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ORIGINAL CONTRIBUTION

The effect of fatty or lean fish intake on inflammatory gene expression in peripheral blood mononuclear cells of patients with coronary heart disease

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Abstract
Background Little is known about the effect of fish consumption on gene expression of inflammation-related genes in immune cells in coronary heart disease (CHD).
Aim of the study We sought to evaluate the effect of a fatty fish (FF) or a lean fish (LF) diet on the modulation of inflammatory and endothelial function-related genes in peripheral blood mononuclear cells (PBMCs) of subjects with CHD, and its association with serum fatty acid (FA) profile and lipid metabolic compounds.
Methods Data from 27 patients randomized into an 8-week FF ($n = 10$; mean ± SD: 4.3 ± 0.4 portions of fish per week), LF ($n = 11$; 4.7 ± 1.1 portions of fish per week), or control diet ($n = 6$; 0.6 ± 0.4 portions of fish per week) were analyzed. The mRNA expression was measured using real-time PCR.
Results The effect of the intervention on the mRNA expression of the genes studied did not differ among groups. In the FF group, however, the decrease in arachidonic acid to eicosapentaenoic acid (AA:EPA) ratio in cholesterol ester and phospholipid fractions strongly correlated with the change in IL1B mRNA levels ($r_s = 0.60$, $P = 0.06$ and $r_s = 0.86$, $P = 0.002$, respectively). In the LF group, the decrease in palmitic acid and total saturated FAs in cholesterol esters correlated with the change in intercellular cell adhesion molecule-1 (ICAM1) expression ($r_s = 0.64$, $P = 0.04$ for both). Circulating levels of soluble ICAM-1 decreased only in the LF group ($P < 0.05$).
Conclusions The intake of FF or LF diet did not alter the expression of inflammatory and endothelial function-related genes in PBMCs of patients with CHD. However, the decrease in AA:EPA ratio in serum lipids in the FF group may induce an anti-inflammatory response at mRNA levels in PBMCs. A LF diet might benefit endothelial function, possibly mediated by the changes in serum FA composition.

Keywords Fish · Fatty acids · Gene expression · Inflammation · Endothelial function · ICAM · IL-1β

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Introduction

Immune cells, such as lymphocytes and monocytes, the major cell types present in peripheral blood mononuclear cells (PBMCs), play a crucial role in the development of

atherosclerotic lesion [17]. The cells of the immune system are the main source of cytokines, and they can also secrete cell adhesion molecules (CAMs) [8, 20]. Moreover, PBMCs can migrate through the blood circulation and infiltrate various tissues, such as the endothelium [38]. Immune cells may therefore play an important role in the development of inflammation, endothelial dysfunction, and cardiovascular disease [14].

Dietary long-chain n-3 polyunsaturated fatty acids (PUFAs: eicosapentaenoic acid, EPA and docosahexaenoic acid, DHA) are mostly derived from fatty fish and are associated with a decreased risk of coronary heart disease (CHD) mortality [6]. Possibilities for the cardioprotective effect of these n-3 PUFAs include improvement of inflammation and endothelial markers [32]. While the hypotriglyceridemic effect of very long-chain FAs is well documented, reports about their effects on inflammation have been far less consistent. The effects of n-3 PUFAs focusing on markers of immune and inflammatory modulation have previously been performed in healthy subjects or patients with chronic inflammatory diseases [32]. Moreover, data are lacking about the effect of n-3 PUFAs, derived from fish, on inflammatory markers in conditions, such as CHD.

The PBMCs have been used to address expression of genes related to inflammation [5, 35] and can reflect insulin metabolism [4]. However, gene modulation in PBMCs has not sufficiently been investigated in the context of inflammation, CHD, and diet. PBMCs are also convenient for gene expression studies because they can be easily collected in contrast to other tissues.

The aim of the present study was to address whether a dietary intervention of fatty fish (FF) or lean fish (LF) modulates mRNA expression of a subset of genes related to inflammation and endothelial function in PBMCs in a homogeneous group of subjects with CHD. We also examined the serum inflammatory markers after these interventions, and assessed the correlations between the serum fatty acid (FA) composition in cholesterol ester (CE) and phospholipid (PL) fractions with the gene expression changes. Our secondary aim was to investigate any associations among the changes in serum PUFA composition, gene expression, and metabolic compounds related to insulin resistance (IR) [11] more specifically in the FF group.

