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Effect of supplementation with long-chain ω-3 polyunsaturated fatty acids on behavior and cognition in children with attention deficit/hyperactivity disorder (ADHD): A randomized placebo-controlled intervention trial ☆

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ABSTRACT

Objective: To determine whether supplementation with the long-chain omega-3 polyunsaturated fatty acids eicosapentaenoic (EPA) and docosahexaenoic acid (DHA) affects behavioral symptoms and cognitive impairments in children 6–12 years of age diagnosed with attention-deficit/hyperactivity disorder (ADHD).

Study design: The randomized, double-blind placebo-controlled 16 weeks trial was conducted with 95 children diagnosed with ADHD according to DSM-IV criteria. Behavior was assessed by parents, teachers and investigators using standardized rating scales and questionnaires. Further outcome variables were working memory, speed of information processing and various measures of attention. For a subgroup of 81 participants, erythrocyte membrane fatty acid composition was analyzed before and after the intervention.

Results: Supplementation with the omega-3 fatty acid mix increased EPA and DHA concentrations in erythrocyte membranes and improved working memory function, but had no effect on other cognitive measures and parent- and teacher-rated behavior in the study population. Improved working memory correlated significantly with increased EPA, DHA and decreased AA (arachidonic acid).

1. Introduction

Attention-deficit/hyperactivity (ADHD) disorder is one of the most prevalent developmental childhood disorders with a worldwide prevalence rate of 5.29% [1]. It is characterized by the inability to concentrate over a long period of time, to pay attention to tasks and to control impulsive and hyperactive behavior [2,3].

Despite convincing evidence for the efficacy of pharmacologic interventions such as methylphenidate (MPH) on ADHD symptom reduction, there is substantial interest in other evidence-based treatment procedures [4].

Not all children experience symptom reduction by pharmacologic treatment with methylphenidate. Although the responder rate to MPH is high (65–70%), supplementation with omega-3 polyunsaturated fatty acids might be an option for non-responders or treatment-resistant children [5]. In addition omega-3 fatty acids could be an alternative for families concerned about adverse effects caused by stimulant medication such as loss of appetite, stunted growth and increased blood pressure and heart rate [6].

During the last years the effect of nutrition on behavior and learning problems in children has attracted the attention of parents, educators and therapists. In this context, research has focussed on long-chain polyunsaturated fatty acids (LC-PUFAs), especially the n-3 fatty acids eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), as a possible treatment option for ADHD [7–9].

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Trial Registration: ClinicalTrials.gov: NCT 01055119 http://clinicaltrials.gov.

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Long-chain polyunsaturated fatty acids of the omega-3 series (EPA, DHA) and omega-6 series (AA; arachidonic acid) cannot be synthesized de novo by humans. They must be provided directly by the diet. Alternatively, they can be synthesized from the short-chain precursor molecules alpha-linolenic acid for the omega-3 series or linoleic acid for the omega-6 series. Omega-3 LC-PUFAs are found in high amounts in marine algae and oily marine fish and seafood such as tuna, mackerel and salmon. Due to the preference of meat and meat products, vegetable oils rich in omega-6 fatty acids and processed foods containing those vegetable oils, the uptake of omega-6 LC-PUFAs exceeds the provision with omega-3 LC-PUFAs, especially in Western societies [10,11].

PUFAs are found in high amounts in the phospholipids of neuronal membranes. Due to their chemical structure, they increase membrane fluidity and alter the function of integral and membrane-bound proteins such as enzymes and transporters [7,10,11].

Experiments with alpha-linolenic deficient rats result in reduced vesicular dopamine and serotonin pools in the frontal cortex [12]. Since these neurotransmitter systems are involved in the etiology of neuropsychiatric diseases such as depression, dementia and ADHD, supplementation with omega-3 fatty acids might result in symptom reduction.

Studies comparing children with ADHD to healthy control groups, report that children affected by the disorder have lower LC-PUFA levels in erythrocyte membranes and plasma than healthy controls [13–16]. Subsequently a limited number of controlled randomized supplementation trials were conducted to examine the efficacy of LC-PUFA supplementation on ADHD symptom reduction.

Results however are inconsistent and the benefit of PUFA supplementation on behavior and cognition needs further investigation. While most studies use standardized parent- and teacher-rated questionnaires for the selection of the study population, investigators not always apply all DSM-IV criteria for syndrome diagnosis. Studies also vary in supplement composition and trial duration. Some of the randomized controlled studies used fatty acid profiles as biomarkers for intervention efficacy and study compliance [17–22].

Results concerning the effect of PUFA supplementation on behavioral ratings are mixed [8,9,23]. Gillies et al. found little evidence for parent- and teacher-rated symptom reduction in children and adolescents with ADHD in their systematic review including 13 trials with 1011 participants [23].

Two meta-analysis of randomized controlled intervention trials found that supplementation with LC-PUFAs results in small but significant improvements of parental ADHD symptom ratings [24,25].

Besides behavioral problems, children with ADHD exhibit various cognitive impairments. Conducting a meta-analysis Martinussen et al., found evidence for working memory impairments in children with ADHD [26].

The effect of PUFA supplementation on cognition in children with ADHD was investigated in several controlled trials. In a study conducted by Hirayama, visual short term memory and continuous performance improved in the control group but not in children consuming DHA enriched foods [27]. Sinn et al. showed that provision with an EPA, DHA, GLA (gamma-linolenic acid; omega-6) supplement led to an increased ability to switch and control attention [28].

The present double-blind placebo-controlled intervention study was conducted to investigate the effect of omega-3 fatty acid supplementation on behavior and cognition in children with ADHD. Inclusion of participants followed DSM-IV criteria and a wide variety of cognitive and behavioral as well as biochemical measures were employed to detect significant changes caused by the intervention.

2. Materials and methods

2.1. Study design

The study was a randomized, double-blind placebo-controlled trial involving parallel treatment for 16 weeks.

2.1.1. Randomization and blinding

A computer-generated random sequence was used to allocate the participants either to the supplement or the placebo group. Participants, parents and those assessing outcome measures were blind to the intervention condition. Blinding was maintained until data analysis was completed.

2.2. Participants and procedure

Children of both sexes 6–12 years of age, meeting DSM-IV criteria for the ADHD combined subtype (hyperactive–inattentive), the primarily inattentive or the hyperactive/impulsive subtype were included in the trial.

2.2.1. Questionnaires

FBB ADHS parent-rated and teacher-rated questionnaires (DISYPS-II) were used for participant selection. Each questionnaire comprises 20 items corresponding to the ICD-10 and DSM-IV diagnostic criteria for ADHD. The items add up to the total score and the 3 subscale scores for attention deficit (9 items), hyperactivity (7 items) and impulsivity (4 items). Each of the items has to be answered on a 4-point scale ranging from "not at all true" (0) to "very much true" (3) with higher scores indicating greater ADHD-related behavior. Participants had to show at least six out of nine symptoms of inattention (score 2 or 3) and/or six out of eleven symptoms of hyperactivity/impulsivity (score 2 or 3) [29]. In addition the maladaptive behavior had to be inconsistent with the developmental level and cause problems in two major settings such as home and school. The onset of problems had to occur before age 7 and persisted for at least 6 month prior to diagnosis.

