Habitual Dietary Intake of n-3 and n-6 Fatty Acids in Relation to Inflammatory Markers Among US Men and Women

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Background—Polyunsaturated fatty acid intake favorably affects chronic inflammatory-related diseases such as cardiovascular disease; however, high intake of n-6 fatty acids may attenuate the known beneficial effects of n-3 fatty acids.

Methods and Results—We investigated habitual dietary n-3 fatty acid intake and its interaction with n-6 fatty acids in relation to the plasma inflammatory markers C-reactive protein, interleukin 6, and soluble tumor necrosis factor receptors 1 and 2 (sTNF-R1 and R2) among 405 healthy men and 454 healthy women. After adjustment for other predictors of inflammation, intake of the n-3 fatty acids eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) was inversely associated with plasma levels of sTNF-R1 and sTNF-R2 ($P=0.03$ and $P<0.001$, respectively) and somewhat less so for C-reactive protein ($P=0.08$). n-3 α-linolenic acid and n-6 cis-linoleic acid were not significantly related to the inflammatory markers. We found little if any association between n-3 fatty acid (EPA+DHA) intake and tumor necrosis factor receptors among participants with low intake of n-6 but a strong inverse association among those with high n-6 intake ($P=0.04$ and 0.002 for interaction of n-3 with n-6 on sTNF-R1 and sTNF-R2, respectively).

Conclusions—These results suggest that n-6 fatty acids do not inhibit the antiinflammatory effects of n-3 fatty acids and that the combination of both types of fatty acids is associated with the lowest levels of inflammation. The inhibition of inflammatory cytokines may be one possible mechanism for the observed beneficial effects of these fatty acids on chronic inflammatory-related diseases. (***Circulation*. 2003;108:155-160.**)

Key Words: fatty acids ■ inflammation ■ nutrition ■ risk factors

Dietary intake of polyunsaturated fatty acids (PUFAs) favorably affects cardiovascular disease (CVD).[1] The improvement in blood lipid levels by dietary PUFAs only partially explains these beneficial effects. Inflammation may be essential in the origin of atherosclerosis, and inflammatory markers such as C-reactive protein (CRP), interleukin (IL)-6, and tumor necrosis factor (TNF)-α are independent risk factors for CVD.[2,3] n-3 fatty acids have antiinflammatory properties and are frequently used clinically to treat symptoms of inflammatory diseases, such as rheumatoid arthritis or Crohn's disease.[4] Only a few studies have investigated the effects of n-3 fatty acid intake on plasma IL-6 and TNF-α levels in humans in vivo, but these have been small and inconsistent.[5–7] Competition with n-6 fatty acids may be the reason for the observed discrepancies of the effects of n-3 fatty acids on cytokines.[8] Both n-3 and n-6 fatty acids are substrates for human eicosanoid production, and they share the same enzymes for the synthesis of prostaglandins and leukotrienes (Figure 1). Eicosanoids derived from n-3 fatty acids have fewer inflammatory properties than those derived from n-6 fatty acids. Thus, the ratio of n-3 to n-6 fatty acid intake may be crucial to inflammatory processes. The aim of the present study was to investigate the habitual dietary n-3 and n-6 fatty acid intake in relation to the inflammatory markers CRP, IL-6, and soluble TNF receptors 1 and 2 (sTNF-R1 and sTNF-R2), markers of TNF-α activity.

Methods

Health Professionals Follow-Up Study

The Health Professionals Follow-up Study (HPFS) is a prospective cohort investigation among 51 529 US male health professionals aged 40 to 75 years at baseline in 1986. Health information and disease status are assessed biennially by a self-administered questionnaire, and diet is assessed every 4 years by a 131-item self-administered semiquantitative food-frequency questionnaire (SFFQ). Between 1993 and 1995, a blood sample was requested from all subjects and returned from 18 225 participants. This cohort and the method for blood collection have been described in detail previously.[9] The men who provided samples were somewhat younger but otherwise similar to those who did not provide samples. Among those who returned the blood sample, subjects were excluded who did not have any information on diet, cigarette smoking, alcohol