Materials and methods

Subjects

Subjects who were part of this study volunteered for the original study as previously described [7]. Briefly, patients who had been in the Kuopio University Hospital due to myocardial infarction or unstable ischemic attack during the previous 3–36 months entered the study. Thirty-five eligible subjects were randomized to one of the three intervention groups [7]. Subjects gave written consent for participation in the study which was approved by the Research Ethics Committee, Hospital District of Northern Savo (registered in *clinicaltrials.gov*, NCT00720655). Two of the subjects dropped out from the study due to medical reasons.

Intervention

Originally, subjects were randomized to one of the following 8-week diets: a FF diet ($n = 11$), a LF diet ($n = 12$), or a control diet ($n = 10$) [7]. Out of these 33 subjects randomized, PBMC samples were available from 27 (FF: $n = 10$, LF: $n = 11$, and control group: $n = 6$). Subjects followed a diet recommended for CHD patients [33] with group specific instructions about use of fish, fish products, meat and meat products (FF diet: salmon, rainbow trout, Baltic herring, whitefish, vendace, or tuna; LF diet: pike, pike-perch, perch, saithe, or cod; and control group: lean meat (beef or pork) or skinless chicken, less than 1 fish meal per week). All patients were advised to replace sources of saturated fat in food preparation by unsaturated fat products. Patients' habitual dietary intake was estimated by a 4-day food record at the baseline and compliance was monitored by using a 7-day food record performed at weeks 3 and 7. Subjects continued to use the medications prescribed by their physicians.

Blood samples for the serum markers and gene expression studies were drawn after a 12-h overnight fast at baseline (week 0) and 8 week. FA composition in CE and PL fractions were measured as described earlier [7]. Data on plasma ceramides (Cer) and diacylglycerols (DG) assessed by lipidomics analyses which were modulated by FF [16], and are suggested to be related to IR [11] are presented here with the intention of testing their associations with FAs and gene expression variables, and also with IR as fasting plasma insulin (FPI) and the homeostasis model assessment of IR: HOMA-IR index [(FPI (mU/l) $\times$ fasting plasma glucose (mmol/l)/22.5)].

Markers of inflammation and endothelial function

Concentrations of hsCRP were determined by Immage® Immunochemistry System (Immulite 2000 DPC, Los Angeles, LA) with an analytical range of 0.1–250 mg/l and a sensitivity of 0.2 mg/l. The concentration of soluble vascular and intercellular CAMs (sVCAM-1 and sICAM-1), chemokine (C–C motif) ligand 2 and 5 (CCL2 and CCL5), soluble P- and E-selectins were measured by solid-phase enzyme-linked immunosorbent assays (Quantikine and Elisa Kits; R&D Systems, Minneapolis, MN), and high-sensitivity enzyme-linked immunosorbent assay kits were used for TNF-α, IL-1β, and IL-6 measurements.

Isolation of PBMCs and gene expression measurements

The PBMCs were isolated from the blood samples collected in a BD Vacutainer CPT Cell Preparation Tube with Sodium Citrate (BD, Franklin Lakes, NJ) according to the manufacturer's instructions. Total RNA was extracted using the RNeasy Mini Kit (Qiagen, Valencia, CA). The RNA concentration and the A_{260}/A_{280} ratio was measured using a NanoDrop spectrophotometer (NanoDrop Technologies, Wilmington, DE), an acceptable ratio being 1.9–2.1. The integrity of the RNA was assessed using agarose gel electrophoresis.

The RNA was reverse transcribed into cDNA using High-Capacity cDNA Archive Kit (Applied Biosystems, Foster City, CA). Quantitative real-time PCR was performed, with TaqMan chemistry using ready made assays purchased from Applied Biosystems for TNF, IL1B, IL6, CCL2, CCL5, ICAM1, VCAM1, SELE, and SELP (genes encoding E- and P-selectin, respectively). The amplification was performed and relative quantities were analyzed in triplicates with ABI Prism 7500 SDS software (TaqMan Gene Expression Assays; Foster City, CA). A standard curve with the points of 0.025, 0.075, 0.3, 0.9, 1.8 ng/μl of cDNA (total RNA equivalent), respectively, and calibrator at a concentration of 0.3 ng/μl were used on every plate. Quantities on each plate were first corrected by the calibrator on the plate and further normalized to the endogenous control GAPDH gene. The mRNA expression of the genes IL6, VCAM1, and SELE were not consistent or not detectable.

