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The effects of omega-3 polyunsaturated fatty acids and genetic variants on methylation levels of the interleukin-6 gene promoter

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Abstract

Scope—Omega-3 PUFAs (*n*-3 PUFAs) reduce *IL-6* gene expression, but their effects on transcription regulatory mechanisms are unknown. We aimed to conduct an integrated analysis with both population and in vitro studies to systematically explore the relationships among *n*-3 PUFA, DNA methylation, single nucleotide polymorphisms (SNPs), gene expression, and protein concentration of *IL6*.

Methods and results—Using data in the Genetics of Lipid Lowering Drugs and Diet Network (GOLDN) study and the Encyclopedia of DNA Elements (ENCODE) consortium, we found that higher methylation of *IL6* promoter cg01770232 was associated with higher IL-6 plasma concentration ($p = 0.03$) and greater *IL6* gene expression ($p = 0.0005$). Higher circulating total *n*-3 PUFA was associated with lower cg01770232 methylation ($p = 0.007$) and lower IL-6 concentration ($p = 0.02$). Moreover, an allele of *IL6* rs2961298 was associated with higher

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cg01770232 methylation ($p = 2.55 \times 10^{-7}$). The association between *n*-3 PUFA and cg01770232 methylation was dependent on rs2961298 genotype ($p = 0.02$), but higher total *n*-3 PUFA was associated with lower cg01770232 methylation in the heterozygotes ($p = 0.04$) not in the homozygotes.

Conclusion—Higher *n*-3 PUFA is associated with lower methylation at *IL6* promoter, which may be modified by *IL6* SNPs.

Keywords

DNA methylation; Gene-by-environment interaction; Genetic variant; Interleukin-6; *n*-3 polyunsaturated fatty acids

1 Introduction

Omega-3 PUFAs (*n*-3 PUFAs) ameliorate systematic inflammation through reduction of plasma concentration of IL-6 [1–3], for which higher concentrations are associated with cardiovascular disease (CVD) [4]. Further, supplementation with both eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) for 8 wk was found to reduce not only the plasma concentration of IL-6 but also its gene expression in adipose tissue [5].

Gene expression of interleukin-6 (*IL6*) has been shown to be regulated by DNA methylation of its promoter region [6–9]. The observed effect of *IL6* methylation on its gene expression may be related to the presence of potential binding sites for the methyl CpG binding protein 2 (MeCP2), which are located from positions –666 to –426 relative to the transcription start site [9]. Therefore, the effect of *n*-3 PUFA on *IL6* promoter methylation as a mechanism to reduce concentration of this inflammatory biomarker is intriguing.

Adding to the complexity of the modulation of *n*-3 PUFA on *IL6* methylation is the role of genetic sequence variation. For example, single nucleotide polymorphisms (SNPs) were found to affect DNA methylation across the whole genome [10]. Moreover, *IL6* SNPs were shown to interact with different environmental factors, including diet [11], smoking [12,13], and social position to determine plasma IL-6 concentration [14]. This evidence demonstrates the modifying role of *IL6* sequence variants on the effect of environmental factors on plasma IL-6 concentration. Therefore, the objective of this study is to explore the relationships between *n*-3 PUFA, genetic variants, DNA methylation, and gene expression at *IL6* locus.

To achieve this objective, we first identified the methylation site relevant to both transcription and translation within the *IL6* locus based on the DNA methylation-expression relationship across all 17 human cell types available with both methylation and gene expression at *IL6* from the Encyclopedia of DNA Elements (ENCODE) consortium, and the DNA methylation–protein relationship across 848 participants of the Genetics of Lipid Lowering Drugs and Diet Network (GOLDN) study. We further investigated in GOLDN participants the association between *n*-3 PUFA and methylation levels of the identified CpG site, and evaluated whether the observed association could be further modified by *IL6* SNPs.

