Effects of docosahexaenoic acid–rich n−3 fatty acid supplementation on cytokine release from blood mononuclear leukocytes: the OmegAD study[1–3]

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ABSTRACT

Background: Dietary fish or fish oil rich in n−3 fatty acids (n−3 FAs), eg, docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA), ameliorate inflammatory reactions by various mechanisms. Whereas most studies have explored the effects of predominantly EPA-based n−3 FAs preparations, few have addressed the effects of n−3 FAs preparations with DHA as the main FA.

Objective: The objective was to determine the effects of 6 mo of dietary supplementation with an n−3 FAs preparation rich in DHA on release of cytokines and growth factors from peripheral blood mononuclear cells (PBMCs).

Design: In a randomized, double-blind, placebo-controlled trial, 174 Alzheimer disease (AD) patients received daily either 1.7 g DHA and 0.6 g EPA (n−3 FAs group) or placebo for 6 mo. In the present study blood samples were obtained from the 23 first randomized patients, and PBMCs were isolated before and after 6 mo of treatment.

Results: Plasma concentrations of DHA and EPA were significantly increased at 6 mo in the n−3 FAs group. This group also showed significant decreases of interleukin (IL)-6, IL-1β, and granulocyte colony-stimulating factor secretion after stimulation of PBMCs with lipopolysaccharide. Changes in the DHA and EPA concentrations were negatively associated with changes in IL-1β and IL-6 release for all subjects. Reductions of IL-1β and IL-6 were also significantly correlated with each other. In contrast, this n−3 FA treatment for 6 mo did not decrease tumor necrosis factor-α, IL-8, IL-10, and granulocyte-macrophage colony-stimulating factor secretion.

Conclusion: AD patients treated with DHA-rich n−3 FAs supplementation increased their plasma concentrations of DHA (and EPA), which were associated with reduced release of IL-1β, IL-6, and granulocyte colony-stimulating factor from PBMCs. This trial was registered at clinicaltrials.gov as NCT00211159. *Am J Clin Nutr* 2008;87:1616–22.

INTRODUCTION

n−3 Fatty acids (n−3 FAs), eg, eicosapentaenoic acid (EPA; 20:5n−3) and docosahexaenoic acid (DHA; 22:6n−3), present in marine oils, modulate inflammatory reactions and ameliorate symptoms of several autoimmune and other inflammatory disorders (1–3). In addition, EPA and DHA administration reduces cardiovascular morbidity and mortality, eg, from ventricular arrhythmias (4). Recently, high fish intake or dietary supplementation with DHA and EPA has been linked to reductions in the risk of developing Alzheimer disease (AD) (5–7) and to delayed cognitive decline in patients with very mild AD (8).

n−3 FAs are considered to exert anti-inflammatory effects on several cellular levels, including surface receptor modulation, ion pumps, G-proteins, binding to transcription factors [eg, nuclear transcription factor κB (NFκB) and other signaling systems], gene interactions, and generation of several proinflammatory cytokines (9–11). Previously, dietary supplementation with mainly EPA to humans or laboratory animals has been found to reduce release of various cytokines, eg, interleukin (IL)-1β and tumor necrosis factor (TNF)-α, from isolated blood mononuclear cells after stimulation ex vivo with, eg, endotoxin [lipopolysaccharide (LPS)] (12–18). In vitro additions of EPA or DHA to various cells have similar effects (19, 20). However, many of the human studies have evaluated the effects of only short-term dietary EPA supplementation to healthy individuals. Few studies have been performed on the long-term effect of n−3 FAs treatment (21). Moreover, there are only 4 short-term studies on the effects of treatment with oils containing much more DHA than EPA (22–25), without congruent results. Effects of DHA, which is beneficial for brain function and vision as well as for regulation of the inflammatory response, differ from those of EPA (26, 27).

We present results of a trial, the OmegAD study, where a product rich in DHA was given as dietary supplementation for 6 mo to patients with mild to moderate AD. The goal of the OmegAD study was, inter alia, to see if this n−3 preparation would reduce the cognitive deterioration (8). In the present report of the OmegAD trial, we evaluated long-term effects of the DHA-rich n−3 supplementation on ex vivo cytokine and growth factor

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production from isolated peripheral blood mononuclear cells (PBMCs).