Statistical analyses

Baseline variables were compared among groups using One-Way ANOVA followed by the Bonferroni correction for multiple comparisons or χ^2 test as appropriate. The effects of the treatments on serum circulating markers were compared by using GLM univariate analysis using the treatment as a fixed factor, the change between the weeks 0 and 8 measurements as the dependent variable. When a significant difference among the groups was found, the effect of treatment in each group between the beginning and the end of treatment was compared further by use of Student's paired *t* test. Pearson product correlations (*r*) were computed to determine cross-sectional associations among the variables of interest. Variables with a skewed distribution were log or square root transformed before the analyses and are reported as median (interquartile range). Because of the high variation of the gene expression responses among treatments and small number of samples, non-parametric tests were used for testing the difference among groups (Kruskall–Wallis) and for correlation analyses (Spearman coefficient: r_s). A *P* value of <0.05 was considered to be significant. Analyses were performed using SPSS software version 14.0 (SPSS Inc., Chicago, IL).

Results

Subjects

The detailed information on the study subjects has been described earlier [7]. For the present study ($n = 27$), the mean (±SD) age and BMI of the subjects in each intervention group at screening were, respectively: FF group: 62.7 ± 6.3 years and 27.0 ± 3.2 kg/m^2; LF group: 60.6 ± 5.3 years and 27.8 ± 2.2 kg/m^2; and control group: 59.2 ± 7.6 years and 26.4 ± 3.5 kg/m^2, and they did not differ among the groups ($P > 0.10$). The patients' lipid profile at screening and fasting plasma glucose and insulin concentrations assessed at baseline were also similar among groups (Table 1). All the subjects were using betablockers and statins, and continued their medications

Table 1 Biochemical characteristics of the patients

Variable	Fatty fish ($n = 10$)	Lean fish ($n = 11$)	Control ($n = 6$)	P^a
Total cholesterol (mmol/l)	3.99 ± 0.60	4.06 ± 0.70	4.22 ± 0.92	0.88
LDL cholesterol (mmol/l)	2.09 ± 0.39	2.30 ± 0.60	2.39 ± 0.67	0.65
HDL cholesterol (mmol/l)	1.53 ± 0.50	1.35 ± 0.36	1.40 ± 0.30	0.55
Triglycerides (mmol/l)	1.22 (0.75–1.56)	1.03 (1.00–2.08)	1.47 (1.24–2.37)	0.43
FPG (mmol/l)	6.03 ± 0.98	5.66 ± 0.39	5.82 ± 0.27	0.75
FPI (mU/l)	11.0 (6.7–13.9)	9.4 (5.7–14.0)	10.3 (7.2–19.1)	0.69
HOMA-IR index[b]	2.89 (1.53–4.0)	2.46 (1.50–3.61)	2.78 (1.88–2.78)	0.64

Values are mean ± SD or median (interquartile range)

FPG fasting plasma glucose, *FPI* fasting plasma insulin, *HOMA-IR* homeostasis model assessment of insulin resistance

[a] ANOVA

[b] HOMA-IR index as: (FPI (mU/l) × FPG (mmol/l)/22.5)

throughout the study, with the exception of one subject in the control group who stopped using a statin. The use of other medications (aspirin, ACE inhibitors, calcium antagonists, oral anticoagulant, and nitrates) did not differ among the groups ($P > 0.10$).

Dietary intake and serum lipid FA composition

The nutrient intake and the serum FA composition in CE and PL fractions at baseline and at the end of the study are described in Table 2. In FF group, EPA and DHA

Table 2 Nutrient intake and serum fatty acid composition in cholesterol ester (CE) and phospholipid (PL) fractions according to the study interventions