2 Materials and methods

2.1 Study population

The GOLDN study, designed to evaluate genetic factors that modulate lipid responses to diet and fenofibrate treatment, recruited participants from the National Heart, Lung, and Blood Institute Family Heart Study [15]. The study design and methodology were described previously [16]. Study protocol was approved by the Human Studies Committee of Institutional Review Board at the University of Minnesota, University of Utah, and Tufts University/New England Medical Center. All participants provided written informed consent. The current analysis consisted of 848 individuals (459 men and 389 women) after removing those with missing variables and those taking hormone replacement therapies because of their mixed effects on IL-6 [17].

2.2 Biochemical measurements

Blood samples from each participant were collected, stored frozen at -70°C , and analyzed at the same time to eliminate interassay variability. IL-6, IL-2-soluble receptor α , tumor necrosis factor (TNF) α , and monocyte chemoattractant protein 1 (MCP-1) were measured using quantitative sandwich enzyme immunoassay techniques (ELISA kit assays, R&D System Inc., Minneapolis, MN, USA) as described previously [18]. High-sensitivity C-reactive protein was measured using a latex particle enhanced immunoturbidimetric assay (Kamiya Biomedical Company, Seattle, WA, USA) as described previously [19]. Plasma adiponectin was measured using competitive RIA (Linco Research, St Charles, MO, USA) as described previously [20]. Fatty acids in the erythrocyte membranes were measured by a capillary Varian CP7420 100 m column with a Hewlett Packard 5890 gas chromatograph equipped with a HP6890A autosampler [21]. The measurements of fatty acids were reliable and have been validated against a diet history questionnaire [22,23].

2.3 Genotyping and DNA methylation in GOLDN

The region of interest, referred as *IL6* locus, was defined as the region from 1 kb upstream of the CpG island of *IL6* promoter to 1 kb downstream of the 3' untranslated region of *IL6* (Fig. 1A). At *IL6* locus, genotypes for 39 SNPs were obtained using the Affymetrix Genome-Wide Human SNP array 6.0 (Affymetrix, Santa Clara, CA, USA) with the genomic DNA extracted from blood samples using Gentra Puregene Blood Kits (Gentra Systems, Inc., Minneapolis, MN, USA).

Two CpG sites (cg01770232 and cg26061582) were included into the analysis because they are located within the potential binding site for the MeCP2 [9]. Their methylation levels were measured using Infinium Human Methylation 450K BeadChip (Illumina, San Diego, CA) [24] with 500 ng sodium bisulfite treated DNA (Zymo Research Corporation, Irvine, CA, USA) extracted from CD4⁺ T-cells (Qiagen, Germantown, MD, USA) [24], which were isolated from the stored buffy coats collected at the same time erythrocyte fatty acids and plasma cytokines were measured. CD4⁺ T cells were selected because of its relevance to the proinflammatory cytokines and the established temporal stability [25].

2.4 Gene expression and DNA methylation data from ENCODE

In ENCODE, methylation and gene expression data at *IL6* locus were available for 17 human cell types, including one human primary hepatocyte and 16 human cell lines (Supporting Information Table 1), and all of these 17 available human cell types were included into the correlation analysis. Methylation data were downloaded (1 September, 2014) from UCSC genome browser HAIB Methyl450 track (<http://hgdownload.cse.ucsc.edu/goldenPath/hg19/encodeDCC/wgEncodeHaibMethyl450/>). Gene expression data were downloaded (1 September, 2014) from UCSC genome browser Duke Affymetrix Exon Array track (<http://hgdownload.cse.ucsc.edu/goldenPath/hg19/encodeDCC/wgEncodeDukeAffyExon/>).