2.2.2. Recruitment

Parents of children diagnosed with, or suspected of ADHD were informed about the possibility to participate in the study by pediatricians, child psychiatrists and other health professionals such as psychologists, speech therapists and physiotherapists. Teachers specifically qualified to recognize learning disabilities and developmental childhood disorders were asked to inform concerned parents about the study. Leaflets outlining the study were handed out to various support groups and distributed at community centers. Furthermore children were recruited through an advertisement in a free of charge regional newspaper specifically addressing parents.

Study eligibility, pre- and post-behavioral ratings and cognitive functions were assessed by child psychologists during four visits at the Division of Social Pediatrics and Child Neurology University Children's Hospital, Ulm, Germany.

2.2.3. Visit 1 and 2 (pre-intervention)

An extensive anamnestic interview with the child, already diagnosed (38 children) or suspected of having ADHD (72 children), and the caretakers was used to address behavioral symptoms and explore the social and medical background of the child. General cognitive ability (IQ) and attentional performance were assessed by the psychologist. In addition the behavior of the child during the study visits was rated by the psychologist using four symptom-specific items (hyperactivity, attention, impulsivity and

self-reflexive working) on a four level scale. All parents were asked to complete a background questionnaire assessing family history, childhood diseases as well as psychological/ psychiatric problems of the child.

2.2.4. Visit 3 (pre-intervention)

Only children fulfilling the DSM-IV criteria were invited to study visit 3. Parents and children were informed about the results of the preceding testing sessions. A medical examination including height, weight, blood pressure, pulse measurements, auditory and visual testing, cardiovascular status and gross and fine motor skills was carried out by a pediatrician. This excluded the possibility that the behavioral impairments were caused by other conditions such as auditive and/or visual problems. A blood sample for fatty acid analysis of erythrocyte membranes was taken by the pediatrician.

The families were asked to fill out a questionnaire addressing omega-3 rich fish and seafood consumption (yes/no) during the previous four weeks.

Capsules and intake protocols for the entire intervention period were handed out to the parents. Children and caregivers were instructed about supplement use and asked to return unused capsules and intake protocols at the post-intervention visit.

2.2.5. Visit 4 (post-intervention)

After 16 weeks of intervention children and caretakers returned to the study site. Behavior had again been rated by parents and teachers completing the described standardised questionnaires approximately one week before the appointment.

Post-intervention behavioral ratings and cognitive assessments were repeated by the same professional who had done the pre-intervention testing.

The families were again asked to fill out the fish and seafood questionnaire addressing omega-3 rich fish and seafood consumption (yes/no) during the previous four weeks.

A post-intervention blood sample was taken for biochemical analysis.

Exclusion criteria were IQ $\leq$ 70, use of stimulant medication and other psychoactive medication as well as fatty acid supplements used within the previous 6 months. Children with allergies against fish or fish products were also excluded from participation.

Parents/caregivers of 334 children inquired about study participation. All parents/caregivers were contacted and informed about the investigation. If children had not been previously diagnosed according to DSM-IV standards by a psychologist, parents and teachers were provided with the appropriate questionnaires (DISYPS-II). If both teacher and parent ratings were inconspicuous about the diagnostic procedure was not carried any further and the child was excluded from participation.

Patients meeting DSM-IV diagnostic criteria of ADHD by scoring above the norm in at least 6 out of 9 criteria for attention deficiency (DISYPS-II parent- and/or teacher-rated) were invited to attend two more testing sessions. If only either the parent or the teacher rating was above the norm, the child was further assessed by a trained professional who decided on participation based on behavioral observations and objectively assessed attentional ratings.

The study was explained to 334 interested families. Of those 92 declined participation: in 17 cases families declined participation due to the possibility of obtaining placebo, 5 families had objections against pork gelatine, 2 children were unable to swallow capsules, 11 children were not willing to participate and 57 families gave no explanation.

Two hundred forty two children were assessed on the basis of the study criteria. Of those 132 did not meet the study criteria for the following reasons: 62 children were not fulfilling the DSM-IV criteria, 35 were taking stimulant medication, 13 did not fulfill the age requirement, in 8 cases symptom severity did not permit study participation, 7 children were already taking a PUFA supplement, 5 children had an IQ below 70 and 2 reported that they were allergic to fish.

One hundred ten children were included in the study population after the medical examination. All but 3 children were stimulant medication naïve.

Thirteen (12%) children did not return to the follow-up assessment. In two cases the families were lost to follow-up. Eleven discontinued the intervention. Of those: five children had problems swallowing the provided capsules. In 4 cases symptom severity required stimulant medication. Two families were unable to cope with the study protocol.

Ninety seven children, 75 boys and 22 girls completed the intervention and returned for post-intervention testing.

Two participants of the intervention group were excluded post-hoc. According to the intake protocols (compliance data were available from 93 participants) one child had taken only 57% of the provided capsules. The fatty acid profile of a second child showed no increase of the EPA and DHA concentrations at the end of the intervention period (Fig. 1).

Child psychiatrists informed the caretakers of 56 participants about the possibility to participate in the study. Eighteen families were informed by pediatricians or general practitioners. Only 21 children were recruited via the community (teachers, leaflets, advertisements).

2.3. Behavioral assessment

At baseline and after the 16 weeks intervention period, behavior was assessed by parents and teachers using the following standardized questionnaires, describing different aspects of child behavior

FBB ADHS parent-rated and teacher-rated questionnaires (DISYPS-II): Total score and the 3 subscale scores for attention deficit (9 items), hyperactivity (7 items) and impulsivity (4 items).

Symptom scores (attention, hyperactivity, impulsivity, hyperactivity/impulsivity, total score) were obtained before and after the intervention. They represent the sum of the individual item scores ranging from 0 to 3 divided by the number of items.

Child Behavior Checklist 4–18 y (CBCL; German version of the Child Behavior Checklist): The first part of the parent questionnaire assesses different abilities such as academic performance and adaptive functioning of the child/adolescent. The second part consists of 120 items assessing behavioral, emotional and somatic problems. These items are subsumed in the following 8 syndrome scales: Withdrawal, Somatic Problems, Anxiety/Depression, Social Problems, Thought Problems, Attention Problems, Delinquent Behavior and Aggressive Behavior. Withdrawal, Somatic Problems, Anxiety/Depression comprise the Internalizing Domain, Delinquent and Aggressive Behavior the Externalizing Domain. The Total Score comprises all 8 syndrome scales. Results of all scales and domains are gender- and age-adjusted t-scores. [30].

Teacher's Report Form 5–18 y (TRF; German version of the Teacher's Report Form): The TRF is used to obtain the teacher's perception of the child's behavioral and emotional problems as well as the academic performance. The 113 problem behavior items comprise three broad-band scales: Internalizing, Externalizing and Total Problems and eight syndrome scales: Withdrawal, Somatic Problems, Anxiety/Depression, Social Problems, Thought Problems, Attention Problems, Delinquent Behavior and Aggressive Behavior. Total raw and t-scores are available for Internalizing Behavior, Externalizing Behavior and Total Behavior Problems and each of the eight syndrome scales [31].