	Fatty fish ($n = 10$)		Lean fish ($n = 11$)		Control ($n = 6$)		P for intervention versus group[b]
	Baseline	During the study[a]	Baseline	During the study[a]	Baseline	During the study[a]	
Dietary intake							
Fat, % energy	31.4 ± 6.5	31.7 ± 5.3	30.7 ± 4.6	26.2 ± 3.4	31.2 ± 8.3	27.8 ± 3.9	0.14
Saturated fat, % energy	10.9 ± 3.7	9.8 ± 2.3	10.3 ± 3.6	7.7 ± 2.1	11.5 ± 3.7	9.5 ± 1.7	0.53
Monounsaturated fat, % energy	10.9 ± 2.8	10.6 ± 2.5	10.1 ± 1.2	8.9 ± 1.5	10.7 ± 2.7	9.6 ± 1.9	0.64
Polyunsaturated fat, % energy	5.7 ± 1.1	7.2 ± 1.1[c]	6.5 ± 1.4	6.4 ± 0.9	5.4 ± 2.3	5.6 ± 1.4	<0.01
EPA + DHA, g/day	0.37 (0.23, 0.59)	0.89 (0.73, 1.37)[c]	0.29 (0.17, 0.52)	0.43 (0.36, 0.50)	0.27 (0.08, 0.90)	0.18 (0.07, 0.34)	<0.01
		End of the study		End of the study		End of the study	
Fatty acids in CE (mmol%)							
Palmitic acid	14.4 ± 1.1	14.8 ± 1.2	14.4 ± 1.0	13.6 ± 0.7[c]	13.1 ± 1.3	13.4 ± 1.3	0.01
Total SFAs	16.6 ± 1.4	16.9 ± 1.3	16.6 ± 1.0	15.6 ± 0.7[c]	15.4 ± 1.5	15.4 ± 1.7	0.03
AA	6.1 ± 1.2	5.6 ± 1.2	6.2 ± 1.5	6.3 ± 1.3	5.4 ± 1.6	5.6 ± 1.2	0.20
EPA	1.61 (1.24, 2.17)	2.80 (2.46, 3.23)[c]	1.36 (1.11, 1.75)	1.30 (1.06, 1.39)	0.98 (0.72, 1.89)	0.86 (0.57, 1.53)	<0.001
DHA	0.42 ± 0.13	0.56 ± 0.17[c]	0.44 ± 0.13	0.43 ± 0.09	0.29 ± 0.11	0.28 ± 0.09	0.01
Total n-3 PUFAs	2.90 ± 0.61	4.34 ± 1.39[c]	2.67 ± 0.72	2.64 ± 0.94	2.32 ± 0.75	2.07 ± 0.58	0.001
AA:EPA ratio	3.87 ± 1.22	2.04 ± 0.76[c]	4.58 ± 1.71	5.05 ± 1.78	5.28 ± 2.15	6.98 ± 4.32	<0.01
P:S ratio	3.47 ± 0.55	3.46 ± 0.38	3.51 ± 0.34	3.77 ± 0.28	3.76 ± 0.58	3.85 ± 0.64	0.16
Fatty acids in PL (mmol%)							
Palmitic acid	33.1 ± 1.8	32.6 ± 1.7	33.0 ± 1.2	32.8 ± 1.2	32.6 ± 1.7	32.3 ± 0.90	0.74
Total SFAs	48.9 ± 1.1	48.6 ± 1.0	48.7 ± 1.3	48.2 ± 1.1	48.7 ± 1.6	48.4 ± 0.9	0.94
AA	9.0 ± 1.5	7.9 ± 1.6[c]	9.1 ± 1.1	9.1 ± 1.2	8.8 ± 1.9	9.0 ± 1.9	<0.01
EPA	2.37 ± 0.80	4.35 ± 1.91[c]	2.18 ± 0.97	2.00 ± 1.11	1.48 ± 0.61	1.81 ± 0.49	<0.01
DPA	0.68 ± 0.11	0.74 ± 0.12	0.72 ± 0.10	0.69 ± 0.20	0.64 ± 0.12	0.62 ± 0.16	0.35
DHA[d]	4.64 ± 0.89	5.77 ± 0.91[c]	4.22 ± 1.05	4.14 ± 0.71	3.14 ± 1.08	3.15 ± 0.98	<0.01
Total n-3 PUFAs	7.9 ± 1.3	11.5 ± 2.40[c]	7.4 ± 2.0	7.1 ± 1.6	5.6 ± 1.6	5.9 ± 1.1	<0.01
AA:EPA ratio	4.28 ± 1.73	2.16 ± 0.96[c]	4.97 ± 2.28	5.37 ± 1.93	6.47 ± 2.04	5.13 ± 1.02	<0.01
P:S ratio	0.76 ± 0.04	0.77 ± 0.03	0.76 ± 0.04	0.78 ± 0.05	0.76 ± 0.05	0.76 ± 0.03	0.91

All values are mean ± SD or median (interquartile range); *EPA* eicosapentaenoic acid, *DHA* docosahexaenoic acid, *SFAs* saturated fatty acids, *AA* arachidonic acid, *PUFAs* polyunsaturated fatty acids, *P:S* PUFAs to SFAs, *DPA* docosapentaenoic acid

Serum fatty acids in CE and PL fractions are presented as molar percentages of total fatty acids

[a] Mean of intake at weeks 3 and 7 for dietary data

[b] GLM

[c] $P < 0.05$ for baseline versus during the study (dietary intake) or versus end of the study (serum fatty acid composition) after Paired t test

[d] $P < 0.05$ for the difference in baseline value among groups, $P < 0.05$ for fatty fish versus control after Bonferroni correction for multiple comparisons

increased in both lipid fractions. In PLs, the arachidonic acid (AA) significantly decreased after FF intervention. We also found that the AA to EPA (AA:EPA) ratio in the CE and PL fractions decreased after the FF diet. After the LF intervention, palmitic acid and total saturated fatty acids (SFAs) in the CE fraction decreased. For more details on dietary data see ref. [7].

