

Effects of n-3 FA supplementation on the release of proresolving lipid mediators by blood mononuclear cells: the OmegAD study^S

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Abstract Specialized proresolving mediators (SPMs) induce resolution of inflammation. SPMs are derivatives of n-3 and n-6 PUFAs and may mediate their beneficial effects. It is unknown whether supplementation with PUFAs influences the production of SPMs. Alzheimer's disease (AD) is associated with brain inflammation and reduced levels of SPMs. The OmegAD study is a randomized, double-blind, and placebo-controlled clinical trial on AD patients, in which placebo or a supplement of 1.7 g DHA and 0.6 g EPA was taken daily for 6 months. Plasma levels of arachidonic acid decreased, and DHA and EPA levels increased after 6 months of n-3 FA treatment. Peripheral blood mononuclear cells (PBMCs) were obtained before and after the trial. Analysis of the culture medium of PBMCs incubated with amyloid- β 1–40 showed unchanged levels of the SPMs lipoxin A₄ and resolvin D1 in the group supplemented with n-3 FAs, whereas a decrease was seen in the placebo group. The changes in SPMs showed correspondence to cognitive changes. Changes in the levels of SPMs were positively correlated to changes in transthyretin. **■** We conclude that supplementation with n-3 PUFAs for 6 months prevented a reduction in SPMs released from PBMCs of AD patients, which was associated with changes in cognitive function.— Wang, X., E. Hjorth, I. Vedin, M. Eriksdotter, Y. Freund-Levi, L.-O. Wahlund, T. Cederholm, J. Palmblad, and M. Schultzberg. **Effects of n-3 FA supplementation on the release of proresolving lipid mediators by blood mononuclear cells: the OmegAD study.** *J. Lipid Res.* 2015. 56: 674–681.

Supplementary key words amyloid β • Alzheimer's disease • clinical trials • docosahexaenoic acid • fish oil • inflammation • lipoxin • nutrition • peripheral blood mononuclear cell • resolvin • fatty acid

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Many diseases of the brain display signs of inflammation, including Alzheimer's disease (AD), the most common type of dementia. The association between inflammation and AD is evidenced from many different disciplines of research. Postmortem studies have revealed increased levels of proinflammatory cytokines in the AD brain (1–3), particularly around the amyloid plaques, and further support is provided by analysis of clinical samples from AD patients, including elevated proinflammatory markers in cerebrospinal fluid (CSF) (4) and plasma/serum (5–7) samples. Epidemiological studies have shown that long-term use of nonsteroidal anti-inflammatory drugs (NSAIDs) confers reduced prevalence of AD (8). However, prospective clinical trials based on NSAIDs have not been successful in reducing the cognitive decline in AD (8, 9).

Consumption of PUFAs, especially the n-3 FAs DHA and EPA, is well known for beneficial effects in the regulation of inflammation (10). PUFAs can modulate the inflammatory response by changing cell membrane fluidity and composition, leading to effects on the function of receptors, and the conductance of ion channels involved in immune activation. In recent years, the concept of resolution of inflammation has received attention due to the discovery of specialized proresolving mediators (SPMs). SPMs are lipid mediators (LMs) derived from PUFAs and play a key role in resolution, in which the tissue is restored by removal of cellular and molecular debris, and regeneration/

Abbreviations: AA, arachidonic acid; A β , amyloid β ; A β ₄₀, amyloid- β 1–40; AD, Alzheimer's disease; CSF, cerebrospinal fluid; LM, lipid mediator; LTB₄, leukotriene B₄; LXA₄, lipoxin A₄; MMSE, mini-mental state examination; PBMC, peripheral blood mononuclear cell; RvD1, resolvin D1; SPM, specialized proresolving mediator; TTR, transthyretin.

