Effects of Peroral Omega-3 Fatty Acid Supplementation on Cerebrospinal Fluid Biomarkers in Patients with Alzheimer's Disease: A Randomized Controlled Trial—The OmegAD Study

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Abstract

Background: Studies have suggested a connection between a decrease in the levels of polyunsaturated fatty acids (PUFAs) and Alzheimer's disease (AD). We aimed to assess the effect of supplementation with omega-3 fatty acids (n-3 FAs) on biomarkers analyzed in the cerebrospinal fluid (CSF) of patients diagnosed with AD. ‘

Objective: To investigate the effects of daily supplementation with 2.3 g of PUFAs in AD patients on the biomarkers in CSF described below. We also explored the possible correlation between these biomarkers and the performance in the cognitive test Mini-Mental State Examination (MMSE).

Methods: Thirty-three patients diagnosed with AD were randomized to either treatment with a daily intake of 2.3 g of n-3 FAs (n = 18) or placebo (n = 15). CSF samples were collected at baseline and after six months of treatment, and the following biomarkers were analyzed: Aβ 38, Aβ 40, Aβ 42, t-tau, p-tau, neurofilament light (NfL), chitinase-3-like protein 1 (YKL-40), acetylcholinesterase (AChE), butyrylcholinesterase (BuChE), soluble IL-1 receptor type II (sIL-1RII), and IL-6.

Results: There were no significant differences between the groups concerning the level of the different biomarkers in the CSF at baseline. Within the treatment group, there was a small but significant increase in both YKL-40 ($p = 0.04$) and NfL ($p = 0.03$), while the other CSF biomarkers remained stable.

Conclusion: Supplementation with n-3 FAs had a statistically significant effect on NfL and YKL-40, resulting in an increase of both biomarkers, indicating a possible increase of inflammatory response and axonal damage. This increase in biomarkers did not correlate with MMSE score.

Key words: Dementia. Inflammation. MMSE. Neurodegeneration. Neurofilament light. YKL-40.

Abbreviations

ACh: Acetylcholine

AChE: Acetylcholinesterase

AD: Alzheimer's disease

Aβ: Amyloid beta

BuChE: Butyrylcholinesterase

CI: Confidence interval

CSF: Cerebrospinal fluid

DHA: Docosahexaenoic acid

ELISA: Enzyme-linked immunosorbent assay

EPA: Eicosapentaenoic acid

IL: Interleukin

MMSE: Mini-mental state examination

n-3 FA: Omega-3 fatty acid

NfL: Neurofilament light

p-tau: Hyperphosphorylated tau-protein

PUFA: Polyunsaturated fatty acid

sIL-1RII: Soluble interleukin-1 receptor type II

t-tau: Total tau-protein

YKL-40: Chitinase-3-like protein 1

Introduction

Alzheimer's disease (AD) is the most common type of dementia with an estimated prevalence among those aged 60+ of 40.2 per 1000 [1]. Two well studied biomarkers in AD are 1) amyloid β $(Aβ)_{42}$ which, due to the formation of amyloid plaques, has lower detectable levels in the cerebrospinal fluid (CSF) of AD patients compared to controls [2–4], and 2) hyperphosphorylated tau-protein (p-tau), which seems to be a more specific biomarker for AD as compared to the total amount of tau-proteins (t-tau) [5,6]. It is also believed that the formation of $Aβ_{42}$ leads to an increase in p-tau in the CSF [7], supported by the finding of higher CSF-levels of p-tau in AD patients [3,6].

Other Aβ-peptides, such as $Aβ_{38}$ and $Aβ_{40}$, have also been studied, but the findings have not been as conclusive as those for $Aβ_{42}$, with some studies finding a connection with AD [8–11], and others not [4,12,13].

Inflammation is an important part of the neuropathology in AD, characterized by astrocyte proliferation and microglial activation as well as increased levels of inflammatory markers such as pro- and anti-inflammatory cytokines. Thus, cytokines such as interleukin (IL)-1α [14], IL-1β [15] and IL-6 [15,16] are increased in the AD brain. Several studies implicate the role of inflammation and cytokines in the pathophysiology of AD [17–20]. Despite this, findings regarding the levels of these cytokines in the CSF of patients with AD have been discordant, showing either an increase, a decrease or no difference compared to controls [21].