Effect of dietary interventions on gene expression

Data on the changes in mRNA expression levels after the dietary interventions are depicted in Table 3. There were no significant differences among the groups in the mRNA expression change of TNF, IL1B, CCL2, CCL5, ICAM1, and SELP.

Effect of dietary interventions on serum inflammatory or endothelial markers

The serum inflammatory and endothelial markers results are described in Table 4. The change in sICAM-1 levels was different among the groups, and decreased only after the LF diet. Although an effect of group was seen, serum E-selectin did not significantly respond to any of the interventions. No effect of intervention was observed on the other markers.

Correlations between serum FA composition and the mRNA expression of the target genes

Among the correlations assessed in the FF group, we found that the change in the AA:EPA ratio in CE and PLs significantly correlated only with the change in IL1B expression (Table 5). No correlations were found, however, between the changes in mRNA expression levels of IL1B and the changes in the n-3 PUFAs measured in CE or PL fractions ($P > 0.10$).

Interestingly, in the LF group, we observed that the change in ICAM1 mRNA levels correlated positively with the change in the proportions of palmitic acid and total SFAs in CE, and inversely correlated with the change in the ratio of the total PUFAs to total SFAs (P:S ratio) in CE (Table 5). Moreover, the change in CCL2 mRNA expression in PBMCs correlated with the change in palmitic acid in CE fraction (Table 5). The change in the proportion of total SFAs in CE also correlated positively with the change in CCL2 mRNA expression, but the association was not significant ($P = 0.11$ for both).

Table 3 Gene mRNA expression of inflammatory and endothelial dysfunction related molecules before and after fatty fish ($n = 10$), lean fish ($n = 11$) and control diet ($n = 6$) interventions and the relative changes

	Week 0 Mean ± SD	Week 8	Change % Median (interquartile range)	P^a
TNF				
Fatty fish	1.08 ± 0.26	1.04 ± 0.29	−1.5 (−22.5, 19.5)	0.90
Lean fish	1.09 ± 0.22	1.01 ± 0.21	−5.6 (−19.0, 7.8)	
Control	1.01 ± 0.28	0.97 ± 0.27	0.4 (29.4, 30.2)	
IL1B				
Fatty fish	1.10 ± 0.46	1.15 ± 0.44	−14.3 (−26.9, 59.8)	0.83
Lean fish	1.03 ± 0.44	0.94 ± 0.40	−13.9 (−29.3, 55.2)	
Control	0.93 ± 0.15	0.97 ± 0.30	6.4 (−7.8, 17.9)	
CCL2				
Fatty fish	0.90 ± 0.35	0.88 ± 0.61	−5.2 (−30.4, 54.1)	0.92
Lean fish	1.04 ± 0.42	1.00 ± 0.47	0.1 (−27.0, 8.8)	
Control	1.10 ± 0.58	1.16 ± 0.78	0.0 (−26.8, 70.4)	
CCL5				
Fatty fish	0.86 ± 0.20	0.94 ± 0.28	10.9 (−0.5, 23.4)	0.11
Lean fish	1.02 ± 0.35	1.07 ± 0.42	7.9 (−14.5, 21.3)	
Control	1.14 ± 0.34	0.97 ± 0.34	−18.8 (−34.4, 7.9)	
ICAM1				
Fatty fish	1.07 ± 0.14	1.05 ± 0.09	−0.1 (−9.4, 3.3)	0.95
Lean fish	1.09 ± 0.15	1.08 ± 0.15	−0.1 (−6.0, 5.5)	
Control	0.93 ± 0.21	0.94 ± 0.24	−6.3 (−8.1, 16.0)	
SELP				
Fatty fish	1.45 (0.96, 1.62)[b]	1.46 (0.81, 2.05)[b]	16.4 (7.0, 53.3)	0.27
Lean fish	1.23 (0.99, 1.83)[b]	0.94 (0.84, 1.46)[b]	−11.0 (−29.2, 39.3)	
Control	0.70 (0.50, 1.43)[b]	0.67 (0.49, 1.40)[b]	13.8 (−51.8;36.9)	

Values for week 0 and week 8 are expressed as arbitrary values normalized to GAPDH mRNA expression, and values for % change as: ((week 8 – week 0) × 100/week 0)

[a] Kruskal–Wallis test

[b] Data as median (interquartile range)

Table 4 Concentrations of serum inflammatory markers before and after fatty fish, lean fish and control diet interventions