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healing. It is, therefore, likely that many of the beneficial effects of PUFAs are dependent on the formation of SPMs. The known SPMs include arachidonic acid (AA)-derived lipoxins, the DHA-derived resolvin D series, neuroprotectins, maresins, and the EPA-derived resolvin E (RvE) series (11). Upon binding to their specific receptors, SPMs downregulate proinflammatory signals and promote the healing process of the tissue (11). Recent studies have indicated that resolution of inflammation is disturbed in AD (12, 13) and AD-related models (14–16). Treatment with one of the SPMs, lipoxin A₄ (LXA₄), has been demonstrated to rescue synaptic loss and reduce AD-like pathologies in transgenic AD mouse models (17, 18). These studies suggest that stimulating resolution of inflammation with SPMs is a promising new strategy for AD therapy.

The OmegAD study, the first prospective large randomized clinical trial using DHA and EPA to treat AD patients, showed that supplementation with these PUFAs could reduce the rate of cognitive decline in very mild AD cases (19). The mechanism of this effect is unknown but may be due to beneficial modulation of inflammation, and therefore, resolution is implicated. Although it is technically difficult to directly investigate the function of microglia, the resident cells with immune function in the brain in live patients, peripheral blood mononuclear cells (PBMCs), represent a useful model to assess the effect of treatments on general aspects of immune function. PBMCs, such as T lymphocytes and monocytes, can in certain circumstances infiltrate into the AD brain, or move along brain vessel walls, and directly participate in the inflammation process (20–25). Moreover, PBMCs can influence amyloid- β (A β) metabolism without infiltration into the CNS (26). A β is the main component of the senile plaques that characterize the brain afflicted by AD and is produced from amyloid precursor protein through the activity of β - and γ -secretase. Of the various lengths produced, amyloid- β 1–40 (A β ₄₀) and amyloid- β 1–42 (A β ₄₂) are the most common forms. A β ₄₀ levels in plasma are higher than those of A β ₄₂ (27). Interestingly, A β ₄₀ was shown to decrease the production of the anti-inflammatory cytokine interleukin (IL)-10 by PBMCs from AD patients (28). IL-10 has been shown to be decreased in the hippocampus of AD patients (13). Thus, the effect of A β ₄₀ on PBMCs indicates its ability to impair anti-inflammatory signaling in a way that is relevant to AD, in addition to its well-known proinflammatory properties.

In the present study, we aimed to investigate whether oral treatment with DHA and EPA in AD patients for 6 months affects the production of SPMs by A β ₄₀-exposed PBMCs, and whether there is a link to the treatment effects on cognition and other related biomarkers.

SUBJECTS AND METHODS

Study design

The OmegAD study enrolled 204 AD patients, with 174 patients completing the trial. This double-blind, placebo-controlled

trial randomized the patients to daily oral treatment of n-3 FA-rich supplementation or placebo for 6 months. The patients in the n-3 FA supplementation group received 1.7 g DHA and 0.6 g EPA (EPAX1050TG; Pronova Biocare A/S, Lysaker, Norway) daily, while patients in the placebo group received daily isocaloric placebo oil containing 1 g corn oil, of which 0.6 g linoleic acids were included. All patients received an equal amount of vitamin E supplementation, which was added into the capsules of EPAX1050TG and placebo. The primary outcome of the OmegAD study has been published previously (19).

In total, 17 patients were involved in the present study, and 15 patients concluded the study (2 dropped out), including 8 subjects (mean \pm SD of age = 72.5 \pm 8.2 years old, 3 females) who received n-3 FA supplementation and 7 subjects (mean \pm SD of age = 70.4 \pm 6.6 years old, 2 females) who received placebo (29). Peripheral venous blood was collected prior to and after the 6 month treatment, in EDTA-coated tubes. Prior to the treatments, there was no difference between the two groups with regard to age, gender, APOE4, cognitive evaluation by mini-mental state examination test (MMSE), plasma AA, DHA and EPA levels, body weight, and intake of aspirin (supplementary Table 1). There was no change in the cell counts of neutrophils, monocytes, and lymphocytes in the blood after the 6 month treatment (29).