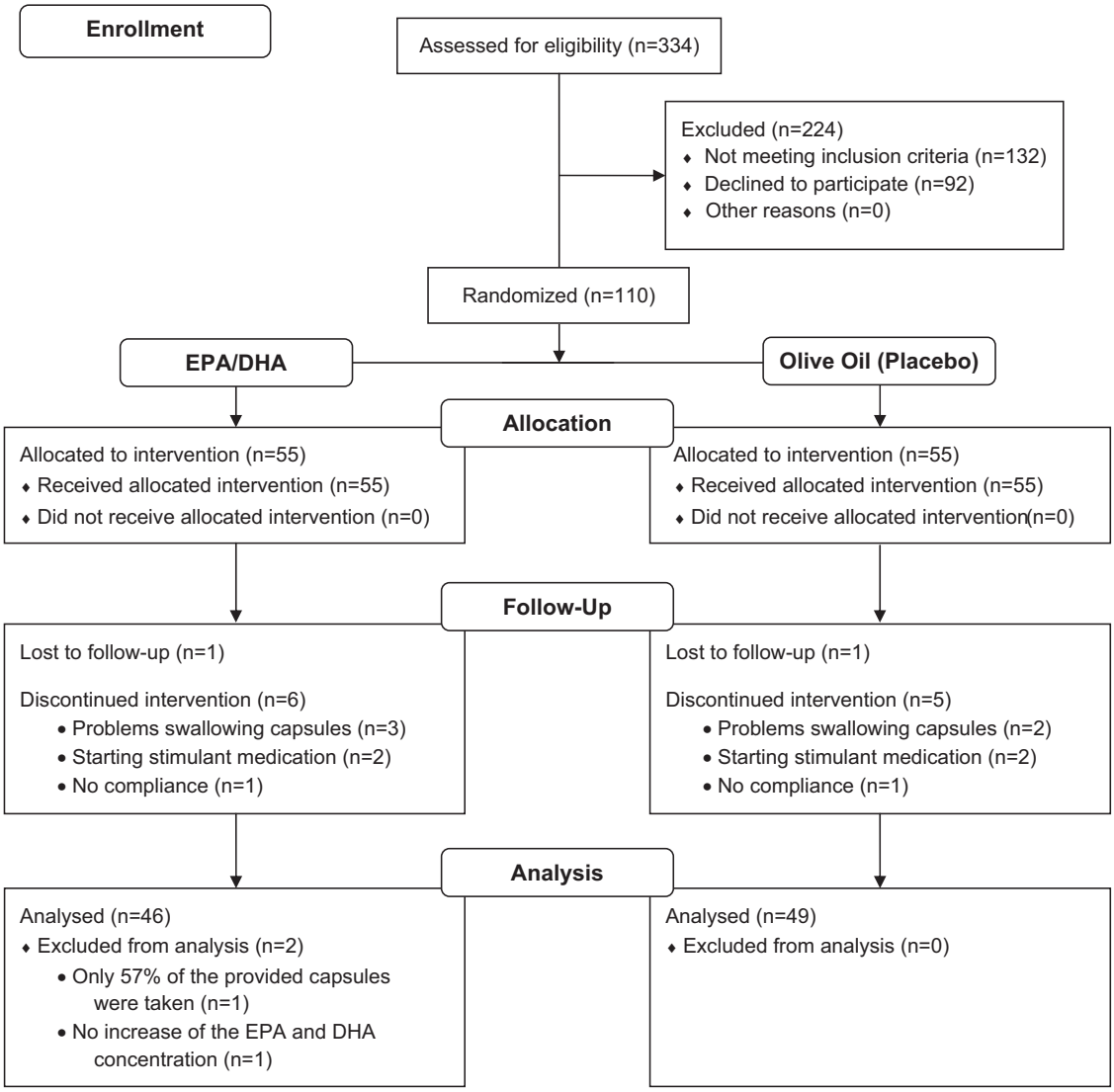

Fig. 1. Flow of participants.

2.4. Cognitive assessment

2.4.1. Cognition (HAWIK-IV)

General cognitive ability was estimated using the Hamburg Wechsler Intelligence Scales for Children–IV [32]. The test assesses cognition of children and adolescents 6.0–16.11 y.

The HAWIK-IV intelligence test comprises four cognitive domains: Verbal Comprehension Index, Perceptual Reasoning Index, Working Memory Index and Processing Speed Index. Full scale IQ is derived from the results of the four domains.

2.4.2. Working memory (HAWIK-IV)

The Working Memory Index Score comprises the performance of the two subtests Digit Span and Letter Number Sequencing. The Digit Span subtest consists of the Digits Forward and the Digits Backward tasks. The Digits Forward task requires the child to repeat numbers in the same order as read aloud by the examiner. The Digits Backward task requires the child to repeat the presented numbers in the reverse order. The Letter–Number Sequencing subtest requires the child to repeat a sequence of letters and numbers and recall the numbers in ascending order and the letters in alphabetical order.

2.4.3. Speed of information processing (HAWIK IV)

The Speed of Information Processing Index Score comprises the HAWIK Number-Symbol and the Symbol Search subtests

The Number-Symbol test requires the child to copy symbols that are paired with simple geometric shapes or numbers. The task has to be accomplished in a limited time.

Symbol-Search test requires the child to scan a search group and indicate whether the target symbol matches any of the symbols in the search group within a specified time limit (2 min).

2.4.4. Attention (KITAP/TAP)

Two age-standardized computerized test batteries, the “Test-batterie zur Aufmerksamkeitsprüfung für Kinder” (KITAP 6–10 y) and the “Testbatterie zur Aufmerksamkeitsprüfung” (TAP 10-18y) were used as measures for attentional performance [33,34]. The different subtests are measures for inhibition (GoNoGo) (KITAP/TAP: reaction time, errors, omissions), divided attention (KITAP/TAP: reaction time, errors, omissions), flexibility (KITAP: total score, reaction time, errors), distractibility (KITAP: reaction time with/without distractors, total score reaction time, errors with/without distractors, total score errors, omissions with/without distractors, total score omissions) and sustained attention (KITAP: reaction time, errors, omissions).

Table 1
Sample demographics.

	EPA/DHA	Placebo	Total
Participants (*N*)	46	49	95
Age (y), Mean ± SD	8.90 ± 1.48	8.92 ± 1.24	8.91 ± 1.35
IQ Estimate, Mean ± SD	99.89 ± 9.35	100.45 ± 9.29	100.18 ± 9.27
Male, *N* (%)	35 (76%)	39 (79%)	74 (77.9%)
Female, *N* (%)	11 (24%)	10 (21%)	21 (22.1%)
ADHD subtype			
Combined, *N* (%)	21 (45.7%)	20 (40.8%)	41 (43.2%)
Inattentive, *N* (%)	24 (52.2%)	28 (57.1%)	52 (54.7%)
Hyperactive/Impulsive, *N* (%)	1 (2.2%)	1 (2.0%)	2 (2.1%)

2.5. Biochemical measures

2.5.1. Erythrocyte fatty acid profiles

Blood samples for fatty acid analysis were collected in EDTA tubes. Samples were spun within one hour after collection and the erythrocytes were frozen at -80 °C until further analysis.

Analysis of erythrocyte fatty acid composition was carried out by Omegametrix, Martinsried, Germany, as described [35]. Results are expressed as fatty acid percentage of total identified fatty acids after response factor correction.

2.6. Supplement

Active treatment consisted of two soft gelatine capsules providing a daily dose of 720 mg omega-3 fatty acids (600 mg EPA, 120 mg DHA) and 15 mg of vitamin E as antioxidant. Placebo treatment consisted of two olive oil-containing capsules per day. Capsules were provided as blister packs. Supplement and placebo were provided by Merck Selbstmedikation (Darmstadt, Germany)

2.7. Compliance

Compliance was defined as taking more than 168 capsules (75% of the provided 224 capsules; two capsules per day for 16 weeks). Using a protocol sheet, the daily capsule intake was recorded by the families. At the end of the treatment period, compliance was assessed through counts of returned capsules. The numbers of returned capsules were compared with the intake protocols.