	Fatty fish ($n = 10$)		Lean fish ($n = 11$)		Control ($n = 6$)		P^a
	Week 0	Week 8	Week 0	Week 8	Week 0	Week 8	
CRP (mg/l)	0.58 (0.36–2.13)	0.76 (0.23–1.60)	1.17 (0.50–2.85)	0.80 (0.54–3.22)	1.63 (0.25–4.53)	1.33 (0.50–4.33)	0.69
TNF-α (pg/ml)	1.27 (0.83–1.59)	1.17 (0.57–1.82)	1.37 (0.78–1.69)	1.16 (0.79–1.32)	1.28 (1.05–1.47)	1.08 (0.89–1.25)	0.81
IL-1β (pg/ml)	0.78 ± 0.08	0.80 ± 0.13	0.83 ± 0.14	0.84 ± 0.16	0.82 ± 0.08	0.79 ± 0.07	0.56
IL-6 (pg/ml)	1.58 (0.91–3.98)	1.57 (0.82–4.13)	1.40 (0.89–2.12)	1.50 (0.90–1.99)	1.44 (0.94–4.12)	1.47 (1.07–1.47)	0.94
CCL2 (pg/ml)	342 (256–377)	344 (227–440)	458 (356–591)	466 (350–549)	266 (203–594)	396 (249–481)	0.42
P-selectin (ng/ml)	75 ± 24	73 ± 26	81 ± 40	79 ± 43	96 ± 46	91 ± 51	0.39
E-selectin (ng/ml)	32 ± 8	33 ± 8	27 ± 12	26 ± 10	41 ± 18	36 ± 13	0.03
VCAM-1 (ng/ml)	618 ± 122	618 ± 132	545 ± 53	555 ± 80	610 ± 80	562 ± 95	0.06
ICAM-1 (ng/ml)	186 ± 49	189 ± 43	208 ± 32	203 ± 32[b]	183 ± 69.0	169 ± 57	0.04
CCL5 (pg/ml)	70,545 ± 13,543	66,984 ± 15,173	82,175 ± 20,910	81,687 ± 21,123	80,355 ± 22,833	83,263 ± 22,262	0.44

Data are mean ± SD or median (interquartile range)

[a] GLM univariate analyses for the group effect on the change as ((week8 − week0) × 100/week 0)

[b] $P < 0.05$ for week 0 vs. week 8 (Paired t test)

Table 5 Correlations (r_s) between the changes[a] in mRNA expression of the target genes and: (1) the changes in serum arachidonic acid to eicosapentaenoic acid ratio (AA:EPA) in cholesterol ester (CE) and phospholipid (PL) fractions in the fatty fish group, and (2) the changes in palmitic acid (16:0), saturated fatty acids (SFAs) and polyunsaturated fatty acids to SFAs ratio (P:S) in CE fraction in the lean fish group

	Fatty fish group ($n = 10$)				Lean fish group ($n = 11$)					
	AA:EPA in CE		AA:EPA in PL		16:0 in CE		SFAs in CE		P:S in CE	
	r_s	P	r_s	P	r_s	P	r_s	P	r_s	P
TNF	0.22	0.53	0.21	0.56	0.46	0.15	0.35	0.30	−0.05	0.89
IL1B	0.60	0.06	0.86	0.002	0.51	0.11	0.43	0.19	−0.21	0.54
CCL2	0.33	0.37	0.55	0.10	0.64	0.03	0.51	0.11	−0.04	0.92
CCL5	−0.41	0.24	−0.09	0.80	0.37	0.26	0.36	0.29	−0.46	0.15
ICAM1	0.14	0.70	0.37	0.29	0.64	0.04	0.64	0.04	−0.70	0.02
SELP	0.29	0.43	0.32	0.37	−0.12	0.73	−0.15	0.67	−0.41	0.21

[a] Changes as: week 8–week 0

At baseline, a higher mRNA expression of ICAM1 was associated with a higher proportion of palmitic acid and total SFAs in CE ($r = 0.56$, $P = 0.003$ and $r = 0.52$, $P = 0.005$), and with a higher proportion of AA in both CE and PL fractions ($r = 0.49$, $P = 0.009$ and $r = 0.40$, $P = 0.04$, respectively). Conversely, mRNA expression of ICAM1 correlated inversely with linoleic acid proportion in both CE and PLs ($r = -0.40$, $P = 0.04$ and $r = -0.56$, $P = 0.002$, respectively). No correlations were found between the baseline mRNA expression of CCL2 and these respective FAs in CE or PL fractions ($P > 0.10$ for all).