The soluble interleukin-1 receptor type II (sIL-1RII) has been described as a decoy receptor for both IL-1α and IL-1β, since binding to this receptor results in a reduction of biological activity of these cytokines [22,23]. Increased levels of sIL-1RII were described in the CSF of AD patients, leading to a hypothesis that the increase could be a compensatory response to counteract the increased levels of IL-1 [24,25].

More recently, neurofilament light (NfL), a protein indicating axonal damage in the white matter of the brain, was found to be elevated in AD as compared to healthy controls [4,26]. Similarly, chitinase-3-like protein 1 (YKL-40) [4,27], secreted by astrocytes and associated with neuroinflammation [28], is elevated in AD.

The decrease in acetylcholinesterase (AChE) measured in CSF [29–31] is another change that has been seen in the brain of AD patients. Findings regarding butyrylcholinesterase (BuChE) are

more contradictory as it has been found to be increased in the brain tissue of AD patients [32,33], but decreased in the CSF [34,35].

Regarding the different findings in AD, studies have also explored the role of the lipid metabolism in the brain and found a connection between decreased levels of polyunsaturated fatty acids (PUFAs), in particular the two types of omega-3 fatty acids (n-3 FA) docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA), and AD [36,37]. This may be of interest since PUFAs seem to act in a neuroprotective manner through an inhibiting effect on production and a reduced accumulation of both $A\beta_{42}$ and $A\beta_{40}$, as shown in preclinical trials [38,39]. The same effect has been seen with regard to t-tau and p-tau [40], IL-1β [41] and IL-6 [42,43]. The effect on $A\beta_{38}$ has not been studied so far to our knowledge, nor has any studies been conducted regarding the effects of PUFA on NfL, YKL-40, sIL-1RII, AChE or BuChE.

Despite the findings in preclinical trials, little is known about the effects of PUFAs on these biomarkers in the CSF of AD patients. In a previous study, based on the same material as this trial, the effects of PUFAs on IL-6, sIL-1RII, t-tau, p-tau and $A\beta_{42}$ were investigated [44]. There was no significant treatment effect, but a positive correlation was found between increased levels of PUFAs in CSF and sIL-1RII, previously shown to be increased in AD [24,25], and of an inverse correlation with p-tau and t-tau [45]. In addition, there was a correlation between sIL-1RII and $A\beta_{42}$ at baseline [44].

In this study we aimed to investigate the effects of daily supplements with 2.3 g of PUFAs consisting of 1.7 g of DHA and 0.6 g of EPA in AD patients on biomarkers in CSF discussed above. We also explored the possible correlation between these biomarkers and the performance in the cognitive test Mini-Mental State Examination (MMSE).

Materials and methods

Patients

This is a *post hoc* study based on a subgroup of patients recruited for the double-blind randomised-controlled trial, the OmegAD [46]. Between December 13th, 2000, and March 25th, 2004, 204 patients diagnosed with AD were recruited at Karolinska University Hospital, S:t Göran hospital and Danderyd's hospital in Stockholm, Sweden. Initially, the 40 first patients recruited for the study were included for analysis of CSF biomarkers, but due to dropouts, only 33 of them participated in the study.

The patients were randomised to either the omega-3 FA treatment group (n = 18) and received four 1g capsules daily, each containing 0.43 g DHA and 0.15 g EPA, making a total of 2.3 g of n-3 FAs per day (EPAX1050TG, Pronova Biocare A/S, Lysaker, Norway), or the placebo group (n = 15) who instead received treatment with four isocaloric 1 g capsules daily, containing 1 g corn oil (including 0.6 g linoleic acid, an omega-6 fatty acid). CSF samples were collected at baseline and at visit two, after six months of treatment. To ensure compliance with the n-3 FA therapy blood samples were collected for quantification of plasma FAs. The amount of n-3 FAs administered to the omega-3 group roughly equals the Nordic Nutrition Recommendations of dietary reference values [47]. All patients also underwent evaluation of cognitive function using MMSE [48] at baseline and after six months of treatment.