2.5 Statistical methods

In ENCODE, Pearson correlation was conducted between methylation of the candidate CpG sites and *IL6* gene expression. In GOLDN, χ^2 tests, analysis of variance, and analysis of covariance analyses were conducted to examine the differences in population characteristics and potential confounding factors by erythrocyte total *n*-3 PUFA, categorized in quartiles. Generalized linear models were applied to test the main associations and interactions among the methylation levels of both CpG sites, plasma IL-6 concentration, genotypes of *IL6* SNPs, and erythrocyte *n*-3 PUFA, including total *n*-3 PUFA, EPA, and DHA. The methylation levels of CpG sites were represented as quartiles for the exposure variables and as continuous variables for the outcome variable. The analysis adjusted for the potential confounding factors including pedigree, principal components of cellular purity [26] and population structure, age, sex, study center, smoking, alcohol intake, total energy intake, physical activity, vitamin B-12 intake, folate intake, acute inflammatory conditions (infection or fever), chronic diseases known to affect IL-6 such as abdominal obesity [27], CVD [4], diabetes [28], and hyper-tension [29], and other inflammatory markers known to affect IL-6, including high-sensitivity C-reactive protein [22], tumor necrosis factor α [30], MCP-1 [31], IL-2-soluble receptor α [22], and adiponectin [32]. Log transformation was performed for those variables not following a normal distribution. All data were analyzed using SAS (version 9.3 for Windows; SAS Institute, Inc. Cary, NC, USA). A two-tail *p*-value of <0.05 was considered statistically significant.

3 Results

3.1 Population characteristics

Population characteristics were compared across quartiles of erythrocyte total *n*-3 PUFA (Table 1). Individuals in the quartiles with higher erythrocyte total *n*-3 PUFA tended to be older, consumed more dietary *n*-3 PUFA and less total energy, and had lower concentration of MCP-1 ($p < 0.05$) than those in the lower quartiles. Also, the quartiles with higher levels of erythrocyte *n*-3 PUFA tended to contain fewer smokers, and more individuals with abdominal obesity than those with lower levels ($p < 0.05$). Individuals in the quartiles with higher *n*-3 PUFA tended to have lower plasma IL-6 concentration ($p = 0.02$).

3.2 Cg01770232 methylation with protein concentration and gene expression of *IL6*

Two CpG sites located within the potential binding site for the MeCP2 (cg01770232 and cg26061582) were included in the analysis (Fig. 1A). Methylation level of cg01770232 was correlated with *IL6* gene expression across 17 human cell types in ENCODE ($r = 0.75$ and $p = 0.0005$; Fig. 1B). In GOLDN, methylation level of cg01770232 was also associated with plasma IL-6 concentration ($\beta = 0.22$ and $p = 0.03$; Fig. 1C). We did not find any significant associations for cg26061582 (data not shown).

3.3 *n*-3 PUFA with protein concentration and cg01770232 methylation of *IL6*

We compared associations of *n*-3 PUFA with the methylation level of cg01770232, and also with plasma IL-6 concentration in GOLDN (Fig. 2). Total *n*-3 PUFA and DHA were negatively associated with plasma IL-6 ($p = 0.02$ and 0.01 respectively), while the negative association with EPA did not reach statistical significance. In parallel with the association patterns observed between total *n*-3 PUFA, EPA, and DHA for the outcome of plasma IL-6 concentrations, the same fatty acids were also negatively associated with methylation level of cg01770232 ($p = 0.007$ and 0.01 for total *n*-3 PUFA and DHA, respectively), and the negative association with EPA reached a borderline significance ($p = 0.05$).

3.4 Rs2961298 with cg01770232 methylation at *IL6*

Since we have test associations between 39 SNPs within the region of interest and methylation of cg01770232, the Bonferroni-adjusted significance level should be 0.001, which was obtained by dividing 0.05 by 39. Evidence for associations between all 39 SNPs within *IL6* locus and the methylation level of cg01770232 is presented in Fig. 3 and Supporting Information Table 2. Rs2961298, an SNP close to the CpG island upstream of *IL6* gene, exhibited the most robust association with the methylation level of cg01770232 ($p = 2.55 \times 10^{-7}$).