Correlations of serum FA composition and gene expression with plasma lipid metabolites

We assessed correlations between the change in serum AA:EPA ratio, and in IL1B mRNA levels with the change in plasma concentration of lipid metabolites potentially involved in insulin metabolism: Cer and DG, which decreased after the FF intake [16]. Considering the data of patients in this present study ($n = 27$), the plasma Cer and DG concentrations decreased only in the FF group ($n = 10$; Cer: 4.64 ± 1.09 vs. 3.07 ± 0.79 μmol/l and DG: 4.88 ± 2.34 vs. 2.74 ± 1.08 μmol/l; $P < 0.05$ for both), but not in LF ($n = 11$; Cer: 3.26 ± 1.38 vs. 3.74 ± 1.21 μmol/l and DG: 4.54 ± 1.56 vs. 4.03 ± 2.03 μmol/l; $P > 0.10$ for both) or control ($n = 6$; Cer: 4.03 ± 1.13 vs. 4.97 ± 1.34 μmol/l and DG: 4.90 ± 1.07 vs. 4.67 ± 2.24 μmol/l; $P > 0.10$ for both) groups. The change in Cer was also different among groups ($P = 0.005$), but not the change in DG ($P = 0.28$).

In the FF group, the changes in the AA:EPA ratio in CE and PL fractions were positively correlated with the change in DG ($r_s = 0.79$, $P = 0.006$ and $r_s = 0.48$, $P = 0.16$),

although the latter was not significant. A positive correlation was found between the changes in the AA:EPA ratio in CE and in plasma Cer when all patients were considered ($n = 27$; $r_s = 0.42$, $P = 0.03$), and also in the FF group ($r_s = 0.55$, $P = 0.10$), but with borderline significance. No correlation was found, however, between the change in the mRNA expression of the IL1B and either the changes in plasma Cer or DG ($r_s = -0.16$ and $r_s = 0.33$, respectively; $P > 0.10$). Also, the change in AA:EPA ratio in PLs did not correlate with the change in plasma Cer in the FF group ($r_s = -0.08$, $P = 0.83$).

Correlations of markers of IR with gene expression in PBMCs and plasma lipid metabolites

At baseline FPI and HOMA-IR were positively associated with plasma Cer ($r = 0.48$, $P = 0.01$ and $r = 0.46$, $P = 0.02$, respectively; $n = 27$). Higher FPI and HOMA-IR at baseline also correlated with higher mRNA levels of TNF in PBMCs ($r = 0.41$, $P = 0.04$ and $r = 0.43$, $P = 0.02$, respectively). In the FF group, FPI and HOMA-IR correlated with the mRNA expression of IL1B ($r = 0.70$, $P = 0.03$ and $r = 0.70$, $P = 0.02$, respectively), TNF ($r = 0.73$, $P = 0.02$ for both) and CCL2 ($r = 0.56$, $P = 0.09$ and $r = 0.65$, $P = 0.04$, respectively) at the end of the study.

Discussion

In the present study, the increase in the median dietary intake of EPA+DHA from 0.37 g to 0.89 g per day derived from a FF diet and the concomitant increase in their respective serum FA proportions after 8 weeks did not significantly modulate the mRNA expression of IL1B, TNF, CCL2, CCL5, ICAM1, and SELP in PBMCs of patients with established CHD. Similar results were observed for the LF diet. However, the change in the serum ratio of AA to EPA in the patients following the FF regimen was positively associated with the change in the mRNA levels of the proinflammatory cytokine IL-1β. Interestingly, circulating levels of sICAM-1 decreased with LF intake. Moreover, the change in serum palmitic acid proportion in the CE in this group was positively associated with the changes in ICAM1 and CCL2 mRNA levels.

In immune cells, FAs are incorporated into the PLs of cell membranes acting as regulators of gene expression. It is therefore expected that changes in FA composition alter gene expression [2, 32]. Because diet-induced changes in plasma PUFA proportions are also reflected in circulating immune cell membranes [9, 19], the serum FA composition measured in our study likely reflects the PUFA profile of the PBMCs. While dietary EPA is rapidly incorporated into PL membranes, the incorporation of DHA might respond more slowly to the increase in n-3 PUFA intake [9]. Although DHA proportion in both serums CE and PL fractions increased in the FF group, more time could have been needed in order for the DHA to be incorporated in the cell membrane composition of the PBMCs. The length of the study could have masked possible additive effects of this FA on modulation of the target genes. Moreover, an insufficient increase in EPA+DHA intake could have also played a role.