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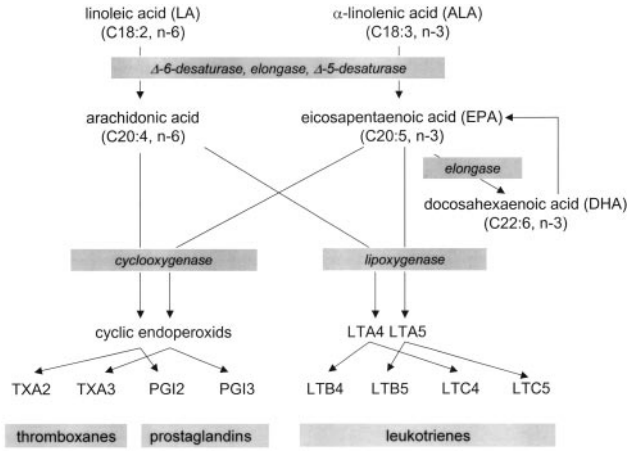

Figure 1. Human eicosanoid synthesis from n-6 and n-3 fatty acids.

consumption, or physical activity between 1986 and 1994. In addition, subjects with a history of myocardial infarction, angina pectoris, stroke, diabetes mellitus, intermittent claudication, gastric or duodenal ulcers, gallbladder disease, liver disease, or cancers (except nonmelanoma skin cancer) before 1994 were excluded. From the remaining men, 468 participants were randomly selected on the basis of different patterns of self-reported alcohol consumption determined by frequency, amount, and time (eg, with meals) of alcohol intake. The purpose of selecting this subset was to investigate the association between alcohol drinking patterns and novel biomarkers of CVD. Except for the exclusion criteria, there is little difference in various characteristics between the 468 men used in the present study and the remaining 17 757 who gave blood.

Average nutrient intake was derived from the SFFQ completed in 1994 and computed with composition values from US Department of Agriculture sources,[10] supplemented with other data. Reproducibility and validity of the SFFQ have been evaluated within the HPFS.[11] The correlation for nutrients estimated by the SFFQs and those measured by 2 weeks of diet recording was 0.60 for eicosapentaenoic acid (EPA) and ranged from 0.37 for polyunsaturated fat to 0.92 for vitamin C with supplements (average 0.65).[11,12] Correlations for whole foods were in a similar range. The correlation of EPA intake and the percentage of EPA in adipose tissue was 0.49 ($P<0.001$).[12]

Body mass index was calculated as the ratio of body weight to body height squared, and physical activity was expressed as metabolic equivalent-hours (MET-hr). One MET-hr is equivalent to sitting quietly for 1 hour.[13] The validity of self-reported weight, physical activity, and alcohol intake has been reported in detail elsewhere.[14]

Nurses' Health Study II

The Nurses' Health Study II is a prospective cohort of 116 671 United States registered female nurses aged 25 to 42 years at baseline in 1989.[15] Health information and disease status are assessed biennially similar to methods described above. Blood samples were obtained between 1996 and 1998 from more than 29 000 participants. The samples were selected from premenopausal women who collected their blood during the luteal phase of their menstrual cycle and who were not taking any hormones. After exclusion of women with any of the previously described preexisting conditions, a subset of 473 women were randomly selected from the remaining participants on the basis of drinking patterns similar to those used for the HPFS. Women who gave blood were very similar to those in the overall cohort; the only difference was a greater percentage of women with a family history of breast cancer in the subgroup.

Average nutrient intake and alcohol consumption were derived from the SFFQ in 1995 and other covariates from the 1997 questionnaire. The validity and reproducibility of information for these nurses is described elsewhere.[14]

Measurement of Biochemical Variables

Blood samples were collected in three 10-mL liquid EDTA blood tubes for the men and sodium heparin for the women, placed on ice packs, stored in Styrofoam containers, and returned to our laboratory via overnight courier. More than 95% of the samples arrived within 24 hours. After arrival, the blood samples were centrifuged and separated into aliquots for storage in the vapor phase of liquid nitrogen freezers (−130°C or colder). Fewer than 15% of the samples were slightly hemolyzed, and very few were moderately hemolyzed (<3%), lipemic (<1%), or not cooled on arrival (<0.5%). Plasma sTNF-R and IL-6 concentrations were measured by ELISA (R&D Systems for sTNF-R1 and IL-6; Genzyme Diagnostics for sTNF-R2 in HPFS; and R&D Systems for sTNF-R2 in the Nurses' Health Study II). CRP was measured with a high-sensitivity assay (Roche Diagnostics). Day-to-day coefficients of variation of all assays were below 10%, and transport conditions did not substantially affect the plasma levels of these inflammatory markers.[16] In a subsample of 82 men from the HPFS who provided blood samples 4 years apart, there were good to excellent intraclass correlations for the inflammatory markers (sTNF-R1, 0.81; sTNF-R2, 0.78; IL-6, 0.47; and CRP, 0.67). All participants gave written informed consent, and the Harvard School of Public Health Human Subjects Committee Review Board approved the study protocol.