SUBJECTS AND METHODS

Study design

This study included 25 patients. They were the first to be randomized in the OmegAD study, described in detail in Freund-Levi, et al (8). In summary, the double-blind, placebo-controlled OmegAD study included a total of 204 patients (73 ± 9 y, 52% women) with mild to moderate AD. Patients were randomized to 6 mo of nutritional supplementation with a marine n−3 fish oil rich in DHA or to placebo. Patients were treated daily with either 1.7 g DHA plus 0.6 g EPA (EPAX 1050TG; Pronova Biocare A/S, Lysaker, Norway) or with an isocaloric placebo oil (1 g corn oil, including 0.6 g linoleic acid). EPAX1050TG is a 60% n−3 fatty acid concentrate in triacylglycerol form, produced according to good manufacturing practices. Four milligrams of vitamin E (tocopherol) was added to each EPAX1050TG and placebo capsule. A total of 174 patients concluded the OmegAD study. Plasma fatty acid profiles and cognition and behavioral data have been published (8, 28).

Based on a pretrial power calculation, with a statistical significance level of $P < 0.05$ and 80% power, a minimum of 20 patients was required to detect a difference of 30% between the n−3 FAs and placebo groups through use of the cytokine assays. Blood samples, for preparation of PBMCs or for plasma for the present study, were obtained from 23 patients before and after 6 mo of treatment because 2 of the 25 patients did not complete the OmegAD trial. Samples from 2 patients had to be excluded because of technical laboratory failure. Thus, 9 (57–82 y; median 75, 3 women) of the remaining patients received the n−3 FAs preparation, and 12 (58–79 y; median 71, 4 women), the placebo capsules.

No changes in peripheral blood neutrophil, monocyte, and lymphocyte cell counts were recorded after 6 mo of n−3 FAs supplementation. Patients were not given any advice on food intake during the study. Food intake in the AD subjects will be reported separately.

Blood sampling

PBMCs were isolated from EDTA anticoagulated venous blood by means of a Lymphoprep (Nycomed Pharma, Oslo, Norway) gradient centrifugation. The cell preparations, obtained before and after treatment with n−3 FAs, contained, on average, 14% monocytes and 85% lymphocytes on both occasions. Corresponding figures for the placebo group were 15% and 85%, respectively. The cell viability in both groups was 96%, as assessed by trypan blue staining.

Experimental design

One million PBMCs were suspended in 1 mL Hank's balanced salt solution with $CaCl_2$ and $MgCl_2$, supplemented with penicillin and streptomycin, Hepes 0.0149 mol/L (GIBCO, Paisley, Scotland, United Kingdom) and 2% inactivated pooled AB serum. The PBMCs were stimulated with LPS from *Escherichia coli* 055:B5, L4005, at 1 or 10 ng/mL (Sigma, St. Louis, MO). Ten nanograms of LPS/mL represents the optimal stimulating concentration, whereas 1 ng/mL was aimed to obtain an ED 50 concentration. Controls were treated with Hank's balanced salt solution alone. Samples were then incubated overnight (22 h) in 37 °C humidified 5% CO_2 atmosphere. Subsequently, cells were centrifuged, and supernatants were collected and stored in −80 °C before cytokine determinations. Cytokines and growth factors were measured using a Human Cytokine Ten-Plex antibody bead kit [for the following cytokines: IL-1β, IL-2, IL-4, IL-5, IL-6, IL-8, IL-10, TNF-α, interferon (IFN)-γ and granulocyte-macrophage (GM) colony-stimulating factor (CSF)] and Human Growth Factor Four-Plex antibody bead kit [vascular endothelial growth factor (VEGF), epidermal growth factor (EGF), basic fibroblast growth factor (FGF), and granulocyte (G)-CSF] for Luminex (BioSource Europe S.A., Nivelles, Belgium) in an array Bio-Plex 100 System-reader (Bio-Rad Laboratories, Inc, the Netherlands). Cytokine release was expressed in ng/mL. We adopted the rationale by Endres et al (12) for giving the results per million PBMCs.

The study was approved by the ethical committee of the Karolinska Institutet (8). The n−3 FAs treatment was safe and well tolerated.