3.5 *n*-3 PUFA, rs2961298, and cg01770232 methylation

Because of the identified associations of both SNP rs2961298 and *n*-3 PUFA with the methylation level of cg01770232, the potential interactions between fatty acids and SNPs were further explored (Fig. 4). Rs2961298 was shown to significantly modify the association between cg01770232 methylation and total *n*-3 PUFA ($p_{\text{interaction}} = 0.02$), EPA ($p_{\text{interaction}} = 0.01$), and DHA ($p_{\text{interaction}} = 0.05$). However, we found a significant lowering effect of *n*-3 PUFA on cg01770232 methylation only in the rs2961298 heterozygotes (AC; $p_{\text{trend}} = 0.04$, 0.06 , and 0.05 for total *n*-3 PUFA, EPA, and DHA, respectively), not in the homozygotes (AA and CC).

4 Discussion

In the current study, we identified a transcriptionally and translationally relevant CpG site within the *IL6* promoter, cg01770232, and its association with *n*-3 PUFA in erythrocyte membranes, which was further modified by *IL6* SNP rs2961298. This is the first study to integrate analysis of datasets from both a population cohort and in vitro experiments to systematically explore the associations between *n*-3 PUFA and different components of *IL6*, including epigenetic modification, genetic variants, gene expression, and protein

concentration. Our consistent association findings provide potential mechanistic hypotheses, calling for further experimental demonstrations.

Our finding of the positive correlation between cg01770232 methylation and *IL6* gene expression is consistent with the observations of those promoters with low content of CG because the CG content within 500 bp of cg01770232 is just 1.4%. It was shown that the hypermethylation status of some of the CG poor promoters was correlated with greater gene expression [33–35], and more active promoter status [36]. Also, the positive correlation between methylation and gene expression shown by the ENCODE datasets was in line with the positive correlation between methylation and IL-6 protein concentration in GOLDN population. Moreover, the parallel negative associations between *n*-3 PUFA and both IL-6 protein concentration and *IL6* methylation in GOLDN population further provided indirect evidence for the observed positive correlation between *IL6* methylation and gene expression. Although a previous publication reported a negative association between methylation and expression [9], our study may provide additional solid evidence based on a greater number and variety of cell types from data in ENCODE, and a more unbiased statistical analysis. Finally, the unbiased nature of the publically available datasets from ENCODE consortium may also help to increase the level of scientific evidence for our findings.

Although we have obtained consistent associations among *n*-3 PUFA, *IL6* methylation, gene expression, and IL-6 protein concentration, we do not have the power to detect whether the effect of *n*-3 PUFA on IL-6 protein concentration is through its effect on *IL6* methylation because of our observational study design. Potential mechanisms to account for the observed association between *n*-3 PUFA and *IL6* methylation are unknown. One possibility may be the potential interactions between *n*-3 PUFA and MeCP2, the protein binding to the methylated cg01770232 site at *IL6* locus. This mechanistic possibility is supported by previous studies reporting protective effect of *n*-3 PUFA on Rett Syndrome, a neurodevelopmental disorder mainly caused by mutations in *MECP2* gene [37, 38]. Of course, there could be also many other possibilities that are beyond the scope of the current study.

Our finding that only subjects with heterozygous geno-type of rs2961298 have the significant lowering effects of total *n*-3 PUFA on *IL6* promoter methylation suggests that another SNP sharing linkage disequilibrium with rs2961298 might be responsible for the differential methylation. The phenomenon of genotype-dependent methylation is widely spread across the whole human genome [10], but the specific mechanism driving the allele-specific differential methylation at *IL6* is unclear. It could be possible that different genotypes may have different affinity for DNA-binding proteins with downstream effects on DNA methylation [39]. It could also be possible that DNA sequence had direct effects on the propensity of DNA methylation [40].

Our study was limited by its cross-sectional design, from which only associations rather than causality can be established. Further longitudinal and intervention studies are needed in order to confirm and solidify these findings. Although we have analyzed all the publically available cell types in ENCODE to remove the potential of selection bias to some degree, we admit that there could still be some remaining selection bias because not all the cell types

were included in ENCODE. Lack of replication and inability to directly assess relationships with gene expression in GOLDN should also be taken into consideration. Additionally, our findings can be appropriately generalized only to those populations with similar intakes of *n*-3 PUFA.