2.8. Data analysis

Data were analyzed per protocol since the aim of the present study was to investigate whether the used supplement affects behavior and cognition rather than assessing overall treatment efficacy. Twelve % of the randomized participants were lost to follow-up. With the exception of one additional child having swallowing problems in the active treatment group, equal numbers of children were lost to follow-up in both groups. In both groups (active, placebo) 2 children required stimulant medication due to the severity of problems (Fig. 1).

2.8.1. Behavior

Data distribution was not normal. Two Mann-Whitney U tests (two-tailed) were used for comparisons between active and placebo group. The dependent variables for the pre-post comparisons are the differences between post and pre intervention measurements. The dependent variables for the pre intervention comparisons are the baseline measurements of both groups.

2.8.2. Erythrocyte fatty acid composition

Data distribution was not normal. Two Wilcoxon tests (two-tailed) were used for comparison between active and placebo group. The dependent variables for the pre-post comparisons are the differences between post and pre intervention measurements. The dependent variables for the pre intervention comparisons are the baseline measurements of both groups.

Two Wilcoxon tests (two-tailed) were used to determine within group effects from baseline to follow-up

2.8.3. Cognition

t-tests were used to compare if active and placebo group differed at baseline in Working Memory Index Scores, working memory subtest (Digit Span, Letter Number Sequencing) and task scores (Digits Forward, Digits Backward), information processing scores and various measures of attention. The effect of omega-3 supplementation on cognitive measures (time by treatment) was investigated by using repeated-measures analysis of variance.

2.8.4. Sample demographics

χ^2 analysis was used to compare if active and placebo group differed in gender. Unpaired t-tests were used for age and IQ comparison.

2.8.5. Study compliance

χ^2 analysis was used to assess differences in dropout rates between active and placebo group.

2.8.6. Fish and seafood consumption

χ^2 analysis was used to assess whether active and placebo group differed in the number of fatty fish consuming families before the start of the intervention. χ^2 analysis was used to assess whether the number of families consuming omega-3 rich fish changed in active and placebo group during the intervention period.

2.8.7. Correlation and regression analysis

Associations between changes in fatty acid measures and changes in behavioral and cognitive measures in the total study population were analyzed by Pearson/Spearman correlation analysis.

Regression analysis was used to test whether AA, EPA and DHA baseline fatty acid levels were predictors of working memory performance at baseline and post intervention.

2.8.8. Sensitivity analysis

Multiple imputation (5) followed by linear mixed modeling analysis considering all 110 cases (intention-to-treat analysis) was used to test the robustness of the two significant findings (working memory, parent-rated thought problems).

Number of missing values pre/post Intervention Cognition and Behavior: Working Memory Index Sore: 1/15, Digit Span: 18/22, Letter Number Sequencing: 18/22, Digits Forward: 37/22, Digits Backward: 37/22; parent-rated thought problems (CBCL): 2/18.

All values are missing completely at random [36].

The level of significance was set at $p < 0.05$. Statistical analysis was done with SPSS statistical software 21 (SPSS Inc. Chicago, Ill).

Table 2
Parent and teacher-rated behavior at baseline and after 16 weeks of EPA/DHA versus placebo treatment in children with ADHD.

a) DISYPS-II questionnaire

Parent rating	**Baseline**				**Follow-up**			
	EPA/DHA $N=44$, * $N=45$ Mean ± SE	**Placebo** $N=48$, * $N=49$ Mean ± SE	***Z***	***p***	**EPA/DHA** $N=45$ Mean ± SE	**Placebo** $N=47$ Mean ± SE	***Z***	***p***
Attention	2.02 ± 0.08	1.98 ± 0.07	−0.15	0.88	1.72 ± 0.08	1.71 ± 0.08	−0.85	0.39
Hyperactivity	1.27 ± 0.11	1.20 ± 0.10	−0.49	0.62	0.89 ± 0.11	0.84 ± 0.09	−0.40	0.69
Impulsivity	1.58 ± 0.15	1.45 ± 0.14	−0.64	0.52	1.21 ± 0.13	1.30 ± 0.13	−1.25	0.21
Hyperactive/ Impulsive	1.43 ± 0.12	1.33 ± 0.11	−0.61	0.54	1.05 ± 0.11	1.07 ± 0.10	−0.88	0.38
Total	1.68 ± 0.08*	1.64 ± 0.07*	−0.55	0.58	1.35 ± 0.08	1.34 ± 0.07	−0.65	0.52
Teacher rating	**EPA/DHA** $N=44$, * $N=45$ Mean ± SE	**Placebo** $N=48$, * $N=49$ Mean ± SE	***Z***	***p***	**EPA/DHA** $N=39$ Mean ± SE	**Placebo** $N=45$ Mean ± SE	***Z***	***p***
Attention	1.75 ± 0.10	1.88 ± 0.10	−0.97	0.33	1.45 ± 0.11	1.59 ± 0.11	−0.12	0.90
Hyperactivity	0.89 ± 0.12	0.79 ± 0.12	−0.75	0.46	0.65 ± 0.12	0.59 ± 0.09	−1.13	0.26
Impulsivity	0.93 ± 0.14	0.88 ± 0.13	−0.33	0.74	0.70 ± 0.14	0.81 ± 0.13	−0.73	0.46
Hyperactive/ Impulsive	0.91 ± 0.12	0.83 ± 0.11	−0.58	0.56	0.68 ± 0.12	0.70 ± 0.10	−1.09	0.27
Total	1.31 ± 0.09*	1.31 ± 0.08*	−0.03	0.97	1.04 ± 0.10	1.11 ± 0.08	−0.20	0.84

(b) Child Behavior Checklist (CBCL)

Parent rating	**Baseline**				**Follow-Up**			
	EPA/DHA $N=46$; * $N=45$ Mean ± SE	**Placebo** $N=49$; *$N=48$ Mean ± SE	***Z***	***p***	**EPA/DHA** $N=45$; *$N=44$ Mean ± SE	**Placebo** $N=47$; *$N=46$ Mean ± SE	***Z***	***p***
Withdrawal	61.13 ± 1.29	61.22 ± 1.30	−0.01	0.99	59.18 ± 1.40	57.43 ± 1.12	−1.03	0.30
Somatic problems	60.85 ± 1.44	57.61 ± 1.11	−1.58	0.12	58.04 ± 1.30	55.81 ± 1.19	−0.29	0.76
Anxiety/ depression	62.41 ± 1.48	60.16 ± 1.31	−1.03	0.30	59.47 ± 1.43	57.94 ± 1.16	−0.27	0.79
Social problems	63.17 ± 1.56	60.82 ± 1.42	−1.21	0.23	61.89 ± 1.51	58.30 ± 1.20	−0.97	0.33
Thought problems	55.07 ± 1.11*	56.81 ± 1.19*	−1.11	0.27	55.34 ± 1.18*	53.74 ± 0.92*	−2.10	0.03
Attention problems	70.50 ± 1.21	68.82 ± 1.04	−1.07	0.29	68.60 ± 1.17	66.04 ± 1.18	−0.98	0.32
Deliquent behavior	59.74 ± 0.96	59.14 ± 1.10	−0.67	0.50	58.93 ± 1.06	57.19 ± 1.11	−0.55	0.58
Aggressive behavior	64.65 ± 1.45	62.96 ± 1.40	−0.77	0.44	62.51 ± 1.46	60.15 ± 1.47	−1.05	0.29
Externalizing	62.89 ± 1.47	61.41 ± 1.42	−0.85	0.40	60.27 ± 1.73	57.68 ± 1.64	−0.78	0.43
Internalizing	63.11 ± 1.47	60.33 ± 1.49	−1.07	0.28	58.69 ± 1.66	56.77 ± 1.46	−0.59	0.55
Total problems	65.89 ± 1.30	63.86 ± 1.34	−1.13	0.26	62.36 ± 1.47	60.15 ± 1.38	−0.02	0.98

c) Teacher's Report Form (TRF)