All the patients and their caregivers were informed and gave the written consent before being enrolled in the clinical trial. The studies were approved by the Southern Ethical committee at Karolinska Institutet.

Plasma FA measurements

The levels of plasma FAs before and after the treatment were analyzed by gas chromatography using a TR-Frame column of 30 m length \times 0.32 mm diameter \times 25 μ m film (Thermo Scientific, Waltham, MA). The results were presented as the relative abundance of each FA, as described previously (30).

Plasma transthyretin analysis

Plasma samples of all 174 patients finally included in the OmegAD study have been analyzed previously for transthyretin (TTR) levels (31). Standard nephelometrical assay of TTR using Immage system (Beckman Coulter, Bromma, Sweden) was performed in the Laboratory of Clinical Chemistry, Karolinska University Hospital.

PBMC preparation and ex vivo experiment

PBMCs were isolated from the peripheral venous blood by gradient centrifugation using Lymphoprep solution (Nycomed Pharma, Oslo, Norway). The isolated PBMCs contained an average of 15% monocytes and 85% lymphocytes, before and after treatment, in both treatment groups (29). The cell viability was measured by the trypan blue exclusion assay, and the number of viable cells was \sim 96% in both treatment groups (29).

Two millions of isolated PBMCs from each patient were resuspended in 1 ml Hank's balanced salt solution (Life Technologies, Paisley, Scotland, UK), containing CaCl₂ and MgCl₂, but without phenol red. The culture medium was supplemented with 0.0149 M HEPES agent (Life Technologies). Penicillin and streptomycin were added into the culture medium to prevent biological contamination. A β ₄₀ peptide (Bachem, Heidelberg, Germany) was dissolved in DMSO (Sigma, Stockholm, Sweden) and added to the cultures at a final concentration of 7 μ M. This concentration was chosen to be in the range of concentrations used in previous studies (32–36). It was shown that 7 μ M A β ₄₀ did not induce significant cell death (33) but could increase the lipid peroxidation (34) that may influence SPM production. A preparation of 7 μ M A β ₄₀, similar to the one used in the present study, results in

monomers and dimers in the culture medium (33). The same concentration (1%) of the solvent (DMSO) used for A β ₄₀, which was present in the cultures treated with A β ₄₀, was also present in the control (vehicle) cultures. After 22 h incubation at 37°C with 5% CO₂, cell cultures were centrifuged, and the supernatants were collected for further analysis of LMs.

Analysis of LMs

Supernatants from the cell cultures were extracted as described previously (13). Briefly, the supernatants were diluted with methanol and water and then acidified to pH 3.5. C₁₈ columns were preconditioned with methanol and then washed with water. The acidified supernatants were immediately applied to the C₁₈ columns at a speed of 0.5 ml/min, after which the columns were washed with water and then hexane. Subsequently, lipids were eluted with methyl formate, which was further evaporated under a nitrogen gas stream. The residue containing lipid contents was resuspended with extraction buffer supplied with the LXA₄ enzyme immunoassay (EIA) kit (Oxford Biomedical Research, Oxford, MI).

The extracted lipids were analyzed by EIA assays of the SPMs LXA₄ and resolvin D1 (RvD1; Cayman Chemical, Ann Arbor, MI), as well as leukotriene B₄ (LTB₄; Cayman Chemical). The assays were performed according to the manufacturers' instructions.

Statistics

All statistical analyses were performed using the SPSS software. Analyses across the two treatment groups were performed by the Mann-Whitney *U*-test. Wilcoxon signed rank test was applied for analysis of dependent data of paired samples. Correlation analysis was performed with the nonparametric Spearman's rho test. *P* < 0.05 was considered as statistically significant.