The inclusion criteria for the study were as follows: 1) patients diagnosed with mild to moderate AD according to the classification stated by the Diagnostic and Statistical Manual of Mental Disorders, fourth edition, 2) a score between 15 and 30 on the MMSE scale, and 3) at least three months of treatment with a stable dose of AChE inhibitor, prior to the study start. Further details regarding the materials and methods have been described previously [45,46].

The ethical committee at Karolinska University Hospital approved the study protocol (291.00) and the local ethical committee gave approval for 40 patients to undergo lumbar puncture. Written informed consent was provided by all patients and caregivers.

Sample collection and analysis of CSF biomarkers

Lumbar puncture was performed according to standard methods [44,46] on 33 patients for analysis of the following biomarkers in CSF: AD-associated biomarkers $A\beta_{38}$, $A\beta_{40}$, $A\beta_{42}$, t-tau and p-tau; decomposition biomarkers AChE, BuChE and NfL; inflammatory biomarkers YKL-40, sIL-1RII and IL-6. The n-3 FAs EPA and DHA were also analysed in the CSF samples. The collected CSF samples were frozen for storage immediately after collection and kept in freezers in -70°C. All analyses were performed at the same time.

The concentrations of $A\beta_{38}$, $A\beta_{40}$, and $A\beta_{42}$ were analysed using a commercially available Abeta Triplex immunoassay with electrochemiluminescence detection (MSD, Rockville, MD). Commercially available enzyme-linked immunosorbent assays (ELISAs) were employed for analysis of t-tau and p-tau (Fujirebio, Ghent, Belgium) and IL-6 and sIL-1RII (R&D Systems, Abingdon, UK). The detection range for IL-6 and sIL-1RII was 0.16-10 ng/l and 31.3-2 000 ng/l, respectively. The levels of NfL were analysed using a commercially available NF-Light kit

(UmanDiagnostics, Umeå, Sweden) and the YKL-40 levels were analysed using a commercially available assay (MSD, Rockville, MD). AChE and BuChE activities were analysed as previously described [49].

The extraction of n-3 FAs from the CSF samples was performed according to the methods of Blight and Dyer [50], before they were converted to methyl esters [51]. The n-3 FAs were analysed by a gas chromatographic method designed for detecting low concentrations.

Statistical analysis

Per protocol analyses were performed and the data are presented as mean and 95% confidence interval (CI), unless otherwise stated. Shapiro Wilk's test of normality was performed on all of the variables, as well as visual assessment of histograms and box-plots, showing significance for BuChE in the placebo group, IL-6 in the placebo group, t-tau in the placebo group and p-tau in both the omega-3 and placebo group. Therefore, the non-parametric Wilcoxon signed-rank test was used on the non-normally distributed variables to assess differences from baseline to visit two within the groups, whilst paired student's t-test was used on the rest of the variables, which were normally distributed.

For differences between the groups, unpaired student's t-test was used since all variables showed homogeneity of variance in Levene's test of homogeneity. For the nominal variable gender, chi-2 test was used.

Pearson correlation coefficient was used to analyse correlations between changes in the levels of variables in both the omega-3 and placebo group as well as with changes in the levels of the n-3 FAs and score on the MMSE.

A p-value of < 0.05 was considered statistically significant for all the analyses. The analyses were performed using IBM SPSS Statistics for Windows, version 26 (IBM Corp., Armonk, N.Y., USA).

Results

Regarding the demographic data, there were no significant differences between the groups concerning gender and age (Table 1).

Similarly, there were no differences between the groups concerning levels of the different biomarkers in the CSF, neither at baseline nor after six months of treatment (Table 2). In contrast, there was a significant increase in both YKL-40 ($p = 0.04$) and NfL ($p = 0.03$) within the omega-

3 group from baseline to visit two, and a significant decrease in MMSE score in the placebo group ($p = 0.01$), but not in the omega-3 group (Table 2). Correlation analysis was performed between EPA, DHA, NfL and YKL-40, to assess if the increase in NfL and YKL-40 had any connection with changes in the levels of EPA and DHA in the CSF. The results did not show significant correlations (Table 3). Analysis of correlation between YKL-40, NfL and MMSE scores did not show any significance (Table 3). Descriptive statistics of the n-3 FAs can be found in Table 3, further details have been published previously [45].