An important strength of the current study is that it provides evidence for potential mechanisms by which long-term intake of *n*-3 PUFA is modulating genotype-based variability in disease biomarkers, because the erythrocyte measurement of *n*-3 PUFA reflects long-term intake [41] and the measurements were validated to be correlated with dietary intake (Table 1). While previous studies reported the interactions between sequence variants and environmental factor that modulate plasma IL-6 concentration [11–14], our work suggests that the modification effect of *IL6* SNPs on the relationship between *n*-3 PUFA and IL-6 may occur through *IL6* methylation and gene expression. Specifically, SNP rs2961298 interacted significantly with total *n*-3 PUFA to modulate methylation status of the identified functional CpG site, cg01770232. When evaluated in the same human population, *n*-3 PUFAs were found to be associated with methylation level of cg01770232 in a genotype-specific manner. Our findings may lead to novel mechanistic explanations of the link between *n*-3 PUFA and IL-6, and could identify potential new diagnostic and therapeutic targets for disease prevention.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

Y.M. designed research including project conception and development of overall research plan; C.E.S., and J.M.O. designed research including study oversight; Y.M., C.Q.L., M.R.I., and Y.C.L. conducted research; Y.M. analyzed data; Y.M. and C.E.S. wrote the paper. D.K.A. and D.A. provided essential data. L.D.P., L.D.P., S.A., M.Y.T., I.B.B., and E.K.K. contributed to manuscript editing. Y.M. had primary responsibility for final content.

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Abbreviations

CVD	cardiovascular disease
DHA	docosahexaenoic acid
ENCODE	Encyclopedia of DNA Elements
EPA	eicosapentaenoic acid
GOLDN	Genetics of Lipid Lowering Drugs and Diet Network
IL2sR-α	interleukin 2 soluble receptor α

MCP-1	monocyte chemoattractant protein 1
MeCP2	methyl CpG binding protein 2
n-3 PUFA	omega-3 PUFA
SNP	single nucleotide polymorphism
TNF-α	tumor necrosis factor α

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Figure 1.

Methylation of CpG site cg01770232 within *IL6* promoter is associated with both plasma IL-6 concentration and *IL6* gene expression. (A) The genomic structure of *IL6* locus, in which exons, CpG island, potential binding site for MeCP2, CpG sites, and SNPs are represented by black box, gray box, gray circle, black dots, and black bars, respectively. (B) The Pearson correlation between methylation level of cg01770232 and *IL6* gene expression across 17 human cell types in ENCODE, in which each black dot represents one cell type and the gray line represents the corresponding regression line, and the correlation coefficient (r) and its corresponding P -value are shown at the bottom right corner. (C) The association of quartiles of methylation level of cg01770232 with plasma-logged IL-6 concentration (geometric means $\pm$ 95% CI) in GOLDN. P -Value is obtained from the generalized linear model adjusting for first four principal components for cellular purity and population structure, age, sex, center, pedigree, smoking, alcohol intake, total energy intake, physical activity, intake of vitamin B-12 and folate, acute inflammatory conditions (infection or fever), chronic diseases (abdominal obesity, CVD, diabetes, and hypertension), other inflammatory markers (MCP-1, adiponectin, high-sensitivity C-reactive protein, tumor necrosis factor α , and IL-2-soluble receptor α). TSS, transcription start site; UTR, untranslated region.

Figure 2.