RESULTS

MMSE

The primary outcome of the OmegAD study with regard to MMSE scores was previously reported (19). The pretrial MMSE score in the n-3 FA supplementation group, included in the present study, was 26.0 ± 2.9 (mean ± SD), while that in the placebo group was 24.4 ± 1.9 (mean ± SD) (*P* > 0.05 when comparing the two groups). There was a drop by 3.1 (= mean) in MMSE scores in the placebo group after 6 months compared with pretrial scores, whereas there was no change in MMSE scores in the n-3 FA supplement group after 6 months (Fig. 1).

Plasma FAs

Prior to the oral supplementation of n-3 FAs or placebo, there was no difference between the treatment groups with regard to plasma AA, DHA, or EPA (Fig. 2). After 6 months of treatment, the n-3 FA supplement group had significantly lower levels of AA and higher levels of DHA and EPA compared with the baseline levels before treatment (Fig. 2). The plasma levels of AA decreased 0.5 percentage units, while EPA and DHA levels increased with 2.4 and 3.5 percentage units, respectively.

The ratio between changes in EPA and DHA was 2.4%:3.5% = 0.69:1 in plasma, significantly higher than

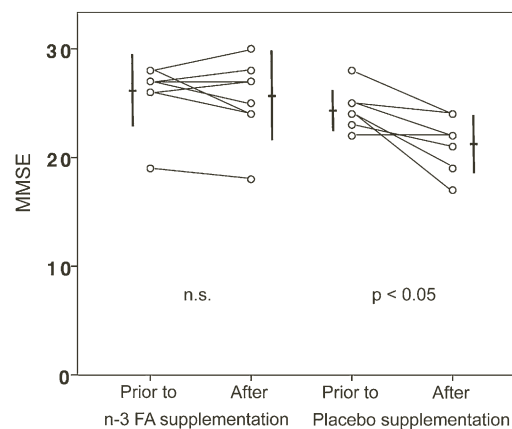


Fig. 1. MMSE scores before and after the 6 month trial. Paired individual values are flanked by mean ± SD. No statistical significance was found in the n-3 FA supplementation group, but a decrease was found in the placebo group after the 6 month trial.

the EPA:DHA ratio (0.6 g:1.7 g = 0.35:1) in the supplemented preparation (EPAX).

There was no change in AA, DHA, or EPA after the 6 month trial in the placebo group (Fig. 2A–C). In this group, however, there was an outlier patient showing significantly increased levels of plasma DHA and EPA after 6 months (Fig. 2). Analysis of all the results in the present study, with and without data from this patient, resulted in the same statistical significances. Thus, all of the results presented in this study included the outlier.

LMs released by PBMCs

LXA₄ and RvD1 levels. Upon A β ₄₀ exposure, the release of LXA₄ and RvD1 from PBMCs was reduced after 6 months in the placebo supplementation group compared with baseline, while in the n-3 FA supplementation group the levels remained similar to that observed at baseline (Fig. 3A, B).

During vehicle (DMSO) conditions, the levels of LXA₄ and RvD1 released from PBMCs did not change after 6 months in any one of the two treatment groups (supplementary Fig. 1A, B). There was no statistically significant difference in LXA₄ or RvD1 levels between DMSO and A β ₄₀ conditions (supplementary Fig. 2A, B).

LTB₄ levels. The levels of LTB₄ in the supernatant of PBMCs showed no difference after 6 months of treatment with either n-3 FAs or placebo, compared with baseline. This was the case in both cultures of PMBCs treated with A β ₄₀ (Fig. 3C) or vehicle (supplementary Fig. 1C). The levels of LTB₄ were lower upon incubation with A β ₄₀ as compared with vehicle (DMSO) conditions (supplementary Fig. 2C).