Significant correlations between biomarkers are depicted in Fig. 1, and results showing no significance are not shown. There was a positive correlation between AChE and YKL-40, between $A\beta_{38}$ and $A\beta_{40}$, and between t-tau and p-tau in both the omega-3 and placebo group, whilst the positive correlation between AChE and NfL, and between YKL-40 and NfL were only significant in the placebo group. Likewise, the negative correlation between sIL-1RII and both t-tau and p-tau showed significance only in the placebo group (Fig. 1).

Discussion

In this study we analysed several novel biomarkers in the CSF of 33 patients with AD who were randomised to six months of treatment with either n-3 FAs or placebo. We found that treatment with n-3 FAs conferred an increase in the CSF levels of YKL-40 and NfL, two biomarkers found to be elevated in patients with AD [4,26,27], indicating neuroinflammation [28] and axonal damage [26], respectively. This would suggest that n-3 FAs increase the degenerative process in the AD brain and intensifies neuroinflammation, which is opposite of what previous studies have shown, where supplementation with n-3 FA resulted in a decrease of NfL [52,53]. However, neither IL-6 nor sIL-1RII, two proteins also involved in neuroinflammation, nor any of the Aβ-peptides or tau-proteins showed any significant increase. This could indicate that of the different inflammatory pathways that are active, n-3 FA affect some of them but not all, which could be why not all inflammatory markers are elevated, strengthened by the fact that there does not seem to be any correlation between YKL-40 and IL-6 or sIL-1RII.

Whether these findings are of clinical significance is hard to say, though studies have shown that elevated levels of NfL are associated with cognitive impairment [54,55], a finding also seen regarding YKL-40 [56], the difference being that these studies had longer follow up time, ranging from one to six years. Our correlations analyses, however, did not show any significant correlations

between YKL-40, NfL and MMSE score. What we could see on the other hand was that there was a significant decrease of MMSE score in the placebo group, indicating that omega-3 has a protective impact on cognition, despite leading to an increase of YKL-40 and NfL. But since we have a relatively small sample size and a short follow up time, this finding should be interpreted with care.

The neuroprotective effect of PUFAs that has been seen in preclinical trials [38–40,42,43] could not be replicated in this study in the way that we could not see any significant effect of PUFAs on any of the Aβ-peptides, the tau-proteins or AChE and BuChE. It is difficult to say if this is because there is no such effect, or if it is because of the relatively small sample size, since the p-value for some of the biomarkers was low but not significantly so. For instance, one could speculate that since there is a positive correlation between AChE and YKL-40 and YKL-40 showed a significant increase in the omega-3 group and the increase of AChE had a p-value of 0.10 that a larger study population would result in a significant p-value for AChE as well. But further studies are necessary to evaluate if this could be true.

Looking at the correlations, it is possible that the reason that some of the correlations are significant in the placebo group and not in the omega-3 group is because of outliers in both groups. Since removing the outliers could alter the results, we chose to include them in the analyses. But in this case as well, a larger study population might give more significant results since a few outliers will then have less impact on the results. It might be possible to find other correlations significant as well in that case. On the other hand, one could hypothesize that for example, the negative correlation between sIL-1RII and the tau-proteins in the placebo group but not in the omega-3 group is because of the anti-inflammatory features of n-3 FAs [57], where treatment with n-3 FAs leads to lessened inflammation and therefore a decrease of sIL-1RII but does not affect the tau-proteins, thus affecting the correlation between the changes in these proteins.

Another interesting finding is the strong positive correlation between YKL-40 and NfL, which gives further strength to the idea that astrocytic activation is involved in axonal damage, a finding previously seen in other neurodegenerative diseases [58]. Astrocytic activation could also be a reason for the strong correlation between YKL-40 and AChE, since it seems that astrocytes also secrete AChE [59], while it could just as well be because both are markers of inflammation [60].