Teacher rating	**Baseline**				**Follow-Up**			
	EPA/DHA $N=46$ Mean ± SE	**Placebo** $N=49$; * $N=48$ Mean ± SE	***Z***	***p***	**EPA/DHA** $N=40$; * $N=39$ Mean ± SE	**Placebo** $N=45$ Mean ± SE	***Z***	***p***
Withdrawal	59.00 ± 1.31	59.82 ± 1.37	−0.22	0.83	56.80 ± 1.12	57.31 ± 1.04	−0.85	0.40
Somatic problems	55.70 ± 1.11	55.20 ± 1.06	−0.39	0.69	53.63 ± 0.91	53.42 ± 0.79	−0.56	0.58
Anxiety/ depression	55.22 ± 0.95	54.69 ± 0.79	−0.20	0.84	53.30 ± 0.84	54.20 ± 0.89	−0.09	0.93
Social problems	57.83 ± 1.01	57.57 ± 0.97	−0.62	0.53	55.88 ± 0.84	56.82 ± 0.96	−0.91	0.36
Thought problems	53.98 ± 1.03	53.40 ± 0.91*	−0.40	0.69	50.41 ± 0.29*	53.40 ± 1.22	−1.54	0.12
Attention problems	62.28 ± 1.01	62.53 ± 1.16	−0.10	0.92	58.93 ± 1.01	60.27 ± 1.05	−0.33	0.74
Delinquent behavior	55.43 ± 0.95	54.88 ± 0.92	−0.66	0.51	54.08 ± 0.81	54.36 ± 0.76	−0.25	0.80
Aggressive behavior	59.43 ± 1.36	57.80 ± 1.09	−0.70	0.48	56.35 ± 1.31	56.84 ± 1.12	−1.73	0.08
Externalizing	57.87 ± 1.37	55.80 ± 1.32	−0.83	0.41	54.38 ± 1.44	54.69 ± 1.36	−1.12	0.26
Internalizing	55.24 ± 1.42	55.14 ± 1.31	−0.15	0.88	51.48 ± 1.46	53.27 ± 1.32	−0.20	0.84
Total problems	59.83 ± 0.93	58.76 ± 0.98	−0.79	0.43	55.80 ± 1.09	56.82 ± 1.16	−0.49	0.62

Reduction of scores corresponds to an improved symptom rating.
SE: Standard error.
Mann–Whitney, 2-tailed.
The dependent variables for the pre intervention comparisons are the baseline measurements of both groups.
The dependent variables for the pre-post comparisons are the differences between post and pre intervention measurements.

Table 3
Erythrocyte membrane fatty acid composition at baseline and after 16 weeks of EPA/DHA versus placebo treatment in children with ADHD.

Fatty acid	Baseline				Follow-Up			
	EPA/DHA $N=43$ Mean ± SE	**Placebo** $N=45$ Mean ± SE	***Z***	***P***	**EPA/DHA** $N=38$ Mean ± SE	**Placebo** $N=44$ Mean ± SE	***Z***	***P***
C18:2n-6	14.32 ± 0.24	14.89 ± 0.31	−1.49	0.14	13.79 ± 0.25	14.63 ± 0.28	−1.59	0.11
C18:3n-3 (LNA)	0.17 ± 0.01	0.18 ± 0.01	−1.15	0.25	0.18 ± 0.01	0.16 ± 0.01	−1.34	0.18
C18:3n-6	0.14 ± 0.01	0.13 ± 0.01	−0.67	0.50	0.11 ± 0.01	0.15 ± 0.01	−4.02	< 0.00
C20:2n-6	0.25 ± 0.00	0.25 ± 0.01	−0.60	0.55	0.25 ± 0.01	0.25 ± 0.01	−1.57	0.12
C20:3n-6	1.99 ± 0.06	2.00 ± 0.07	−0.16	0.87	1.54 ± 0.05	1.91 ± 0.06	−5.31	< 0.00
C20:4n-6 (AA)	15.47 ± 0.24	14.81 ± 0.23	−2.44	0.01	13.14 ± 0.20	15.13 ± 0.23	−6.15	< 0.00
C20:5n-3 (EPA)	0.62 ± 0.03	0.58 ± 0.02	−1.19	0.23	3.02 ± 0.08	0.56 ± 0.03	−7.73	< 0.00
C22:4n-6	2.81 ± 0.08	2.78 ± 0.09	−0.84	0.40	1.75 ± 0.06	2.85 ± 0.08	−7.12	< 0.00
C22:5n-6	0.79 ± 0.02	0.78 ± 0.02	−0.07	0.94	0.39 ± 0.01	0.80 ± 0.02	−7.63	< 0.00
C22:5n-3	1.97 ± 0.06	1.81 ± 0.04	−1.86	0.06	3.70 ± 0.06	1.88 ± 0.05	−6.53	< 0.00
C22:6n-3 (DHA)	3.51 ± 0.09	3.47 ± 0.10	−0.30	0.77	4.80 ± 0.08	3.47 ± 0.11	−6.82	< 0.00

Results are expressed as fatty acid percentage of total identified fatty acids after response factor correction.
LNA: alpha-linolenic acid, AA: arachidonic acid, EPA: eicosapentaenoic acid, DHA: docosahexaenoic acid.
SE: Standard error.
Wilcoxon, 2-tailed.
The dependent variables for the pre intervention comparisons are the baseline measurements of both groups.
The dependent variables for the pre-post comparisons are the differences between post and pre intervention measurements.

2.9. Ethics statement

Written, informed consent was given by the participating children and the parents/caregivers. The study was approved by the Ethics Committee, Ulm University 07/09 – UBB/se.

3. Results

3.1. Study population

The total study population consisted of 95 children, 74 boys (78%) and 21 girls (22%). Average age at start of the intervention period was 8.86 y for boys and 9.10 y for girls. 54.7% of the children were diagnosed as being inattentive, 43.2% as inattentive/hyperactive (combined phenotype) and 2.1% as predominantly hyperactive/impulsive (Table 1).

Active and placebo group did not differ significantly in age ($T=-0.054$, $df=93$, $p=0.96$), gender ($\chi^2=0.17$, $p=0.68$) and total IQ ($T=-0.29$, $df=93$, $p=0.77$).

3.2. Baseline characteristics

3.2.1. Fish consumption

The two groups did not differ in the number of fatty fish consuming families before the start of the intervention ($\chi^2=0.29$, $p=0.59$).