LXA₄/LTB₄ ratio. In the supernatant of vehicle-treated cells, the ratio between LXA₄ and LTB₄ was not altered after 6 months of treatment with n-3 FAs or placebo (supplementary Fig. 1D). When PBMCs were exposed to A β ₄₀, the LXA₄/LTB₄ ratio in the placebo group was reduced by 13% (mean) compared with baseline but was similar to

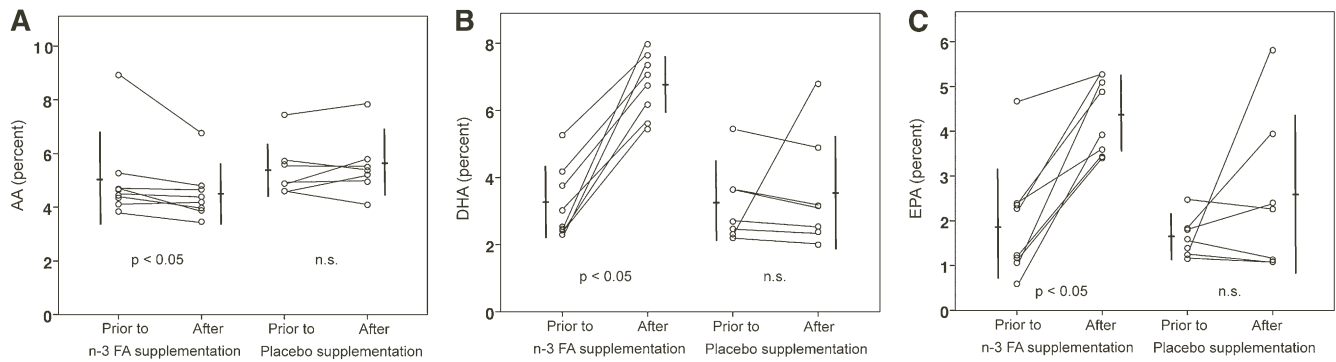


Fig. 2. A–C: Levels of plasma AA, DHA, and EPA before and after the 6 month clinical trial. Paired individual values are flanked by mean \pm SD. Supplementation of n-3 FAs significantly decreased plasma AA levels (A) and increased DHA (B) and EPA (C) levels in plasma. There were no changes over time in the placebo group.

the ratio observed at baseline in the n-3 FA treatment group (Fig. 3D). The LXA_4/LTB_4 ratio was higher upon incubation with $A\beta_{40}$ compared with that in vehicle (DMSO) conditions (supplementary Fig. 2D).

Correlation analysis

Correlation analysis was performed to relate the change in the levels of SPMs from $A\beta_{40}$ -exposed PBMCs before

and after 6 months of treatment with n-3 FA or placebo, with changes in MMSE scores, and plasma FAs and TTR. The data on TTR in all 174 patients have been published previously (31) and showed decreased levels in the placebo group after 6 months, but no significant change in the n-3 FA group. There was no correlation between changes in the levels of SPMs and MMSE scores, neither between plasma levels of AA, DHA, and EPA. A positive