Exclusions

We excluded participants who did not provide information on body mass index, physical activity, diet, or smoking status on the questionnaires returned in 1994 (men) and 1995/1997 (women), respectively, which resulted in a sample size of 859 subjects (405 men and 454 women). Our subset included 16 men and 3 women who reported rheumatoid arthritis and 5 men and 5 women who reported ulcerative colitis/Crohn's disease. Exclusion of these subjects from our analysis did not substantially affect our results.

Statistical Analyses

Multivariable linear regression analyses with robust variance were performed to evaluate the association between PUFA intake and plasma levels of inflammatory markers without the need of normal distribution assumptions.[17] We adjusted for age (5-year categories), gender, smoking status (never, past, current 1 to 14 cigarettes/d, and current ≥15 cigarettes/d), physical activity, alcohol intake, intake of nonsteroidal antiinflammatory drugs, body mass index (<20, 20 to 24, 25 to 29, 30 to 34, and ≥35 kg/m^2), total calories, protein, saturated fat, monounsaturated fat, cholesterol intake, and the remaining PUFAs. Nutrients under investigation were considered as continuous variables and covariates as categorical variables (quintiles). In additional models, we included a cross-product term to evaluate the interaction between n-3 and n-6 fatty acids. All probability values presented are 2-tailed, and probability values below 0.05 were considered statistically significant.

Results

The characteristics and biomarker levels according to gender and percentile of EPA+DHA intake are presented in Table 1. In both men and women, individuals with higher intake of EPA and DHA had lower levels of sTNF-R1 and sTNF-R2. In the category with the highest intake of EPA+DHA, sTNF-R1 levels were 14% lower in men and 6% lower in women, and sTNF-R2 levels were 19% lower in men and 5% lower in women than in the lowest quintile. The overall results changed only slightly after multivariate adjustment (Table 2); intake of EPA+DHA was significantly inversely associated with plasma levels of sTNF-R1 ($P=0.03$) and sTNF-R2 ($P<0.001$) and modestly inversely related to CRP levels ($P=0.08$). α-Linolenic acid (ALA), the other main dietary n-3 fatty acid, and cis-linoleic acid (cis-LA), the main dietary n-6 fatty acid, were not significantly related to the

TABLE 1. Characteristics of the Study Population (n=859)

	Percentile of EPA+DHA Intake (% Energy)					
Characteristics	0–20	20–40	40–60	60–80	80–90	90–100
Men						
EPA+DHA, % energy	0.022	0.060	0.100	0.147	0.220	0.454
n	81	81	81	81	40	41
Age, y	59.4	59.8	59.9	59.7	59.6	62.5
BMI, kg/m^2	25.0	25.2	25.8	25.7	25.3	26.1
Current smoker, %	7.4	5.0	4.8	4.2	9.4	6.6
Alcohol, g/d	19.6	22.2	25.6	23.6	22.9	25.8
Total physical activity, METs/wk	33.1	38.4	34.0	45.7	38.2	61.7
Total calories, kcal/d	2157	2138	2258	1946	1999	2225
Total fat, % energy	30.9	29.4	30.1	28.9	27.6	27.7
EPA+DHA, g/d	0.055	0.143	0.250	0.317	0.498	1.120
ALA, % energy	0.50	0.52	0.49	0.51	0.46	0.47
cis-LA, % energy	5.4	5.3	5.1	5.2	4.7	4.5
Intake of NSAID, %	40.5	40.3	52.7	56.3	35.5	53.1
sTNF-R1, pg/mL	979	935	934	926	945	845
sTNF-R2, pg/mL	1495	1480	1418	1392	1440	1212
IL-6, pg/mL	1.42	1.44	1.69	1.47	2.72	1.17
CRP, mg/dL	0.19	0.16	0.22	0.18	0.29	0.13
Women						
EPA+DHA, % energy	0.010	0.033	0.058	0.097	0.133	0.242
n	91	91	91	91	45	45
Age, y	41.9	41.7	42.0	42.9	43.5	43.6
BMI, kg/m^2	23.9	23.8	24.4	24.5	24.2	23.8
Current smoker, %	3.5	5.4	10.4	9.9	7.0	10.4
Alcohol, g/d	11.3	10.5	11.5	11.8	10.4	11.9
Total physical activity, METs/wk	20.9	17.4	20.8	25.8	25.7	26.2
Total calories, kcal/d	1836	1960	1862	1892	1758	1705
Total fat, % energy	29.2	29.4	28.4	28.5	26.6	26.3
EPA+DHA, g/d	0.022	0.072	0.121	0.203	0.266	0.471
ALA, % energy	0.47	0.46	0.46	0.48	0.49	0.48
cis-LA, % energy	4.4	4.5	4.3	4.4	4.3	4.3
Intake of NSAID, %	40.3	49.1	48.1	58.8	43.8	53.3
sTNF-R1, pg/mL	1009	1002	986	1034	962	953
sTNF-R2, pg/mL	2127	2151	2128	2123	2025	2029
IL-6, pg/mL	1.32	1.37	1.52	2.08	1.02	1.67
CRP, mg/dL	0.16	0.18	0.16	0.23	0.16	0.12