3.2.2. Fatty acid profiles

With the exception of arachidonic acid (C20:4n-6), omega-3 and omega-6 fatty acid composition in erythrocyte membranes was similar at baseline in both groups (Table 3).

3.2.3. Behavior

Behavioral scores were not different in the two groups before the start of the intervention (Table 2).

3.2.4. Cognition

The two groups did not differ in HAWIK Working Memory Index Score ($T=0.52$, $df=93$, $p=0.60$), Digit Span score ($T=0.28$, $df=64.39$, $p=0.78$), Letter Number Sequencing score ($T=-0.70$, $df=79$, $p=0.49$), Digits Forward score ($T=0.15$, $df=62$, $p=0.88$) and Digits Backward score ($T=0.01$, $df=62$, $p=0.99$) and Information Processing score at baseline ($T=-0.98$, $df=93$, $p=0.33$).

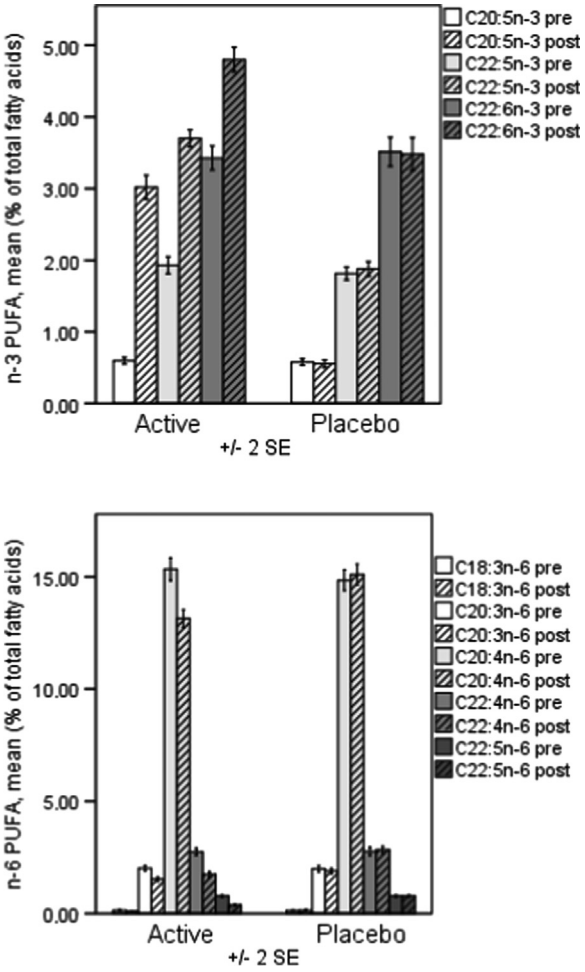

Fig. 2. Erythrocyte membrane n-3 and n-6 polyunsaturated fatty acids (means) pre and post intervention for active (EPA/DHA) and placebo group.
C20:4n-6 (AA), C20:5n-3 (EPA), C22:6n-3 (DHA)
SE: Standard error

Except for TAP GoNoGo total reaction time ($T=-2.59$, df=16, $p=0.020$) and number of errors ($T=-2.71$, df=16, $p=0.015$) attentional measures were not different at baseline.

Table 4
Effect of treatment (time by treatment interaction) with EPA/DHA versus placebo on working memory function in children with ADHD (HAWIK-IV).

	Group	**Pre** (Mean ± SD)	**Post** (Mean ± SD)	***F*** (192)	***p***
Working Memory Index Score $N=94$	EPA/DHA	97.51 ± 10.04	101.78 ± 11.47	5.54	0.019
	Placebo	96.31 ± 9.48	96.92 ± 9.73		
Subtests:	**Group**	**Pre** Mean ± SD	**Post** Mean ± SD	***F*** (176)	***p***
Digit Span $N=78$	EPA/DHA	12.46 ± 2.42	14.11 ± 2.78	9.73	0.003
	Placebo	12.32 ± 1.56	12.68 ± 1.81		
Letter Number sequencing $N=78$	EPA/DHA	14.19 ± 3.86	15.86 ± 3.12	2.92	0.092
	Placebo	14.63 ± 3.36	15.27 ± 2.92		
Tasks:	**Group**	**Pre** Mean ± SD	**Post** Mean ± SD	***F*** (159)	***p***
Digits forward $N=61$	EPA/DHA	6.71 ± 1.51	7.48 ± 1.59	0.55	0.462
	Placebo	6.70 ± 1.15	7.27 ± 1.34		
Digits backward $N=61$	EPA/DHA	5.97 ± 1.35	6.90 ± 1.90	4.88	0.031
	Placebo	5.90 ± 1.06	6.00 ± 1.02		

Repeated measures analysis of variance.

3.3. Intervention effects

3.3.1. Fish consumption

The number of families consuming omega-3 rich fish before and after the intervention did not change significantly in the two groups (active group $\chi^2=0.18$, $p=0.67$; placebo group $\chi^2=0.02$, $p=0.90$)

3.3.2. Erythrocyte fatty acid profiles

Erythrocyte membrane fatty acid profiles differed significantly when comparing blood samples of the EPA/DHA supplement taking group with samples of the placebo taking group after the 16 weeks intervention period. The intervention with the EPA/DHA supplement led to a significant increase of the n-3 fatty acids C20:5n-3 (EPA; 378%), C22:5n-3 (88%) and C22:6n-3 (DHA; 37%). In contrast the intervention with the n-3 supplement led to a decrease of n-6 fatty acids C18:3n-6 (−21%), C20:3n-6 (−23%), C20:4n-6 (−15%), C22:4n-6 (−38%), C22:5n-6 (−51%) (Table 3, Fig. 2).

Within group comparisons showed significant increases for C20:5n-3 (EPA), C22:5n-3 and C22:6n-3 (DHA) and decreases for C20:3n-6, C20:4n-6, C22:4n-6, C22:5n-6 in the EPA/DHA group. Treatment with olive oil for 16 weeks did not lead to significant changes in erythrocyte fatty acid composition in the placebo-taking group (data not shown).

3.3.3. Behavior

Scores for parent-rated (DISYPS-II, CBCL) and teacher-rated (DISYPS-II, TRF) behavior before and after 16 weeks of intervention are shown in Table 2.

Teacher- and parent-rated attention, hyperactivity, impulsivity, hyperactivity/impulsivity and total score (DISYPS-II) did not change significantly during the 4 month intervention period. Except for a significant decrease of parent-rated thought problems of placebo-taking children, parent and teacher ratings showed no significant intervention effect for the CBCL/TRF subscales withdrawal, somatic problems, anxiety/depression, social problems, attention problems, delinquent behavior, aggressive behavior, externalizing, internalizing and total problems (Table 2).

3.3.4. Sensitivity analysis

Intention-to-treat analysis including all 110 randomized cases did not show a significant improvement of parent-rated thought problems for placebo-taking children (Table 7 Supplement)

3.3.5. Association between erythrocyte fatty acid composition and behavior

Correlation analysis showed a positive correlation between changes in C22:6n-3 (DHA) and changes in parent-rated social problems (CBCL) ($r=0.243$, $p=0.032$, $N=78$). None of the other teacher and parent-rated behavioral changes (DISYPS-II parent and teacher-rated, TRF) showed a significant association with changes in AA, EPA and DHA in the total study population (data not shown).