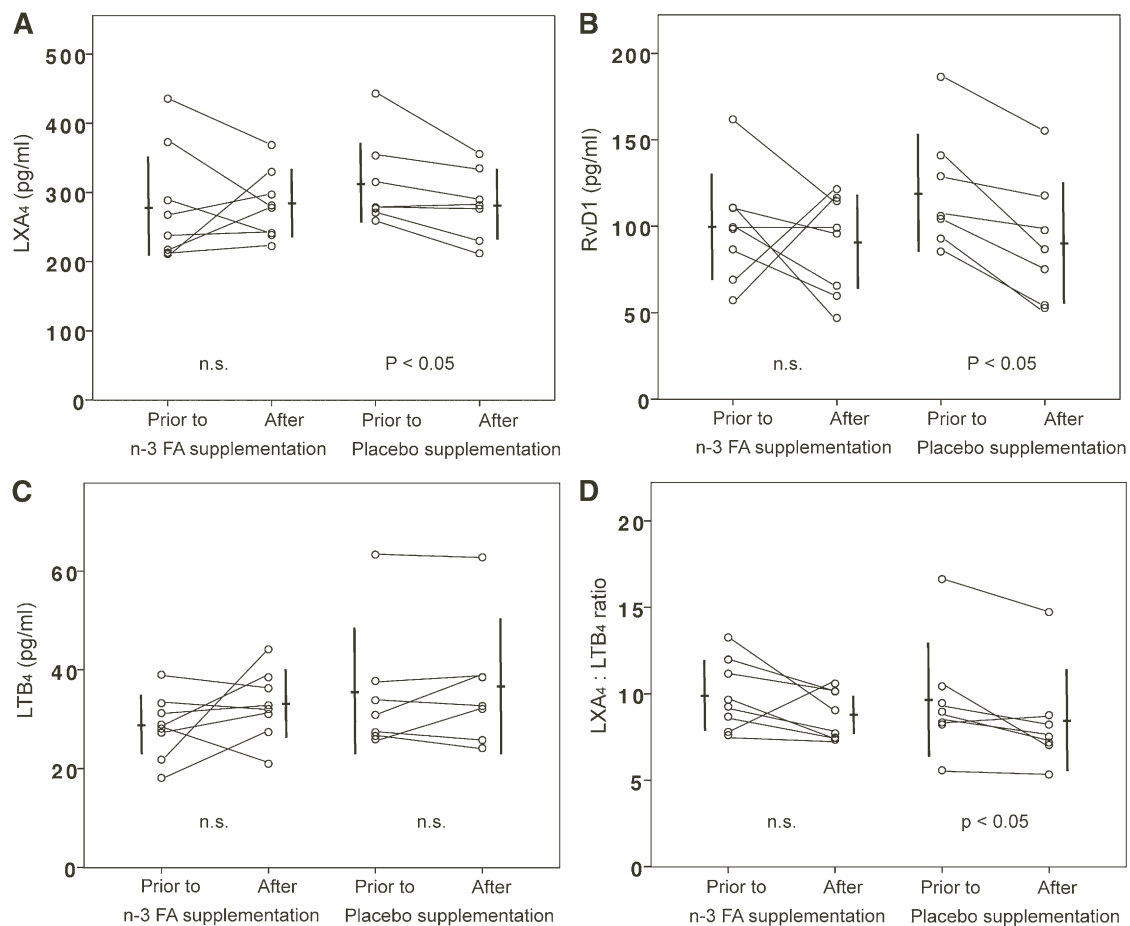


Fig. 3. A–D: Levels of LMs in the medium of PBMCs exposed to $A\beta_{40}$. Paired individual values are flanked by mean \pm SD. A, B: Levels of LXA_4 and RvD1 were unchanged in the n-3 FA supplementation group, but there was a significant decrease in these two SPMs in the placebo-supplemented group ($P < 0.05$). C: Levels of LTB_4 were unchanged in both patient groups over time. D: The ratio between LXA_4 and LTB_4 was not changed in the n-3 FA supplementation group, while it was decreased in the placebo group ($P < 0.05$).

correlation (Spearman's rho test, $r = 0.735$, $P < 0.01$) was found between the change in the levels of LXA₄ and plasma TTR levels (Fig. 4A). The sum of change in the levels of LXA₄ and RvD1 was also correlated to plasma TTR levels, however, to a less extent (Spearman's rho test, $r = 0.556$, $P < 0.05$) (Fig. 4B). No correlation was found between changes in the levels of RvD1 and TTR (Spearman's rho test, $r = -0.021$, $P = 0.94$).

DISCUSSION

In the present study, we report that treatment of AD patients with an oil rich in DHA had a supportive effect on the production of SPMs by PBMCs ex vivo. Upon A β ₄₀ exposure, PBMCs from AD patients of the placebo group secreted less LXA₄ and RvD1 after 6 months of placebo supplementation, compared with the secretion from PBMCs prior to the trial. These data indicate that the ability to produce SPMs by PBMCs decreases with time, during which the disease progresses. Supplementation with n-3 FAs for 6 months prevented this reduction in SPMs, indicating that a defined n-3 FA supplement can hinder age- and AD-related deterioration in proresolving signaling.