BMI indicates body mass index; METs, metabolic equivalent task; and NSAID, nonsteroidal antiinflammatory drugs. Values are means. All values (except age and number of subjects) are age-standardized.

inflammatory markers. When we stratified by gender, the regression coefficients were similar to those presented in Table 2, albeit weaker for the associations between EPA+DHA and sTNF receptors among women. The interaction term for gender and EPA+DHA intake was not significant, which suggests that the weaker association among women may be due to limited variation in dietary intake rather than a true difference between genders.

Because n-3 and n-6 fatty acids compete for the same enzymes in fatty acid elongation and desaturation, we cross-classified individuals on the basis of quartiles of their intake of EPA+DHA and cis-LA (Figure 2). In general, we found little if any association between EPA+DHA and TNF receptors among those with a low n-6 intake but a very strong inverse association among those with high n-6 intake. Participants with the highest EPA+DHA and cis-LA intake had the lowest levels of sTNF-R1 (P interaction=0.04) and sTNF-R2 (P interaction=0.002). However, participants with high cis-LA intake but low EPA+DHA intake had the highest levels of sTNF-R1 and sTNF-R2. We did not find a significant interaction between the n-3 and n-6 fatty acids with regard to IL-6 or CRP levels. When we formally compared

TABLE 2. Multiple Linear Regression Effect Estimates for the Association Between n-3 and n-6-Fatty Acid Intake (1% of Energy) and Inflammatory Markers Among Men and Women (n=859)

		Model 1			Model 2			Model 3		
Biomarkers (Dependent)		EPA+DHA	ALA	cis-LA	EPA+DHA	ALA	cis-LA	EPA+DHA*	ALA†	cis-LA‡
sTNF-R1, pg/mL	β	−171.6‖	−21.3	2.1	−162.5‖	−45.5	−1.1	−156.7‖	−48.5	−0.5
sTNF-R2, pg/mL	β	−453.0¶	−8.6	8.0	−449.4¶	−47.7	2.2	−513.9¶	−7.0	8.0
IL-6, pg/mL	β	0.296	0.763	0.031	0.617	0.784	0.022	0.630	0.438	0.013
CRP, mg/dL	β	−0.098	0.028	0.011	−0.056	0.028	0.008	−0.164§	−0.079	0.004

Model 1 was adjusted for age and gender; model 2 includes all covariates in model 1 and smoking status, physical activity, alcohol intake, intake of nonsteroidal antiinflammatory drugs, and body mass index; and model 3 includes all covariates in model 2 and total calories, protein, saturated fat, monounsaturated fat, and cholesterol intake.

*Model 3 additionally adjusted for cis-LA and ALA.

†Model 3 additionally adjusted for cis-LA and EPA+DHA.

‡Model 3 additionally adjusted for EPA+DHA and ALA.

§$P<0.1$; ‖$P<0.05$; ¶$P<0.001$.

levels of the soluble TNF receptors between groups presented in Figure 2, we found the largest differences in sTNF-R1 (112 pg/mL; $P=0.01$) and sTNF-R2 (285 pg/mL; $P<0.001$) comparing subjects with the highest intake of EPA+DHA and cis-LA to those with the lowest EPA+DHA but highest cis-LA intake.