3.3.6. Cognitive functions

Analysis of variance revealed a significant improvement of working memory function (Index Score) when comparing the EPA/DHA group with the placebo-taking group (time by treatment interaction) (Table 4, Fig. 3).

Analysis of working memory subtest and task performance showed a significant improvement of the Digit Span subtest and the Digits Backward task for the EPA/DHA taking group. In contrast, the intervention with the omega-3 fatty acid mix did not lead to significant changes in the performance of the Letter–Number Sequencing subtest or the Digits Forward task (Table 4, Fig. 3).

Regression analysis showed that C20:4n-6 (AA), C20:5n-3 (EPA), C22:5n-3 (DPA) and C22:6n-3 (DHA) baseline levels were no predictors for pre or post intervention working memory performance (data not shown).

No significant intervention effect was found for speed of information processing (HAWIK-IV). The measures for inhibition, sustained attention, divided attention, flexibility and distractibility were not affected by supplementation with EPA/DHA (data not shown).

Sensitivity analysis: Intention-to-treat analysis including all 110 randomized cases revealed a significant improvement of Working Memory Index Score, Digit Span subtest and Digits Backward task scores in the supplement-taking group (time by treatment) (Table 8 Supplement)

3.3.7. Association between erythrocyte fatty acid composition and cognition

Correlation analysis was used to investigate if changes in working memory performance were associated with changes in the fatty acid composition of the erythrocyte membranes (total study population). Improved working memory (Index Score) correlated with increased C20:5n-3 (EPA), C22:5n-3, C22:6n-3 (DHA) and decreased C20:4n-6 (AA), C22:4n-6, C22:5n-6 (Table 5).

On the working memory subtest level, improved Digit Span scores were correlated with increased C20:5n-3 (EPA), C22:5n-3

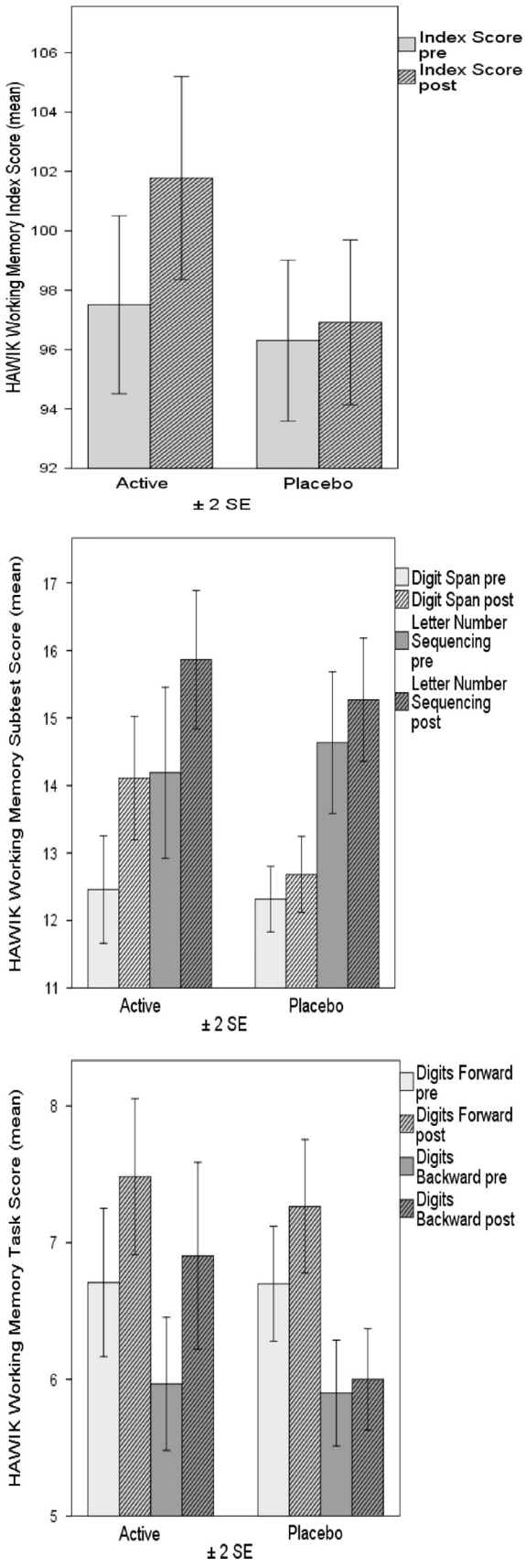

Fig. 3. HAWIK Memory Index Scores, Subtest Scores and Task Scores (means) pre and post intervention for active (EPA/DHA) and placebo group.
SE: Standard error

and C22:6n-3 (DHA) and decreased C18:2n-6. Improved Letter Number Sequencing performance correlated with higher C20:5n-3 (EPA) and lower C20:4n-6 (AA), C22:4n-6 and C22:5n-6 (Table 5).

While improved Digits Backward task scores were significantly correlated with increased C20:5n-3 (EPA) and C22:5n-3 and decreased C18:3n-6, no significant correlations were found for the Digits Forward task (Table 5).

Changes in C20:5n-3 (EPA), C22:6n-3 (DHA) and C20:4n-6 (AA) were not significantly associated with changes of information processing performance (HAWIK-IV) and attention (inhibition, sustained attention, divided attention, flexibility, distractibility) (data not shown).

4. Discussion and conclusions

This study investigated the effect of supplementation with EPA/DHA on erythrocyte membrane fatty acid composition, behavior and cognition in children diagnosed with ADHD according to DSM-IV criteria.

Supplementation with EPA/DHA led to a significant increase of several omega-3 fatty acids including EPA and DHA and decreased levels of omega-6 fatty acids including AA in erythrocyte membranes. Significant changes in fatty acid levels were not observed in the olive oil taking group.

Except for a significant decrease of parent-rated thought problems (CBCL) in the placebo group, supplementation with EPA/DHA did not affect parent- and teacher-rated behavior (DISYPS-II, TRF, CBCL).

Sensitivity analysis, however did not confirm this result and we are hesitant to attach great importance to the observed decrease of thought problems.

The questions comprising this outcome measure are complex. The developers of the CBCL account for the problem by providing extra space to describe the rating in detail. During the study visits we noticed repeatedly, that parents had great difficulties with the items comprising this symptom scale. Despite rating their child's behavior only few parents described the problems. Nonetheless we included all ratings, even if the problem had not been described explicitly, and therefore we cannot be confident that all parents understood the questions. Despite its relevance in a clinical setting, the thought problem subscale might have limitations for research purposes.

Furthermore the effect might be caused by the use of olive oil as placebo. Olive oil contains a high concentration of oleic acid, a precursor of oleamide that has been shown to have psychoactive properties [37]. Daily treatment with 1.2 g olive oil, however did not lead to significant changes in fatty acid profiles as it was seen in an investigation observing positive behavioral effects with 6.4 g olive oil per day as comparison treatment [18].

Supplementation with EPA/DHA leads to improved working memory function in the selected study population. Improvement was associated with an increase of EPA and DHA and a decrease of AA.

Hirayama et al. also investigated short term memory function in a placebo-controlled intervention trial with 40 children (8–12 y). Participants in the active treatment group were provided with fish oil fortified foods (3600 mg DHA, 700 mg EPA per week), while children in the control group obtained food products enriched with olive oil. After 8 weeks of supplementation improvement of short term memory function was observed in the placebo-taking group.