In an earlier study on patients with AD or mild cognitive impairment, and individuals with subjective cognitive impairment, we found a positive correlation between the levels of SPMs in CSF samples and the MMSE score (13). Taken together with the prevention of age-related deterioration in MMSE scores observed upon n-3 FA supplementation, the present data indicate that a deficiency in the ability to produce proresolving mediators may play a role in the cognitive decline in AD. To explain if this is due to a direct neurotrophic effect, or by removal of harmful proinflammatory activities, further investigation is needed. An anti-inflammatory effect that is present ex vivo after treatment with n-3 FAs was shown in previous studies in the OmegAD trial, where n-3 FA supplementation reduced the levels of proinflammatory cytokines and prostaglandin F_{2 α} produced by PBMCs under lipopolysaccharides (LPSs) challenge (29, 37).

Analysis of the levels of LTB₄ showed no change, either in the n-3 supplement group or the placebo group. This finding is in line with previous reports in which n-3 FA supplementation did not alter LTB₄ release (38, 39); however, it is contrary to other studies, where LTB₄ release from PBMCs, or whole blood cells, was reduced by n-3 FA supplementation (40, 41). LTB₄ is considered a proinflammatory mediator and may have detrimental effects that are related to AD (42, 43). AA is the precursor for both LXA₄ and LTB₄, and the ratio between these two LMs produced from AA depends on "class-switching" of enzymes involved in the AA cascade (44). The analysis of this ratio produced results that were along the same line as when analyzing LXA₄ alone, showing a decrease in LXA₄/LTB₄ in A β ₄₀-exposed PBMCs of the placebo group during the study period, which was prevented by supplementation with n-3 FAs.

Because SPMs are biosynthesized from PUFAs, supplementation of the precursors may theoretically increase the products. A previous study showed that supplementation with n-3 PUFAs rich in DHA and EPA significantly increased the levels of RvD1, RvE1, and protectin D1 (PD1) in adipose tissue from patients with obesity (45). Assuming that changes in plasma FAs can contribute to FA composition in PBMCs, as reported previously in healthy subjects (46), similar changes in SPMs released by PBMCs were envisaged. In the present groups of patients, the relative levels of plasma AA were decreased, and the relative DHA levels were increased, by the n-3 FA supplementation. However, we did not observe any accompanying increase in LXA₄ and RvD1 levels released by PBMCs. There were no changes in the relative levels of AA or DHA in the placebo group, but the release of LXA₄ and RvD1 from PBMCs was decreased over the 6 months of the study, indicating that without a change in plasma precursor FAs, deterioration in SPM production occurs in AD patients.

Correlation analysis revealed no association between the changes in SPM levels in the culture medium and their precursors in plasma. The OmegAD study was a trial on AD patients, and it has been shown that levels of lipoxygenases (key enzymes involved in SPM biosynthesis) were