The main limitation of this study is the small sample size, making it hard to know if the results that are not significant reflects the general population or if it would become significant with more

participants. Other limitations include patients' compliance to the treatment, which could affect the results. This is a general problem when it comes to studies where the patients have to follow a specific treatment regimen for a long time, but since these patients all have varying degrees of memory impairment this could pose as an even more marked problem in this case, though measurements were taken to make sure that the patients followed their treatment plan. The relatively short follow-up period could also have an influence on the results.

In conclusion, we have in this study found that supplementation with n-3 FAs have a statistically significant effect on NfL and YKL-40 levels in the CSF, resulting in an increase of both biomarkers. Despite this, the data would seem to suggest a protective effect on cognition since the MMSE score decreased significantly in the placebo group but not in the treatment group. However, the increase in NfL and YKL-4 did not correlate with the MMSE scores.

Table 1. Demographic data at baseline.

	Omega-3 (n = 18)	**Placebo (n = 15)**	**P-value**
Gender, n (%)			
Male	10 (56%)	9 (60%)	0.80
Female	8 (44%)	6 (40%)	
Age, years (SD)	72 (8)	68 (7)	0.13

SD: Standard deviation

Table 2. Analysis of biomarkers in CSF samples from AD patients at baseline and at 6 months of treatment with PUFAs

Variables	Baseline		6 mo		Baseline-6 mo (Within group)		Baseline-6 mo (Between groups)
	Mean (95% CI)	p-value	Mean (95% CI)	p-value	Mean (95% CI)	p-value	p-value
AChE U/l							
Omega-3	62.7 (47.0–78.5)	0.91	66.9 (51.3–82.6)	0.68	3.61 (-0.77–7.99)	0.10	0.10
Placebo	62.2 (43.7–80.7)		59.7 (39.7–79.6)		-2.53 (-9.07–4.01)	0.42	
BuChE U/l							
Omega-3	22.5 (17.4–27.7)	0.84	21.8 (16.9–26.8)	0.98	-0.56 (-2.93–1.82)	0.63	0.73
Placebo	22.4 (16.1–28.7)		21.1 (15.6–26.7)		-0.53[a]	0.60[b]	
YKL-40 ng/ml							
Omega-3	182 (135–228)	0.98	201 (155–246)	0.66	15.5 (1.10–30.0)	**0.04**	0.25
Placebo	186 (147–225)		188 (144–232)		1.97 (-18.5–22.5)	0.84	
NfL ng/ml							
Omega-3	1.23 (0.86–1.58)	0.35	1.38 (1.06–1.70)	0.11	0.15 (0.02–0.27)	**0.03**	0.24
Placebo	1.15 (0.86–1.24)		1.10 (0.91–1.30)		0.04 (-0.08–0.17)	0.47	
$A\beta_{38}$ ng/ml							
Omega-3	1.38 (1.11–1.66)	0.97	1.53 (1.24–1.82)	0.44	0.12 (-0.02–0.25)	0.08	0.16
Placebo	1.40 (1.15–1.64)		1.38 (1.10–1.65)		-0.02 (-0.18–0.14)	0.79	
$A\beta_{40}$ ng/ml							
Omega-3	4.54 (3.80–5.28)	0.92	4.93 (4.04–5.81)	0.59	0.29 (-0.18–0.76)	0.21	0.30
Placebo	4.66 (4.03–5.29)		4.62 (3.92–5.31)		-0.04 (-0.52–0.43)	0.85	
$A\beta_{42}$ ng/l							
Omega-3	350 (290–409)	0.56	357 (316–397)	0.48	3.33 (-30.3–37.0)	0.84	0.96
Placebo	367 (318–415)		369 (327–411)		2.33 (-27.5–32.2)	0.87	
sIL-1RII ng/l							
Omega-3	82.0 (66.9–97.1)	0.81	81.4 (66.0–96.9)	0.81	-0.70 (-10.1–8.65)	0.88	0.94
Placebo	82.8 (65.6–100)		82.6 (59.8–106)		-0.21 (-11.5–11.0)	0.97	
IL-6 ng/l							
Omega-3[c]	3.19 (2.61–3.76)	0.73	3.12 (2.56–3.69)	0.24	-0.06 (-0.46–0.33)	0.74	0.36
Placebo	3.03 (2.30–3.77)		2.71 (2.25–3.16)		-1.31[a]	0.19[b]	
t-tau ng/l							
Omega-3	558 (433–683)	0.68	563 (439–687)	0.78	0.78 (-18.5–20.0)	0.93	0.59
Placebo	631 (538–723)		620 (530–711)		-0.06[a]	0.96[b]	