Plasma fatty acid analyses

Plasma fatty acids were analyzed by gas chromatography (THERMO TR-Fame column 30 m × 0.32-mm ID × 0.25-μm film; Thermo Electron Corp, Waltham, MA) and results are given as the relative abundance of individual fatty acids (29). Data for all 174 patients in the OmegAD study have been given previously (8).

Statistical analyses

All analyses were performed on an intention-to-treat basis. We used the Wilcoxon signed rank test for analyses of dependent data. For comparisons of differences in responses between groups over time, we used a Mann-Whitney *U* test for independent data. However, if data distribution was markedly skewed, values were log-transformed to become normally distributed, and then a *t* test for independent data was employed. For correlation analyses a Spearman's rank correlation was applied. P values < 0.05 were considered significant.

RESULTS

Plasma fatty acids

At study entry, plasma concentrations of EPA and DHA did not differ significantly between the 2 groups (**Figure 1**). In the n−3 FAs group plasma values for DHA as well as for EPA were significantly higher at 6 mo compared with pretrial values (Figure 1). The placebo group displayed no significant reductions of DHA or EPA in plasma compared with pretrial values (Figure 1). The rise of DHA levels was larger than that of EPA in the n−3 FAs group (+3.4 percentage units, corresponding to a 2.1-fold rise, and +2.4 percentage units, or a 2.2-fold rise, respectively). Thus, the EPA values were enhanced more than expected from the ratio of orally supplemented fatty acids, ie, EPA:DHA = 0.35:1.

Cytokine and growth factors

Cytokine and growth factor concentrations in unstimulated supernatants did not vary consistently between groups and over time (if not stated otherwise below); hence, we did not subtract these basal concentrations from values for stimulated samples.

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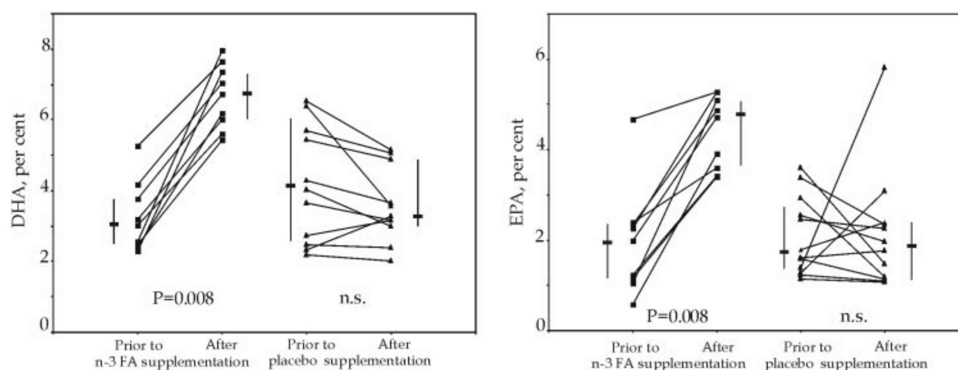

FIGURE 1. Plasma values for docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA) before and after 6 mo of daily supplementation with 1.7 g DHA and 0.6 g EPA (n−3 FAs group) or placebo group. Individual values are flanked by median and 25th–75th percentile values. Significant increases of DHA and EPA were noted in the n−3 fatty acids (n−3 FAs) group after 6 mo.

Interleukin-1β

At baseline, IL-1β release after LPS stimulation did not differ significantly between the 2 groups. n−3 FAs treatment for 6 mo was associated with 35% (mean) lower values of IL-1β when induced by 10 ng LPS/mL and when compared with pretrial values (**Figure 2,** left top panel). The release induced by 1 ng LPS/mL was not significantly lower at 6 mo (**Table 1**).

The placebo preparation group displayed no change of IL-1β release over time (Figure 2, Table 1). The drop of IL-1β induced by 10 ng LPS/mL between pretrial and 6-mo values was significantly larger in the n−3 FAs group compared with the placebo preparation group ($P = 0.039$).

Interleukin-6

At baseline, the 2 groups did not differ significantly as to IL-6 release. IL-6 release from PBMCs, stimulated with 10 ng LPS/mL, from the 9 AD patients given the n−3 FAs preparation were significantly lower (in mean 43%) after 6 mo of treatment (Figure 2, right panel). Also, when the low LPS concentration was used (ie, 1 ng/mL), there were significantly lower IL-6 concentrations at 6 mo (Table 1).