Associations of erythrocyte *n*-3 PUFA with plasma IL-6 and DNA methylation level of cg01770232 in GOLDN. Based on adjusted generalized linear models, predicted plasma concentration of IL-6 (log transformed) is plotted against total *n*-3 PUFA (A), EPA (C), and DHA (E), and predicted methylation level of cg01770232 is also plotted against total *n*-3 PUFA (B), EPA (D), and DHA (F). β - and *p*-values represent the regression coefficients and statistical significance, respectively, and both of them are obtained from the generalized linear model adjusting for first four principal components for cellular purity and population structure, age, sex, center, pedigree, smoking, drinking, total energy intake, physical activity,

intake of vitamin B-12 and folate, acute inflammatory conditions (infection or fever), chronic disease status (including abdominal obesity, CVD, diabetes, and hypertension), other inflammatory markers (MCP-1, adiponectin, high-sensitivity C-reactive protein, tumor necrosis factor α , and IL-2-soluble receptor α).

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Figure 3.

Genetic associations with the methylation level of cg01770232 in GOLDN within the *IL6* locus. SNPs are plotted by position within *IL6* locus against association with the methylation level of cg01770232 ($-\log_{10} p$ -value). Estimated recombination rates (from 1000 Genomes Pilot 1 CEU) are plotted in gray line to reflect the local linkage disequilibrium structure. The SNPs surrounding the most significant SNP (rs2961298), represented by dark gray diamonds, are plotted as gray-coded squares to reflect their linkage disequilibrium r^2 with this SNP, which is estimated based on 1000 Genomes Pilot 1 CEU database.

Figure 4.

Interaction between *n*-3 PUFA and rs2961298 modulating the methylation level of cg01770232 in GOLDN. Based on adjusted generalized linear models, predicted methylation levels of cg01770232 (log transformed) by genotype of rs2961298 is plotted against total *n*-3 PUFA (A), EPA (B), and DHA (C), adjusted for first four principal components for cellular purity and population structure, age, sex, center, pedigree, smoking, alcohol intake, total energy intake, physical activity, intake of vitamin B-12 and folate, acute inflammatory conditions (infection or fever), chronic disease status (including abdominal obesity, CVD, diabetes, and hypertension), and other inflammatory markers (MCP-1, adiponectin, high-sensitivity C-reactive protein, tumor necrosis factor α , and IL-2-soluble receptor α). P -values indicate the statistical significance of the adjusted interaction term and adjusted regression coefficients (represented as betas) in the regression line corresponding to three genotype groups of rs2961298 (diamond for CC, square for AC, and triangle for AA).