Table 2. Analysis of biomarkers in CSF samples from AD patients at baseline and at 6 months of treatment with PUFAs

Variables	Baseline		6 mo		Baseline-6 mo (Within group)		Baseline-6 mo (Between groups)
	Mean (95% CI)	p-value	Mean (95% CI)	p-value	Mean (95% CI)	p-value	p-value
p-tau ng/l							
Omega-3	75.2 (60.7–89.6)	0.87	73.9 (60.1–87.8)	0.90	-1.54[a]	0.12[b]	0.88
Placebo	80.7 (69.5–91.9)		78.0 (66.9–89.1)		-0.63[a]	0.53[b]	
MMSE score[d]							
Omega-3	24.3 (22.4–26.2)	0.63	24.2 (22.3–26.1)	0.06	-0.06 (-1.21–1.10)	0.92	**0.03**
Placebo	23.6 (21.3–25.9		21.6 (19.5–23.7)		-2.00 (-3.45– -0.55)	**0.01**	
EPA ng/ml							
Omega-3	32.4 (25.1–39.8)		82.8 (70.7–94.9)				
Placebo	32.9 (27.3–38.6)		33.4 (23.2–43.6)				
DHA ng/ml							
Omega-3	243 (217–270)		271 (245–297)				
Placebo	237 (200–274)		242 (204–279)				

AD: Alzheimer's disease. **CI:** Confidence interval. **AChE:** Acetylcholinesterase. **Aβ:** Amyloid beta. **BuChE:** Butyrylcholinesterase. **EPA:** Eicosapentaenoic acid. **DHA:** Docosahexaenoic acid. **IL-6**: Interleukin-6. **MMSE:** Mini-mental state examination. **NfL:** Neurofilament light. **p-tau:** Hyperphosphorylated tau-protein. **sIL-1RII:** Soluble interleukin-1 receptor type II. **t-tau:** Total tau-protein. **YKL-40:** Chitinase-3-like protein 1.

- **a.** Z-score based on positive ranks.
- **b.** Calculated through Wilcoxon signed-ranks test.
- **c.** N = 17 due to missing values.
- **d.** 0 - 30 points.

Table 3. Correlations of YKL-40 and NfL levels in CSF samples from AD patients to levels of PUFAs and MMSE scores.

	YKL-40		NfL	
Omega-3 (n=18)	**Pearson's r**	**p-value**	**Pearson's r**	**p-value**
DHA	0.22	0.38	0.08	0.74
EPA	0.03	0.91	0.16	0.54
MMSE	0.33	0.19	0.27	0.27
Placebo (n=15)				
DHA	0.09	0.77	0.10	0.73
EPA	-0.01	0.98	-0.08	0.77
MMSE	0.17	0.55	0.28	0.31

AD: Alzheimer's disease. **DHA:** Docosahexaenoic acid. **EPA:** Eicosapentaenoic acid. **MMSE:** Mini-mental state examination. **NfL:** Neurofilament light. **YKL-40:** Chitinase-3-like protein 1.

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Figure legend

Fig. 1 A – G.

