation. Moreover, P16
394 overexpression and telomerase activity downregulation were not in accordance with the
395 changes of inflammatory cytokines' mRNA expression, probably due to the different and
396 multi-factorial underlying mechanisms involved in inflammatory pathways. Although the
397 primary and classic anti-inflammatory effects of long chain n-3 fatty acids were defined
398 and proven by multiple pathways, including direct PPAR activation or indirect NF-kB or
399 AP-1 pathway inhibition (18), and even though inhibition of the lipopolysaccharide-
400 induced overproduction of pro-inflammatory cytokines including TNF-α and IL-6 through
401 the activation of G protein-coupled receptor 120 (GPR120) signaling was defined for
402 DHA (36), it is likely that, because of the presence and coexistence of T2DM and
403 obesity in the studied population, the proinflammatory pathways were so strong that
404 DHA-enriched fish oil supplementation could not down-regulate the expression of
405 inflammatory cytokines.The c ombination of obesity and insulin resistance leads to an
406 increased quantity of activated macrophages in the blood as well as adipose tissue,

407 which directly affects the production and secretion of inflammatory cytokines such as
408 TNF-α, IL-6, and IL-1b (37). This could account for the lack of differences in mRNA
409 expression of inflammatory cytokines in the current study; as seen by Labonté et al. in
410 2013, DHA+ EPA supplementation by 5 g/d for 8 weeks in obese T2DM patients had no
411 effect on TNF-α or Il-6 intestinal mRNA expression (38).
412 Because of the elevated P16 expression and reduced telomerase activity by DHA-
413 enriched fish oil, the possibility of senescence induction in the present study was
414 proposed. Cellular senescence is defined as irreversible cellular changes such as
415 mitochondrial and endoplasmic reticulum impairment and induction of free radical levels
416 in order for cell-cycle arrest (39). The induced secretion of pro-inflammatory factors by
417 senescent cells leads to a condition called senescence-associated secretory phenotype
418 (40). In other words, the anti-inflammatory effects of DHA-enriched fish oil and the
419 secretion of pro-inflammatory mediators by senescent cells resisted each other and
420 made judging the effects of DHA on mRNA expression of inflammatory cytokines
421 harder. However, this subject needs further investigation.
422 This study had some limitations. One is the small sample size of patients in the
423 polymorphic sub-groups, which could partly account for limited statistical power and
424 insignificant results in telomerase activity changes or P16 mRNA expression between
425 polymorphic sub-groups. A second important limitation of the current study is the lack of
426 access to some senescence-related genes or cell-cycle regulators, such as hTERT,
427 P53, or P38, that could broaden the knowledge about cell-cycle regulation. However, to
428 the best of the authors' knowledge, the present study was the first parallel randomized
429 controlled trial in humans that examined the effects of natural PPAR ligand on P16

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430 levels as a tumor suppressor and cell-cycle regulator based on the interactions of
431 nutrigenomics and nutrigenetics. These primary results may open new horizons on
432 using natural PPAR ligands for some chronic diseases such as T2DM or cardiovascular
433 disease alone or in combination with synthetic PPAR ligands to reduce drug doses or
434 prevent possible drug-associated side effects. Also, P16 as a key target of the present
435 study is one of the main senescence markers involved in some stem or progenitor cell
436 senescence. On the other hand, because the hypothesis of this study was based on
437 telomerase activity or P16 expression established on PPAR-related pathways and with
438 respect to the key points that PPARα was also expressed in great values in vascular
439 endothelial and smooth muscle cells and PPARγ was expressed in white adipose tissue
440 (even more than PBMC), assessing the effects of natural PPAR ligands and PPAR
441 activation on such tissue with the aim of CVD prevention or anti-inflammatory pathway
442 stimulation could be recommended for future studies.

443

444 **Conclusion**

445 Short-term DHA-enriched fish oil increased P16 mRNA levels and decreased
446 telomerase activity in PBMC without modulating the effects of Pro12Ala polymorphism
447 in PPARγ genes. Because of the positive correlation between P16 activity and cellular
448 senescence, the possibility of senescence stimulation by DHA-enriched fish oil could be
449 proposed.

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451 **Authors' contributions**

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Toupchian: Initiated and coordinated the study and finalized the study protocol, developed the dietary intervention, developed the first draft of the manuscript and its subsequent versions,

Mansoori: Initiated and coordinated the study and finalized the study protocol

Koohdani and Sotoudeh: Conceptualized and designed the study and assisted in the development of the study,

Ensieh Nasli-Esfahani, Mahmoud Djalali, Seyyed Ali Keshavarz and Shima Abdollahi edited the final edition of the manuscript,

Ehsan Alvandi and Reza Chahardoli assisted in the telomerase activity assay and real-time procedure.

All the authors contributed to drafts of the manuscript and approved the final version.

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Conflict of interest

The authors of this manuscript have no conflicts of interest to disclose.