In supernatants from the AD patients who were treated with the placebo preparation, IL-6 releases were also lower at 6 mo compared with pretrial values when induced by LPS at 10 ng/mL (Figure 2, right panel) but not at 1 ng/mL (Table 1). The drop of IL-6 between pretrial and 6-mo values was significantly greater in the n−3 FAs group compared with the placebo group at both 1 and 10 ng/mL of LPS concentrations ($P = 0.028$ and 0.039, respectively).

Tumor necrosis factor-α

At baseline, the 2 groups did not differ significantly as to TNF-α release. TNF-α release was not changed in any of the treatment groups, either when cells were stimulated with 1 or with 10 ng LPS/mL (Table 1).

Interleukin-2, -4, -5, -8, and -10 and interferon-γ

Concentrations of spontaneously released IL-2, -4, -5, and -10 in supernatants were not measurable in our system. Spontaneous IL-8 concentrations were slightly lower at 6 mo than at start in the n−3 FAs and placebo groups (from 1.36 to 0.64 and 1.21 to 0.54 ng/mL, respectively).

At baseline, the 2 groups did not differ significantly as to IL-8 or IL-10 release after LPS stimulation.

There were no differences in the released IL-8 and IL-10 and in supernatants from cells stimulated with 1 or 10 ng LPS/mL after treatment with the n−3 or placebo preparations (Table 1).

LPS did not confer any induced release of IL-2, -4, and -5. As for IFN-γ, low or undetectable concentrations of IFN-γ were not significantly changed after stimulation with 1 or 10 ng LPS/mL.

Vascular endothelial growth factor, epidermal growth factor, basic fibroblast growth factor

No measurable levels of VEGF, EGF, and basic FGF were found in supernatants from cells stimulated with LPS.

Granulocyte colony-stimulating factor

At baseline, the 2 groups did not differ significantly as to G-CSF release. n−3 FAs treatment for 6 mo was associated with lower values of G-CSF when induced by 10 ng LPS/mL and when compared with pretrial values (Figure 2, bottom panel).

The placebo preparation group did not display a significant drop of G-CSF release over time (Figure 2). There were no changes of G-CSF release when induced by 1 ng LPS/mL in any group (Table 1). The drop of G-CSF between pretrial and 6-mo values was not significantly larger in the group treated with n−3 FAs formula than in the placebo preparation group ($P > 0.05$).

Granulocyte-macrophage colony-stimulating factor

At baseline, the 2 groups did not differ significantly as to GM-CSF release. There were no significant changes for any of the groups or LPS concentrations for GM-CSF (Table 1).

Correlation analyses

When relating values for released cytokines (IL-1β, TNF-α, IL-6, -8, and -10, G-CSF, and GM-CSF) to plasma concentrations of DHA or EPA, we found that changes in DHA and EPA for all 21 subjects correlated significantly to changes in IL-1β release (induced by 10 ng LPS/mL) (**Figure 3** A and B). In a subgroup analysis, an enhanced DHA (but not EPA) concentration in the n−3 FAs group correlated significantly to a decreased IL-1β release induced by 10 ng LPS/mL ($r = -0.667$; $P = 0.0499$).

Changes in IL-6 release induced by 10 ng LPS/mL were significantly related to changes in DHA and EPA concentrations for