Discussion

In this cross-sectional study of dietary n-3 and n-6 fatty acids and inflammatory markers, we observed statistically significant inverse associations between n-3 fatty acid intake and plasma levels of soluble TNF receptors 1 and 2. The associations were restricted to the long-chain PUFAs EPA and DHA and not ALA. We found similar albeit weaker inverse associations for CRP but not IL-6 levels. These relations were dependent on the intake of n-6 fatty acids, which suggests that at low levels of n-3 fatty acid intake, n-6 fatty acids are associated with high levels of inflammatory markers, yet at high levels of n-3 fatty acid intake, the combination of both types of fatty acids is related to the lowest levels of inflammation.

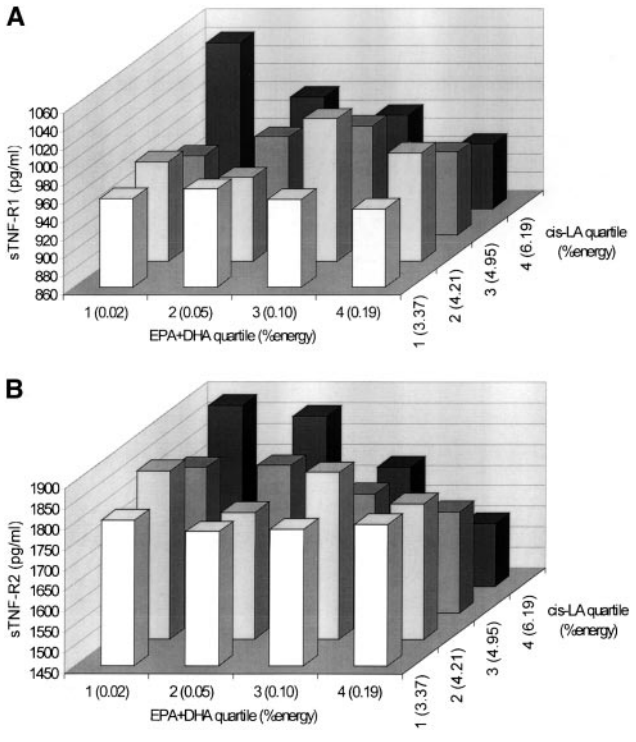

Figure 2. Plasma sTNF-R1 (A) and sTNF-R2 (B) levels in relation to quartiles of EPA+DHA and cis-LA intake. Mean values adjusted for age, gender, smoking status, physical activity, alcohol intake, intake of nonsteroidal antiinflammatory drugs, body mass index, total calories, protein, saturated fat, monounsaturated fat, ALA, and cholesterol intake.

Inflammation plays a central role in development of atherosclerosis, and elevated plasma CRP levels are an important risk factor for CVD and type 2 diabetes.[2] Similarly, plasma levels of TNF-α and IL-6 are also predictive of CVD[2,3]; however, this relationship is less strong than for CRP, which may be due in part to higher variability or lower specificity of these cytokines. For example, it has been shown that IL-6 may induce antagonists to inflammatory cytokines and may therefore play a role in terminating inflammation.[18] The soluble TNF receptors are derived by proteolytic cleavage from TNF cell-surface receptors after induction by TNF or other cytokines such as IL-6, IL-1β, or IL-2, and they have a longer half-life and are detected with a higher sensitivity than TNF.[19] The biological function of these soluble receptors is not entirely clear, and they may bind to TNF and attenuate its bioactivity. However, the soluble receptors also promote formation of complexes, which preserve the active trimeric form of TNF, and thus prevent the decay of TNF into inactive monomeric forms. The receptors may therefore serve as binding proteins and/or as a slow-release reservoir for TNF, thereby prolonging its half-life.[19] Clinically, the soluble TNF receptors are excellent indicators of inflammatory processes[19] and are associated with obesity, insulin resistance, coronary heart disease, and angina severity.[20,21] Similarly, recent studies confirmed that the soluble TNF receptors were more predictive of disease status in heart failure than TNF itself.[22,23]

Previous studies have investigated the effects of n-3 fatty acids on cytokine secretion ex vivo, with quite conflicting results. For example, Blok et al[24] reported 7 animal studies with an increase and 3 with a decrease in TNF-α production after n-3 intake. In contrast, in the 6 cited human studies, 3

found a decrease and 3 found no effect. These experiments may be influenced by cell purification and culturing procedures and depend on cell origin and cell type, because TNF-α and IL-6 are produced by a variety of cells. Therefore, the in vivo plasma cytokine levels measured in the present study may represent the overall subinflammatory process better than cell-type–specific ex vivo expression.