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617 **Table 1:** Real-time PCR primers

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	Forward	Reverse
TNF-α	CCAGGGACCTCTCTCTAATCAG	TGAGGTACAGGCCCTCTGATG
IL-6	GACAGCCACTCACCTCTTCAG	GTGCCTCTTTGCTGCTTTCAC

P16	CTTCCTGGACACGCTGGTG	GCATGGTTACTGCCTCTGGTG
GAPDH*	TGGTATCGTGGAAGGACTCATG	GCTTCACCACCTTCTTGATGTC

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620 *Glyceraldehyde-3-Phosphate Dehydrogenase

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633 **Table 2.** Baseline characteristics of type 2 diabetic patients

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	DHA-enriched Fish oil (n=34)	Control (n=33)	P-value[†]
Age (years, mean±SD)	55.9± 7.8	56± 7	0.9 [a]
Diabetes duration (years, mean±SD)	8.9± 5	11.2± 7.7	0.1 [a]
Sex (female, %)	47.1	54.5	0.5 [b]
Diabetes complications			
CVD event (%)	11.8	15.2	0.7 [b]
Kidney disorders (%)	2.9	3	0.9 [b]
Hepatic disorders (%)	8.8	9.1	0.9 [b]
Neuropathy (%)	14.7	15.2	0.7 [b]

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Retinopathy (%)	14.7	18.2	0.7 [b]
Smoking (%)	5.9	9.1	0.6 [b]
Medication intake			
Metformin (%)	91.2	97	0.7 [b]
Glibenclamide (%)	61.8	63.6	0.8 [b]
Statins (%)	50	48.5	0.9 [b]
BP lowering drugs (%)*	41.2	30.3	0.3 [b]
Aspirin (%)	35.3	27.3	0.7 [b]

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637 Baseline data are given as mean±SD or relative frequency (%) where appropriate.

638 CVD: Cardiovascular disease

639 * Blood pressure lowering drugs

640 †The presented p-values are associated with baseline comparisons of the DHA-
641 enriched fish oil and control groups using the: [a] independent-sample t-test or: [b] chi-
642 square test.

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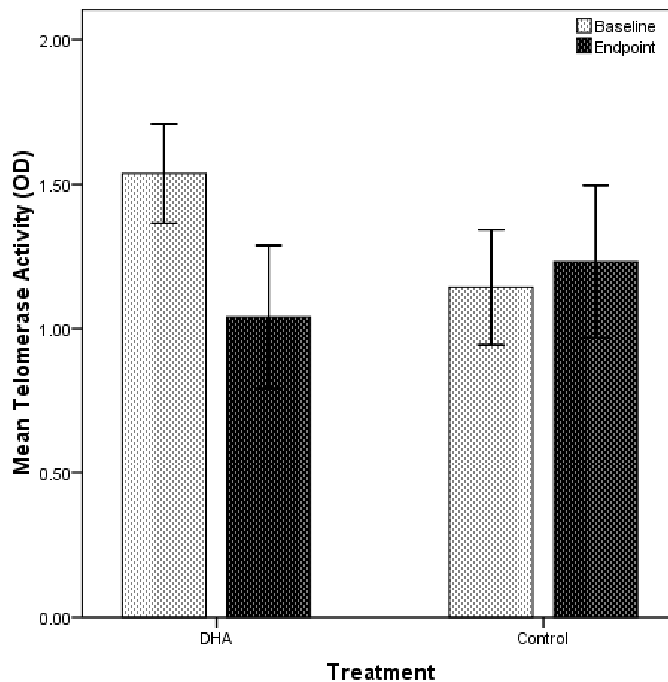

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647 **Fig 1:** Baseline and endpoint values for telomerase activity in peripheral blood
648 mononuclear cells of DHA and control groups. Telomerase activity was down-regulated
649 in DHA-enriched fish oil group during intervention (p=0.001) while in control group it
650 didn't differed from baselines (p=0.5). Between group analyses of mean changes in
651 telomerase activity, conducted using independent sample t-test, also revealed
652 significant differences (p=0.006).

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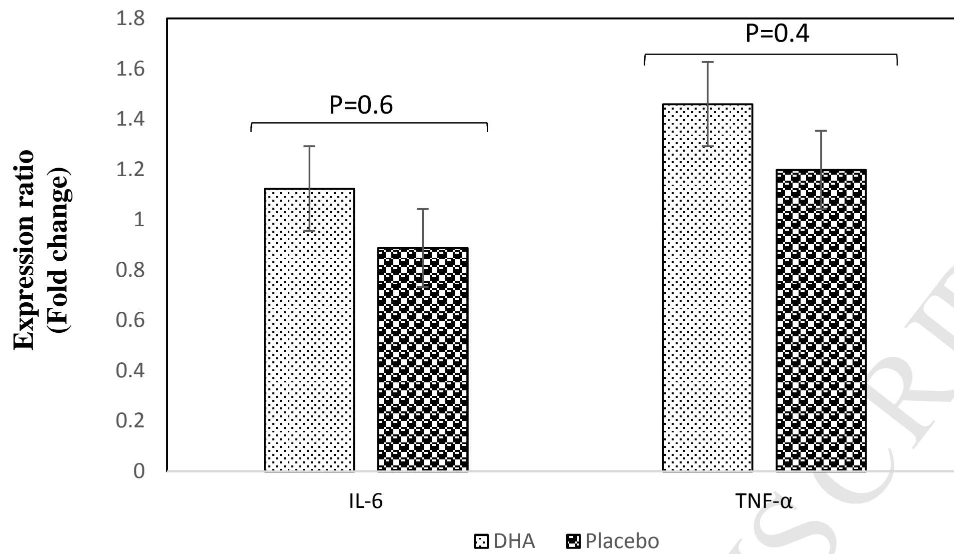