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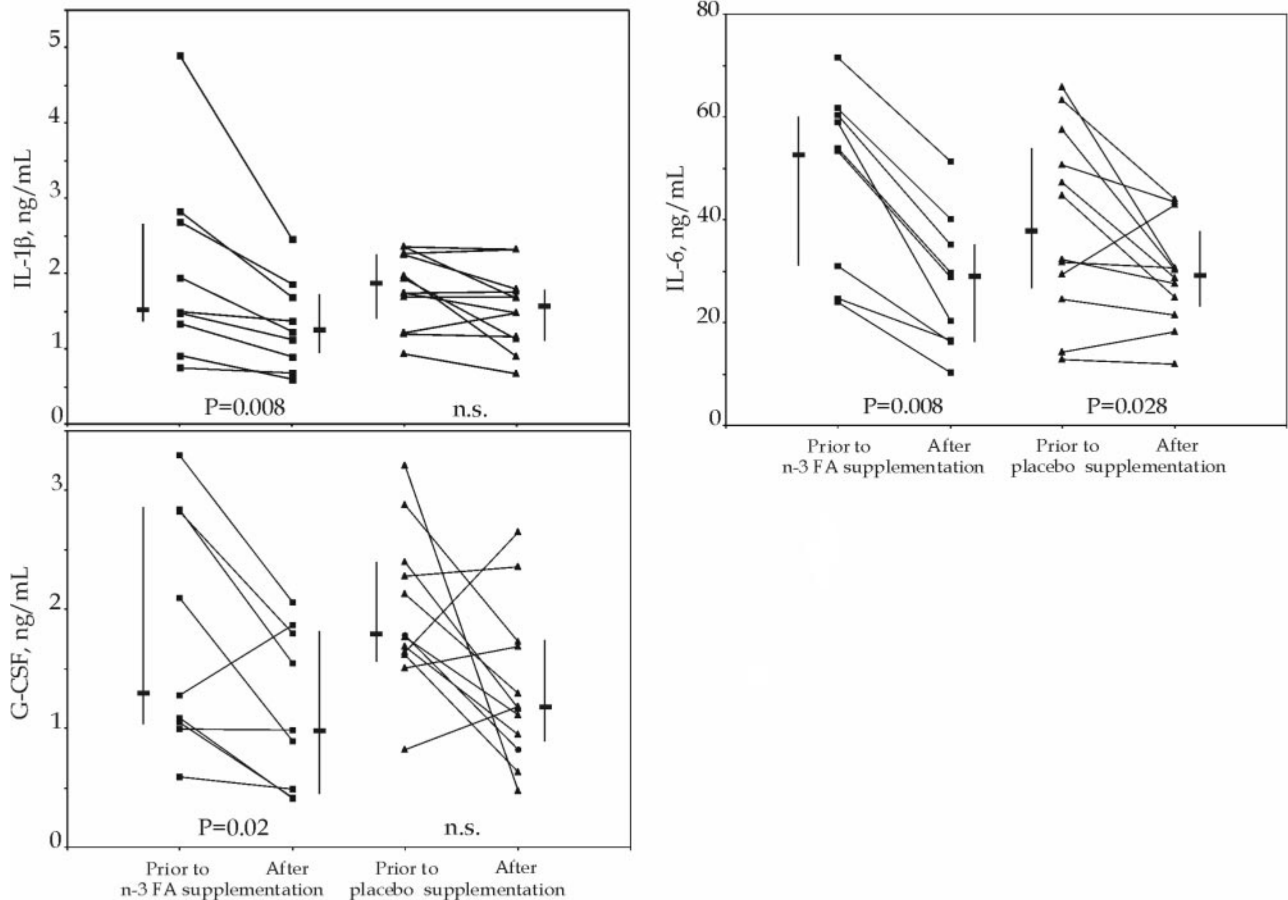

FIGURE 2. Effects of n−3 fatty acid (n−3 FAs) supplementation for 6 mo on interleukin (IL)-1β release from peripheral blood mononuclear cells (PBMCs), on IL-6 release, and on granulocyte colony-stimulating factor (G-CSF) release, all induced by 10 ng lipopolysaccharide/mL. Individual values are flanked by median and 25th–75th percentile values. Significant decreases in IL-1β, IL-6, and G-CSF were noted in the n−3 FAs group after 6 mo, as indicated. Significant differences were noted for changes in IL-1β and in IL-6 between the n−3 FAs group and placebo group ($P = 0.0385$ and $P = 0.0393$, respectively).

all 21 (Figure 3, C and D). Thus, the more DHA (or EPA) concentrations increased, the lower was the release of IL-1β and IL-6. No other significant correlations were found between DHA, EPA, and the cytokines.

Changes of IL-1β related directly to changes of IL-6 for all 21 subjects ($r = 0.509$; $P = 0.018$). Moreover, IL-6 changes related directly to changes of G-CSF ($r = 0.508$; $P = 0.019$) and IL-10 ($r = 0.566$; $P = 0.007$). In a subgroup analysis, IL-6 changes in the n−3 FAs group correlated significantly to IL-10 release induced by 10 ng LPS/mL ($r = 0.767$; $P = 0.016$). No significant correlations were found for any of the other variables.