Table 1

Population characteristics of GOLDN

Variable		Erythrocyte <i>n</i> -3 PUFAs				<i>p</i>
		Q1 (<i>n</i> = 212)	Q2 (<i>n</i> = 212)	Q3 (<i>n</i> = 212)	Q4 (<i>n</i> = 212)	
Erythrocyte <i>n</i> -3 PUFA	Total <i>n</i> -3 PUFA (% total fatty acids) ^{a)}	4.65	5.25	5.96	7.08	
	EPA (% total fatty acids) ^{b)}	0.39 (0.09)	0.45 (0.10)	0.53 (0.12)	0.80 (0.48)	<0.0001
	DHA (% total fatty acids) ^{b)}	2.10 (0.30)	2.59 (0.30)	3.10 (0.32)	4.12 (0.73)	<0.0001
Dietary <i>n</i> -3 PUFA intake	Total <i>n</i> -3 PUFAs (% total energy intake/day) ^{a)}	0.026 (0.02)	0.03 (0.02)	0.04 (0.03)	0.06 (0.05)	<0.0001
	EPA (% total energy intake/day) ^{a)}	0.006 (0.006)	0.008 (0.007)	0.01 (0.009)	0.02 (0.02)	<0.0001
	DHA (% total energy intake/day) ^{a)}	0.017 (0.01)	0.02 (0.01)	0.025 (0.02)	0.04 (0.03)	<0.0001
Demographics	Age (y) ^{b)}	42 (14)	45 (16)	51 (15)	57 (15)	<0.0001
	Men (<i>n</i>) ^{c)}	124 (58)	119 (56)	107 (50)	109 (51)	0.29
Lifestyle parameters	Current smoker (<i>n</i>) ^{c)}	27 (13)	24 (11)	12 (6)	4 (2)	0.0003
	Drinker (<i>n</i>) ^{c)}	114 (54)	112 (53)	93 (44)	93 (44)	0.06
	Total energy intake (kcal/day) ^{b)}	2313.95 (1496.83)	2322.94 (1325.29)	2182.88 (1193.17)	1898.38 (836.63)	0.001
	Vitamin B-12 intake (mcg) ^{b)}	5.62 (3.74)	5.84 (3.76)	5.56 (3.33)	5.18 (3.47)	0.29
	Folate intake (mcg) ^{b)}	423.61 (235.84)	416.24 (263.21)	421.98 (229.78)	404.28 (198.43)	0.83
Disease status	Infection or fever (<i>n</i>) ^{d)}	2 (1)	5 (2)	4 (2)	2 (1)	0.45
	Abdominal obesity (<i>n</i>) ^{d)}	86 (41)	121 (57)	114 (54)	108 (51)	0.01
	Hypertension (<i>n</i>) ^{d)}	30 (14)	52 (25)	54 (26)	83 (39)	0.40
	Diabetes (<i>n</i>) ^{d)}	8 (4)	19 (9)	12 (6)	25 (12)	0.98
	CVD (<i>n</i>) ^{d)}	5 (2)	10 (5)	11 (5)	24 (11)	0.29
Inflammatory markers	IL-6 (pg/mL) ^{e)}	1.75 (1.13)	1.75 (1.12)	1.65 (1.11)	1.57 (1.12)	0.02
	hsCRP (mg/dl) ^{f)}	0.11 (1.09)	0.15 (1.11)	0.11 (1.09)	0.12 (1.09)	0.69
	TNF-α (pg/mL) ^{f)}	2.92 (1.03)	3.08 (1.03)	2.91 (1.03)	3.06 (1.05)	0.57
	MCP-1 (pg/mL) ^{f)}	212.66 (1.02)	210.69 (1.03)	200.22 (1.02)	198.26 (1.02)	0.006
	Adiponectin (ng/mL) ^{f)}	7762.81 (1.05)	6460.1 (1.05)	6930.59 (1.04)	6915.36 (1.05)	0.22
	IL2sR-α (pg/mL) ^{f)}	1000.05 (1.03)	1020.96 (1.03)	959.39 (1.03)	964.97 (1.03)	0.16

hsCRP, high-sensitivity C-reactive protein, IL2sR-α, IL-2-soluble receptor α, TNF-α, tumor necrosis factor.

- a)* Data are medians of each quartile of total *n*-3 PUFAs.
- b)* Data are means (SDs) within each quartile of total *n*-3 PUFAs and *p*-values are obtained from analysis of variance test.
- c)* Data are *n* (%) within each quartile of total *n*-3 PUFAs and *p*-values are obtained from Chi-square test.
- d)* Data are means (SDs) within each quartile of total *n*-3 PUFAs and *p*-values are obtained from logistic regression adjusting for the first four principal components for cellular purity and population structure, age, sex, center, smoking, alcohol intake, total energy intake, physical activity, and intake of vitamin B-12 and folate.
- e)* Data are least squared adjusted means (standard errors) within each quartile of total *n*-3 PUFAs and *p*-values are obtained from analysis of covariance test adjusting for the first four principal components for cellular purity and population structure, age, sex, center, pedigree, smoking, alcohol intake, total energy intake, physical activity, intake of vitamin B-12 and folate, acute inflammatory conditions (infection or fever), chronic disease status (including abdominal obesity, CVD, diabetes, and hypertension), and other inflammatory markers (including hsCRP, TNF- α , MCP-1, IL2sR- α , and adiponectin).
- f)* Data are least squared adjusted means (standard errors) within each quartile of total *n*-3 PUFAs, *p*-values are obtained from analysis of covariance test adjusting for the first four principal components for cellular purity and population structure, age, sex, center, pedigree, smoking, alcohol intake, total energy intake, physical activity, intake of vitamin B-12 and folate.