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664 **Fig 2:** Data is expressed as relative changes in IL-6 and TNF-α gene expressions
665 (normalized to GAPDH gene expression) in peripheral blood mononuclear cells from
666 T2DM patients and comparing DHA-enriched fish oil (n=32) and Placebo (n=28) groups
667 (Independent sample t-test).

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682 **Table 3:** Comparison of mean changes in telomerase activity and P16 mRNA
683 expression in type 2 diabetic patients

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Variable	DHA-enriched Fish oil (n=34) Mean±SD	control (n=33) Mean±SD	ANCOVA P-value	
TA	-0.49±0.8	0.08±0.8	Model 1*	**0.006**
			Model 2†	**0.003**
P16	1.86±1.5	1.1±1.0	Model 1	**0.021**
			Model 2	**0.028**

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688 TA: Telomerase Activity based on optical density (OD)
689 * Unadjusted model
690 † Adjusted for age and sex
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705 **Table 4.** Combined effect of gene and DHA-enriched fish oil supplementation on
706 telomerase activity and mRNA expression in type 2 diabetic patients

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	DHA-enriched Fish oil		Control		P-value †		
	Ala+ (n=10)	Ala- (n=24)	Ala+ (n= 11)	Ala- (n=22)	Intervention	Genotype	Gene*DHA interaction
P16*	1.66± 1.5	1.95± 1.5	1.06± 0.8	1.11± 1.2	**0.06**	**0.4**	**0.2**
IL-6*	1.02± 1.7	1.17± 1.3	1.32± 1.6	1.02± 1.7	**0.09**	**0.2**	**0.2**
TNF-α*	1.5± 1.4	1.42± 1.3	1.45± 1.2	1.03± 0.9	**0.5**	**0.4**	**0.6**

709
710 Data presented as mean± SD
711 * Changes in gene expression relative to glyceraldehyde-3-phosphate dehydrogenase
712 (GAPDH) during the study period
713 † The presented p-values are associated with: net effect of intervention on changes in
714 mRNA expression specified by intervention; net effect of genotype on changes in mRNA
715 expression specified by genotype; and combined effect of genotype and intervention on
716 changes in mRNA expression that specified by gene*DHA interaction

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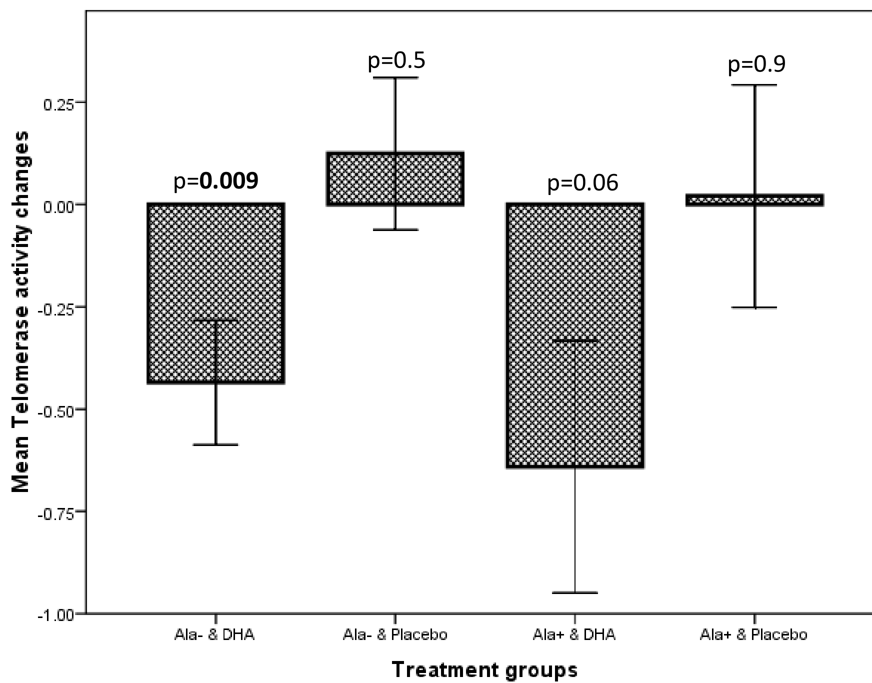

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728 **Fig 3:** Comparison for changes in telomerase activity in PBMC according to Pro12Ala
729 polymorphism in PPAR-γ gene. There were no significant differences between studied
730 groups (p=0.7) and also Ala+*DHA and Ala-*DHA groups (p=0.4). The presented p-
731 value above each column is associated with within-group changes during intervention
732 using paired t-test

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