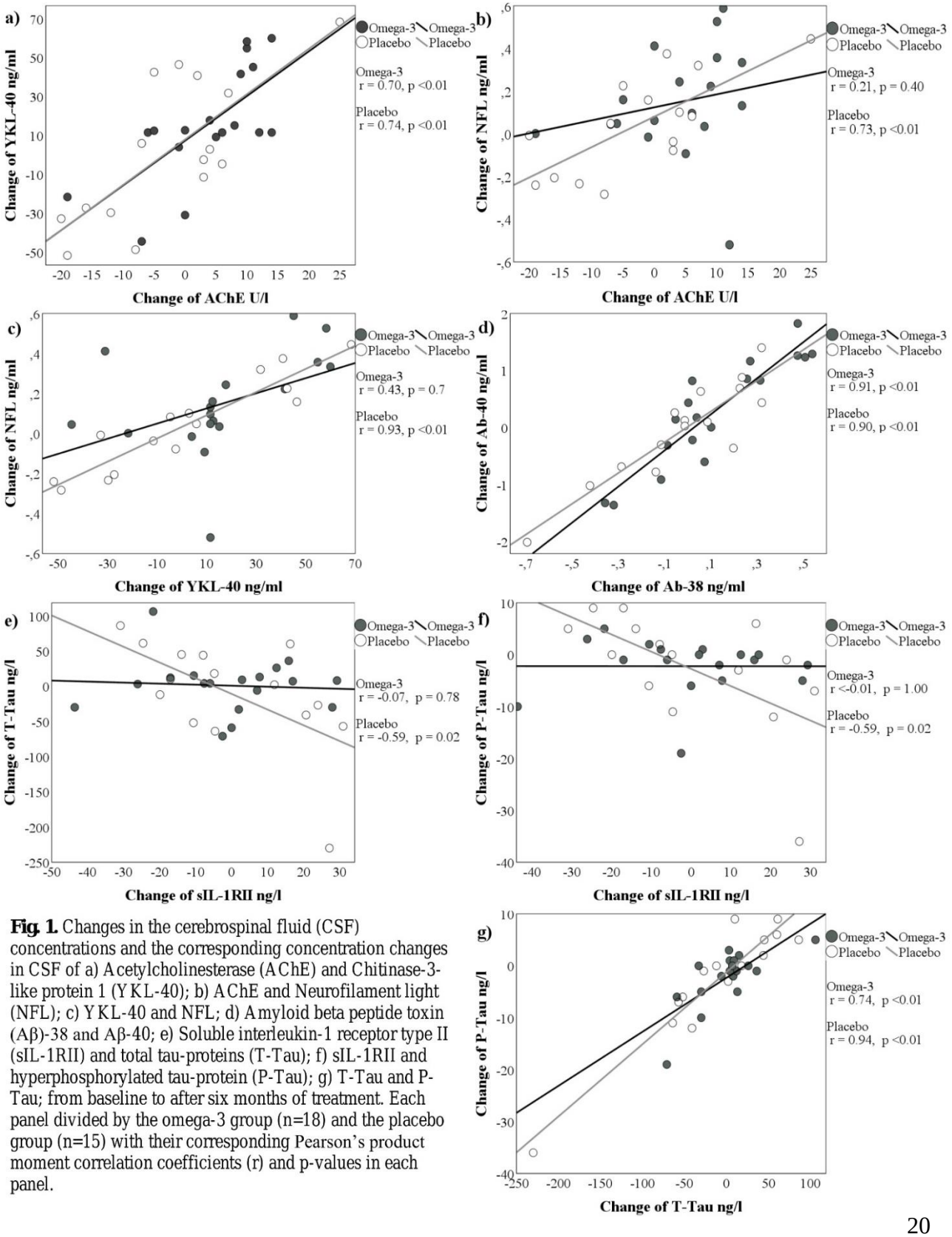

Fig. 1. Changes in the cerebrospinal fluid (CSF) concentrations and the corresponding concentration changes in CSF of a) Acetylcholinesterase (AChE) and Chitinase-3-like protein 1 (YKL-40); b) AChE and Neurofilament light (NFL); c) YKL-40 and NFL; d) Amyloid beta peptide toxin (Aβ)-38 and Aβ-40; e) Soluble interleukin-1 receptor type II (sIL-1RII) and total tau-proteins (T-Tau); f) sIL-1RII and hyperphosphorylated tau-protein (P-Tau); g) T-Tau and P-Tau; from baseline to after six months of treatment. Each panel divided by the omega-3 group (n=18) and the placebo group (n=15) with their corresponding Pearson's product moment correlation coefficients (r) and p-values in each panel.