DISCUSSION

The OmegAD study reported recently that the n−3 FAs supplementation for 6 mo did not reduce the rate of cognitive decline in the 174 AD patients. However, a significant reduction was observed in a subgroup with very mild AD (8). Moreover, there were positive effects on depressive symptoms in non-APOε4 carriers and in non-APOε4 carriers on agitation symptoms (28).

In this part of the OmegAD project, we observed that 6 mo of treatment with a DHA-rich n−3 FAs preparation was associated with clear effects on released cytokines from PBMCs stimulated ex vivo with LPS. We noticed a significant decline of released IL-1β, IL-6, and G-CSF, whereas TNF-α, IL-8, -10, and GM-CSF secretions were not significantly lower at 6 mo. However, the G-CSF response was not significantly different statistically compared with the response of the placebo group.

Although many studies have addressed effects of EPA-rich fish oils on inflammatory reactions, few have investigated effects of DHA-rich fish oils on ex vivo cytokine release. The choice of fatty acid in clinical trials might be of significance because EPA and DHA display partly different modes of action. Thus, DHA binds to the retinoid X receptor, enhances membrane fluidity, and exerts neuroprotection, whereas EPA does not, or at least not to the same extent (10, 26, 27). The choice of n−3 FAs in clinical trials depends also on the EPA:DHA ratio in the targeted organ, eg, the brain, which is rich in DHA but contains virtually no EPA. Nonetheless, EPA and DHA can be metabolized to each other, which is to some extent demonstrated here because EPA plasma levels rose nearly as much as to those of DHA despite administration of 3 times more DHA.

Several studies have examined the effects of fish oils on individual genes and corresponding protein production. In human volunteers 1–6 mo of dietary supplementation with EPA (and some DHA) diminished ex vivo PBMCs production of IL-1β (12, 13, 15, 30), TNF-α (12, 13, 30), and IL-6 (15) in a dose-dependent manner (31). In some studies EPA did not reduce the

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TABLE 1
Effect of 6 mo of treatment with n–3 fatty acids (FAs) and placebo on cytokine release by peripheral blood mononuclear cells[1]

Stimulus/cytokine	FA group: At baseline	FA group: After 6 mo	*P* values	Placebo group: At baseline	Placebo group: After 6 mo	*P* values
LPS (10 ng/mL)						
TNF-α	6.85 (3.83–8.32)	5.52 (4.64–6.64)	NS	7.85 (6.04–8.65)	5.26 (3.28–6.89)	NS
IL-8	7.38 (5.71–10.4)	7.15 (6.45–10.4)	NS	8.47 (6.94–11.4)	7.88 (6.91–8.83)	NS
IL-10	0.42 (0.23–0.46)	0.25 (0.19–0.50)	NS	0.33 (0.25–0.46)	0.23 (0.20–0.40)	NS
GM-CSF	0.56 (0.47–0.62)	0.51 (0.48–0.56)	NS	0.53 (0.47–0.58)	0.54 (0.47–0.56)	NS
LPS (1 ng/mL)						
TNF-α	4.38 (3.03–5.50)	4.62 (3.92–5.44)	NS	4.35 (3.41–7.25)	2.98 (2.55–5.71)	NS
IL-8	7.73 (6.99–9.84)	8.47 (5.64–10.3)	NS	7.36 (6.77–8.86)	7.42 (6.56–8.13)	NS
IL-10	0.08 (0.05–0.22)	0.09 (0.05–0.18)	NS	0.09 (0.06–0.15)	0.09 (0.07–0.16)	NS
GM-CSF	0.52 (0.49–0.61)	0.51 (0.49–0.56)	NS	0.52 (0.47–0.55)	0.51 (0.46–0.54)	NS
IL-1β	1.03 (0.75–1.05)	0.80 (0.74–0.94)	NS	0.89 (0.77–1.08)	0.84 (0.68–0.95)	NS
IL-6	39.9 (23.2–43.0)	23.5 (11.4–28.7)	0.011[2]	25.7 (17.0–35.5)	21.6 (14.6–27.3)	NS
G-CSF	0.88 (0.38–1.42)	0.62 (0.36–0.79)	NS	0.58 (0.53–1.07)	0.61 (0.44–0.91)	NS

[1] Values are medians; 25th–75th percentile values are in parentheses. *P* values are given for changes between baseline and 6-mo values. LPS, lipopolysaccharide; TNF-α, tumor necrosis factor-α; G-CSF, granulocyte colony-stimulating factor; IL, interleukin; GM-CSF, granulocyte-macrophage colony-stimulating factor.