The present results are in accordance with human trials reporting decreased inflammation with n-3 fatty acid intake (as reviewed by Blok et al,[24] Calder,[25] and James et al[26]). However, the present results are in contrast to the customary assumption that high intake of n-6 fatty acids antagonizes the antiinflammatory effects of n-3 fatty acids. One possible explanation for our observation is that n-6 fatty acids lower soluble TNF receptor levels by indirectly reducing insulin resistance.[20,27] Furthermore, the activities of the Δ6 and Δ5-desaturase (which are the rate-limiting steps for arachidonic acid and EPA synthesis in humans) and the activity of the cyclooxygenase are inhibited by both n-3 and n-6 PUFAs.[28–30] Through this mechanism, high intake of both types of fatty acids could reduce inflammatory mediators. PUFAs may also modulate cytokine production or the release of the soluble TNF receptors through eicosanoid-independent pathways, for example, by influencing membrane composition and fluidity,[31] affecting signal transduction processes or second messenger molecules,[32] or binding to or affecting nuclear receptors such as the peroxisome proliferator receptors[33] or nuclear factor-κB.[34]

EPA and DHA are much more effective than ALA in altering membrane composition and eicosanoid production, and it was recently estimated that in humans, only 0.2% of plasma ALA is converted to EPA.[35,36] The conversion rate of LA to arachidonic acid is also very low[37]; however, because of the much higher intake, we speculate that LA still affects arachidonic acid concentrations, whereas ALA, because of its low intake and low conversion rate, has no substantial effect on EPA levels, and thus on inflammation, in the present study population.

It has been argued that the amount of n-6 PUFA in the diet is currently too high in the US population and needs to be lowered to "reduce adverse effects of excesses of arachidonic acid and its eicosanoid products."[38] However, there are no data from human studies that support a detrimental effect of dietary n-6 fatty acids on coronary heart disease or other harmful side effect of n-6 fatty acids.[39] In contrast, several studies found beneficial effects (as reviewed by Hu et al[1]). Specifically, a recent prospective study found similar beneficial effects of n-3 and n-6 fatty acids on cardiovascular risk factors,[40] and a recent cross-sectional study found n-3 and n-6 fatty acid intake combined to be associated with a lower risk of coronary artery disease than either type of fatty acid alone.[41] The present study suggests one possible mechanism for this observed phenomenon.

The cross-sectional design of the present study complicates the drawing of causal inferences, and a single assessment of a biochemical indicator may be susceptible to short-term variation, which would bias the results toward the null. However, the dietary questionnaire has been shown to reflect long-term intake, and the biomarkers of inflammation we measured are stable over time. Measurement error from the use of self-reported dietary intake and lifestyle variables is relatively small[14] and likely does not bias our results, because reporting error is not likely associated with the biological measurements. Although the present study cohorts do not represent random samples of the US population, the ranges of anthropometric parameters and the biological measures are quite broad and are comparable to those of the general population. We cannot exclude the possibility that the observed differences in absolute sTNF-R levels between men and women are due in part to gender differences in kidney function, because sTNF receptor levels may depend on renal function[42]; however, kidney function is not likely associated with PUFA intake. Furthermore, the main analyses were adjusted for gender, and the results were similar across male and female strata.

In conclusion, we found an inverse relationship between n-3 fatty acid intake from fish and plasma soluble TNF receptor levels and less so for other markers of inflammation. This relationship was stronger for higher intakes of n-6 fatty acids. The present results do not support the hypothesis that n-6 fatty acids antagonize the effects of n-3 fatty acids. n-6 fatty acids may raise proinflammatory cytokine levels at low consumption of n-3 fatty acids. However, n-3 fatty acids in combination with n-6 fatty acids may decrease proinflammatory cytokine concentration. This may be one of the possible mechanisms for the observed beneficial effects of n-3 and n-6 fatty acid intake on chronic inflammatory diseases.

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