The study has several limitations. Only 32 children were diagnosed according to DSM-IV criteria. Six children were taking

Table 5
Correlation between Working Memory Index Score change, working memory subtest and task changes (HAWIK-IV) and changes in erythrocyte membrane fatty acids in children with ADHD.

	C18:2n-6 [1])	C18:3n-3 [2])	C18:3n-6 [2])	C20:2n-6 [1])	C20:3n-6 [1])	C20:4n-6 (AA) [1])	C20:5n-3 (EPA) [1])	C22:4n-6 [1])	C22:5n-6 [1])	C22:5n-3 [1])	C22:6n-3 (DHA) [1])
Working memory Index score $N=81$	−0.037	0.113	−0.108	−0.097	−0.097	−0.300**	0.358**	−0.302**	−0.249*	0.315**	0.255*
Subtests:											
Digit span $N=67$	−0.272*	0.059	−0.183	−0.222	−0.100	−0.179	0.358**	−0.205	−0.187	0.385**	0.293*
Letter Number Sequencing $N=67$	−0.085	0.095	0.066	0.001	−0.083	−0.424**	0.274*	−0.320**	−0.271*	0.199	0.134
Tasks:											
Digits forward $N=54$	−0.136	−0.058	0.149	−0.064	0.230	0.007	0.078	0.004	0.078	0.052	0.058
Digits backward $N=54$	−0.243	0.054	−0.301*	−0.190	−0.241	−0.147	0.300*	−0.262	−0.218	0.318*	0.262

AA: arachidonic acid, EPA: eicosapentaenoic acid, DHA: docosahexaenoic acid.
Pearson correlation analysis [1]).
Spearman correlation [2]).
** $p < 0.01$.
* $p < 0.05$.

stimulant medication during the intervention period. The provided dose of EPA (100 mg/day) was low and it is not known how much of the provided food fortified with fish oil or olive oil (placebo) was consumed by the children. In addition the fortification process might have affected the bioavailability of fatty acids. The fatty acid composition of serum or erythrocyte membranes as indicators for pre supplementation omega-3 fatty acid provision, supplementation efficacy and compliance was not determined. Due to the high fish and seafood consumption in Japan it is likely that the children already had high EPA/DHA levels before the start of the intervention [27].

Sinn et al., failed to detect working memory changes using the WISC-III Digits backwards task, while children receiving the PUFA supplement improved their ability to switch and control attention. [28]. Except for an additional 60 mg of GLA per day, supplement composition was similar to the one, used in the present investigation. Although the two study populations are similar in age and gender, children participating in the Australian study were not diagnosed according to DSM-IV criteria and do not represent a clinical sample.

Except for working memory function and parent-rated thought problems we did not observe significant treatment effects for a variety of behavioral and cognitive measures. Despite a lack of significant time by treatment effects, probably due to the small sample size, Milte et al. observed significant correlations between changes in several outcome measures and changes in erythrocyte PUFA levels [38]. Except for a positive association between changes in DHA and parent-rated social problems, no significant correlations for other non significant measures were observed in the present investigation.

Some published studies observed improved behavioral ratings in response to n-3 PUFA supplementation in children with ADHD and coexisting learning problems [38,39]. Few children in the present study sample were officially diagnosed with comorbid disorders and a subgroup analysis to identify possible responders to n-3 supplementation was not possible.

Published studies vary in supplement composition and dosage. According to a meta-analysis (10 trials, 669 children) higher EPA rather than DHA concentrations are associated with symptom reduction in children diagnosed with ADHD [24]. Comparing the results of 13 trials with 1011 participants Gillies et al. concluded that combined omega-3/6 PUFA supplements rather than omega-3 supplements are more likely to cause improvements [23]. In their intervention study Milte et al. observed that increased DHA rather than EPA was associated with better cognitive and behavioral outcome measures [38]. Supplementation with 600 mg EPA, and 120 mg DHA in the present investigation resulted in a large increase of EPA, while the increase of DHA was only modest. An omega-3/6 supplement with a higher DHA concentration might therefore be more suitable to detect behavioral and cognitive intervention effects.

4.1. Strength and limitations

The study population represents a clinical sample. In contrast, some investigations enrolled subjects with a diagnosis based only on parent-ratings without confirming the behavioral deficits in a second setting, such as school [38,40,41].

The majority of caretakers (78%) got the information about this investigation from medical professionals. Child psychiatrists informed 59% of the participating families. Only 22% were recruited via the community. Consultation of a child psychiatrist requires a referral from a pediatrician and makes it unlikely that symptom severity of the study population was low despite meeting DSM-IV criteria.

Fatty acid profiles were used as indicators for treatment efficacy and compliance. Furthermore they allow to investigate whether changes in fatty acid levels correlate with changes in cognitive or behavioral measures.

Parents and children reported no side effects of the used supplement, indicating that composition and dosage were well tolerated by the study population. Five children had problems swallowing the provided capsules and five families decided not to participate since they had objections against pork gelatine containing capsule casings. Hence, use of a liquid or a suspension would have been more suitable for a larger group of children. In addition the use of capsules did not permit weight-adjusted dosage as it was used in an open-label pilot study reporting improved behavior in children with ADHD [42].

Intake protocols and erythrocyte fatty acid profiles are measures of adherence to the study protocol confirming that compliance was high in both groups.

A brief questionnaire addressing fish and seafood consumption before and after the intervention showed that the number of families consuming omega-3 rich fish did not change significantly in either group during the intervention period. This is supported by the observation that fatty acid profiles revealed no omega-3 LC-PUFA increase in the placebo taking group.

The attrition rate was low (12%) compared to other studies [18,39,40].

By using the DISYPS-II questionnaires, the number of DSM ADHD symptoms rather than symptom severity was used for

participant selection in the present study [29]. A comparison with studies using symptom severity [43,44] for participant selection might be limited [28,38,39,41].

The present study is limited by its small sample size. Bloch et al. concluded in their systematic review that a sample size of approximately 330 children would be necessary to detect a supplementation effect in a placebo-controlled intervention trial with omega-3 fatty acids, assuming a medium size effect [24]. Despite 33 month of intense recruitment, only 95 children completed the intervention and returned for the post intervention assessment. Sixty two children had to be excluded from participation because the maladaptive behavior caused problems in only one setting (home or school). Applying the DSM-IV diagnostic criteria made the recruitment of a large sample difficult.

We furthermore can not exclude the possibility that the two findings significant at $p < 0.05$ level might be due to multiple comparisons.

This investigation shows that the used EPA/DHA supplement leads to improved working memory function in the study population.

However, the positive effect of EPA/DHA supplementation on working memory function might not be specific for children diagnosed with ADHD. Zhang et al. used cross-sectional data from the Third National Health and Nutrition Survey, (1988–1994), and found that higher energy intake from PUFAs was associated with lower risks of poor performance on the WISC-R digit span working memory subtest [45]. In addition improved working memory (n-3 back task) after 6 month of supplementation with DHA (750 mg/d) and EPA (930 mg/d) was observed in healthy young adults [46].

Therefore a definite conclusion about the effect of supplementation with EPA/DHA on working memory performance in children diagnosed with ADHD cannot be drawn. A similar intervention study with a healthy control group would be necessary, to investigate whether supplementation with EPA/DHA leads to improvement of working memory function in children with ADHD.

Acknowledgment

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.plefa.2014.04.004.

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