[2] The decrease in IL-6 was significantly larger after 6 mo in the n–3 FAs group than in the placebo group for the 1 ng LPS/mL concentration ($P = 0.028$).

TNF-α secretion (15, 25, 32–34), and after one year of supplementation, TNF-α secretion was still not affected (21). Likewise, some studies did not find an effect on IL-1β or IL-6 releases (25, 34).

Conflicting results exist also demonstrating that low doses of fish oil supplements or of DHA and EPA given to healthy individuals or to children caused a rise in the concentrations of TNF-α, IL-1β, (14, 35), IL-6, and IL-10 secretion (35). Thus, results of EPA supplementation on cytokine release are not unanimous, although a majority indicates attenuations.

As to studies on supplementation with DHA-rich fish oils, one found, using lower DHA doses than we did, a decrease of IL-6 (but not of IL-1β or TNF-α) release after 3 mo (23). Nonetheless, large daily doses of DHA (6 g) for nearly 3 mo decreased TNF-α

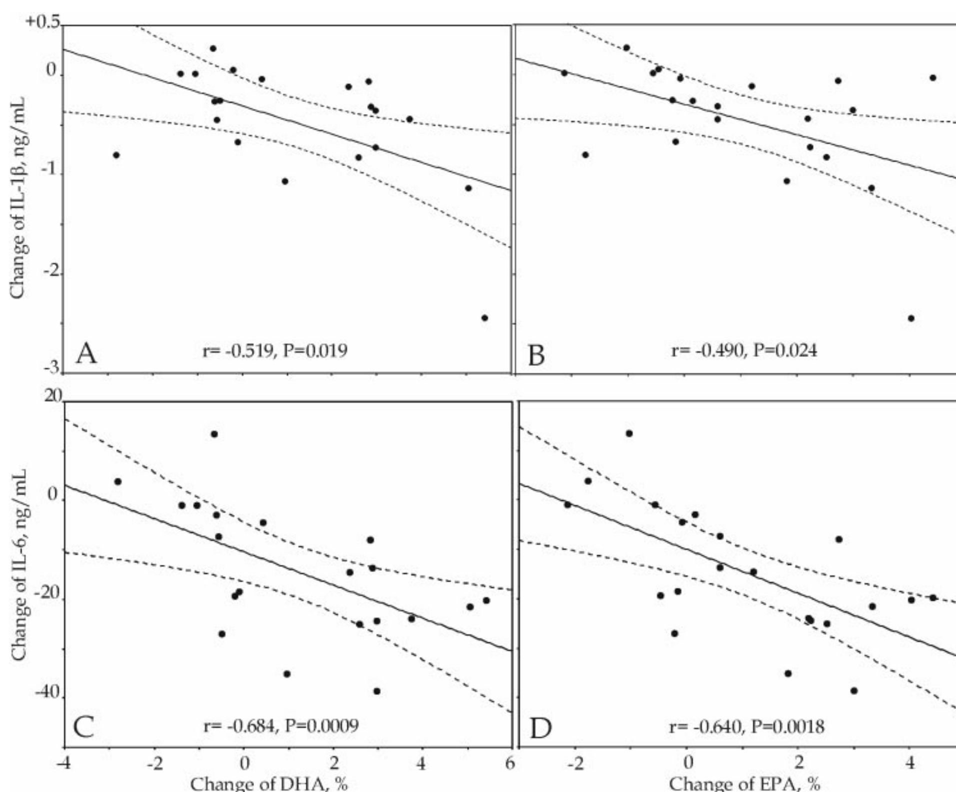

FIGURE 3. Changes in plasma docosahexaenoic acid (DHA; A and C) and eicosapentaenoic acid (EPA; B and D) were related to changes in release of interleukin (IL)-1β (A and B) and IL-6 (C and D) induced by 10 ng LPS/mL. Values are given for all subjects in the study. DHA and EPA changes correlated significantly to changes in IL-1β. Likewise, DHA and EPA changes correlated significantly with changes in IL-6 release.