The change in serum AA:EPA ratio in both CE and PL fractions that resulted from the FF diet was highly and positively correlated with the change in IL1B mRNA expression levels. Inflammatory cell membranes are typically abundant in AA, which in white blood cells is metabolized mostly to leukotrienes that are implicated in inflammatory processes, via the lipoxygenase pathway [23, 27]. In most studies, when EPA is increased by >0.5 g/day in the diet, the characteristic features are a decrease in the proportion of AA and an increase in the proportion of EPA in immune cells [2]. A decrease in the AA:EPA ratio in the membrane of the immune cells and in serum lipids has been shown to decrease IL-1β, TNF-α, and prostaglandin E2 production [3, 21]. The positive correlation found between the change in serum AA:EPA ratio and the changes in plasma DG and Cer levels also indicates that these lipid mediators could contribute to an anti-inflammatory effect in PBMCs. DG is suggested to participate in the inhibition of the insulin action via activation of protein-kinase C and it is also related to inflammation [11, 34]. A decrease in Cer levels and IL1B expression are suggested to improve IR [5, 11]. The cross-sectional correlations found in our study between insulin levels or HOMA-IR and plasma Cer at baseline and with IL1B mRNA levels at the end of the study in FF group also reinforce this concept.

Interestingly, a decrease in sICAM-1 circulating concentration in the LF group, but not in the FF group, was observed. These results confirm recent findings in young healthy subjects [25]. The authors showed that LF (EPA+DHA from fish: $\approx$0.250 g/day), but not FF (EPA+DHA from fish: $\approx$2 g/day) or fish oil (EPA+DHA supplementation: $\approx$1 g/day), decreased serum concentration of sICAM. Other authors have found, however, that 1.2 g/day of EPA+DHA supplementation resulted in a decrease of sICAM levels in older subjects [22].

In the LF group, the change in mRNA levels of ICAM1 positively correlated with the changes in the serum proportions of palmitic acid, total SFAs, and P:S ratio in CE. The change in serum palmitic acid proportion, and, to some extent, the change in SFAs, also positively correlated with the change in CCL2 expression. Palmitic acid is the most abundant SFA derived from the diet. Although not significantly different among groups, pair-wise comparison showed a significant decrease in total SFA intake (10.3 vs. 7.7%, $P = 0.005$) and an increase in P:S ratio in CE

fraction (3.51 vs. 3.77, $P = 0.003$) only in the LF group. Subjects with higher baseline ICAM1 expression levels had higher serum proportions of palmitic acid and total SFAs in the CE. Serum palmitic acid and SFA proportions have previously been shown to be associated with endothelial dysfunction [26, 29]. Moreover, a decrease in SFAs in the diet may improve endothelial function [1, 13]. It is known that endothelial dysfunction plays a role in the pathophysiology of hypertension, cardiovascular diseases, and IR [14]. Furthermore, CCL2 is known to be a proinflammatory chemokine implicated in IR [12, 30], with a suggested role in atherosclerosis [31]. These results also strengthen the relevance of recent findings on the blood pressure lowering effects of the LF diet [7] and indicate a link between SFAs, inflammation and insulin metabolism [28].

All the subjects were using lipid-lowering drugs (statins) commonly prescribed to improve the prognosis of patients with CHD. Statins have shown to alter the levels of hsCRP and TNF-α [24] and cytokine expression response in immune cells [36]. In fact, systemic levels of hsCRP in patients from the present study were relatively low. The ability of fish oil to decrease cytokine production in immune cells might be influenced by polymorphisms in cytokine genes, inherent cytokine production, and use of statins [10, 18, 32]. Nevertheless, other authors have not shown any effect of increasing n-3 PUFAs (2.1 g/day) on serum inflammatory markers of healthy men [37].

The exclusion criteria applied in the present study (diseases, medications, and older age) limited the number of eligible subjects. Therefore, statistical power for detecting significant results was limited. Nonetheless, this selected study population is important to better characterize the effect of the recommended intake of 1 g of long-chain n-3 FAs for secondary prevention of CHD [15]. The lack of the response at mRNA level of the genes studied is likely to be related not only to the ability to incorporate the n-3 PUFAs in PBMC cell membrane, length of the study or high variation on gene expression response among the participants, but also to concurrent medication used for treating CHD and possibly the need for higher doses of n-3 PUFAs.

In conclusion, the intake of FF or LF diet for an 8-week period did not alter the expression of selected inflammatory genes in PBMCs of patients with CHD. However, the decrease in AA:EPA ratio in serum lipids in the FF group may influence an anti-inflammatory response at mRNA levels in these immune cells. We also suggest that a LF diet, along with the reduction of palmitic acid in serum, might benefit endothelial function.

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