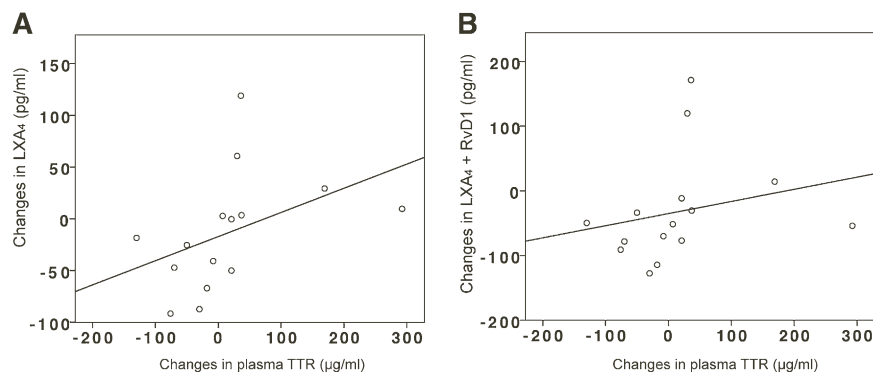


Fig. 4. A, B: Correlation between changes in release of SPMs from A β ₄₀-exposed PBMCs and plasma TTR levels. A: LXA₄ changes were positively correlated to changes in plasma TTR levels (Spearman's rho test, $r = 0.735$, $P < 0.01$). B: The sum of changes in LXA₄ and RvD1 was also correlated to changes in plasma TTR levels (Spearman's rho test, $r = 0.556$, $P < 0.05$).

altered in AD patients (13), thus offering an explanation for the lack of correlation. An implication is therefore that AD patients with an altered capacity to produce SPMs from n-3 FAs may be less sensitive to treatment with the latter. Thus, the levels and functional alterations of lipoxigenases in AD patients need further investigation to understand if and how the ability to produce SPMs is impaired. The trends of changes in SPMs and MMSE scores are similar but lack statistically significant correlation. A possible explanation could be that, unlike in CSF samples, SPM values derived ex vivo from peripheral cells do not reflect the environment in the brain to the same degree. Activities of the immune system have been suggested to be of use as prognostic and diagnostic markers (47), but further investigation is required to determine whether the release of SPMs by PBMCs can be used for this purpose. Moreover, the MMSE scores at baseline revealed that both treatment groups had a modest impairment in cognition, as shown by 26.0 ± 2.9 in the n-3 supplemented group and 24.4 ± 1.9 in the placebo group. Thus, the results presented in this study may not be informative of SPM release from PBMCs obtained from patients with severely affected cognition and a more advanced AD.

A correlation was found between the changes in SPMs secreted by PBMCs and the plasma TTR levels. TTR, originally named prealbumin, was first found to be present in senile plaques (48), and then shown to be able to bind to A β and prevent A β fibrillization (49). There is ample evidence that TTR counteracts the toxic effects of A β in various AD-related models (50–54). LXA₄, and its analog aspirin-triggered LXA₄, have also been shown to ameliorate the detrimental effects of A β in vitro and in vivo (17, 18, 55), probably by increasing phagocytosis, downregulating proinflammatory signaling, and upregulating anti-inflammatory cytokine production (11). RvD1 was also reported to enhance phagocytosis by macrophages (56). Thus, n-3 FA supplementation may favor the capacity of homeostatic functions to handle overabundance of A β , as shown by preserved plasma TTR levels, SPM levels released by PBMCs, and an increased phagocytosis of A β by microglia upon exposure to DHA and EPA in vitro (57).

In conclusion, our study adds further confirmation to the hypothesis that resolution of inflammation is disturbed in AD patients, as shown by the reduced SPM release from PBMCs over time. Supplementation with n-3 FAs prevented this reduction by a yet unknown mechanism, although the increased availability of precursors presents a plausible explanation. The effects of n-3 FA supplementation on SPM release from PBMCs were associated with plasma TTR levels, a marker in plasma for A β clearance, and may have a relation to cognitive status. Whether similar protective effects of n-3 FA supplementation may occur in cells resident in the brain, such as microglia, remains to be further investigated. The n-3 FA supplementation did not increase SPM release from the PBMCs but prevented a reduction in SPM release. A hypothetical impairment, specific for AD, in the enzymatic machinery producing SPMs may blunt the effect of n-3 PUFA treatment and is an important subject of research. Moreover, as the number of

subjects in the present study is limited, firm conclusions will need further confirmation in a larger patient population. A therapeutic strategy using SPMs instead of, or together with, their precursors to treat AD may be further considered. **EU**

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