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and IL-1β release in LPS-stimulated PBMCs (22). The time dependence is of significance because a high dose of DHA (4.9 g/d), but for only one month, had no effects on TNF-α, IL-1β, or IL-6 secretion (25). Thus, our data on IL-1β, IL-6, and TNF-α agree with what has been found previously with several months of supplementation, even when rather low DHA doses were given. Moreover, available data suggest that DHA has anti-inflammatory effects that not only might be time- and dose-dependent but also are comparable with what EPA confers. Similar reactions have been observed in in vitro studies, where TNF-α, IL-1β, and IL-6 production was depressed more with DHA than with EPA (20).

DHA-rich n−3 FAs did not affect the IL-8 and IL-10 release in our study. The same results were observed in a study by Kew et al (25), where supplementation of DHA or EPA had no effect on IL-8 or IL-10 release from LPS-stimulated PBMCs.

The data we have generated on G- and GM-CSF and some other cytokines and growth factors represent novel information in relation to DHA dietary manipulations in humans. As to G-CSF, a neutrophilia-promoting cytokine also involved in AD pathogenesis (36), there was a significant drop of the release in the n−3 FAs group but not in the placebo group. However, because peripheral blood neutrophil counts did not change over time in either the n−3 FAs or the placebo group, we assume that DHA supplementation did not directly interfere with vital host defense mechanisms involving the G-CSF–neutrophil axis. That assumption does not preclude that the drop of G-CSF affected the complex cytokine network in other respects. In contrast, GM-CSF was not significantly changed in the n−3 FAs group, which points to a negligible effect of the DHA-rich preparation on regulatory mechanisms for this cytokine.

Based on the assumption that the plasma FAs concentrations reflect the PBMC FAs composition, we compared plasma fatty acid profiles with the release of IL-6, IL-1β, and TNF-α. Indeed, changes in DHA and EPA for all subjects correlated significantly to released IL-1β. Moreover, we noticed that a decreased IL-1β secretion in the n−3 FAs-treated group correlated specifically to an elevated plasma concentration of DHA, suggesting that the higher the plasma DHA concentration rose, the more the IL-1β release concentration dropped. We also found the IL-6 release for all subjects to be significantly and negatively correlated to changes in DHA and, though less so, to EPA concentrations. Thus, we suggest that it was mainly the administered DHA that affected cytokine release. However, only studies with pure EPA or DHA preparations answer the question of which acid is doing what. Also, the balance between EPA and DHA in a preparation might also be of significance.

Additionally, we compared whether cytokines and growth factors relate to each other. We observed a reduced IL-1β concentration to be related to a decreased IL-6 concentration for all subjects. Furthermore, reductions of IL-6 concentrations were directly related to lower concentrations of the G-CSF and IL-10 concentrations for all subjects. These relations of IL-6 and IL-10 concentrations were also observed in the n−3 FAs subgroup. IL-8 changes did not relate to those of G-CSF. Thus, the coordinated paralleled changes of these cytokines strongly suggest that DHA affected mechanisms common for generation of these cytokines.

Our study emphasizes the close relation between plasma fatty acid concentrations and the cytokine release from blood mononuclear cells as well as the concerted reactions of certain cytokines. However, the question of whether n−3 FAs supplementation is associated with similar attenuation of release of cytokines from AD brain cells remains to be established.

The clinical significance of the cytokines and growth factors analyzed here is further emphasized by the recent report by Ray et al (37), that showed that plasma levels of IL-1, TNF, and G-CSF are strong predictors of development of AD.

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The authors' responsibilities were as follows—IV, TC, JP, YFL, HB, AG, GFI, MEJ, L-OW, and BV: design of the experiment; IV and YFL: collection of data; IV and BV: laboratory analyses; IV, TC, and JP: analysis of data; IV, TC, JP, YFL, HB, AG, GFI, MEJ, L-OW, and BV: preparation of the manuscript; IV, TC, and JP: writing of the manuscript. None of the authors had any conflict of interest.

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