Title Page

Title: Supplementation with oil rich in eicosapentaenoic acid, but not in docosahexaenoic acid, improves global cognitive function in healthy, young adults: results from randomized controlled trials

Running Title: Comparison of EPA and DHA-rich oil Supplementation

Author Names: Michael J. Patan[1], David O. Kennedy[1], Cathrine Husberg[2], Svein Olaf Hustvedt[2], Philip C. Calder[3, 4], Julie Khan[1], Joanne Forster[1], Philippa A Jackson[1]

Author Affiliations:

[1] Brain Performance and Nutrition Research Centre, Northumbria University, Newcastle Upon Tyne NE1 8ST, United Kingdom; [2] BASF AS, Oslo, Norway; [3] School of Human Development and Health, Faculty of Medicine, University of Southampton., Southampton SO16 6YD, United Kingdom; [4] National Institute of Health Research Southampton Biomedical Research Centre, University Hospital Southampton National Health Service Foundation Trust and University of Southampton, Southampton SO16 6YD, United Kingdom

Corresponding author:

Philippa A Jackson
Brain, Performance and Nutrition Research Centre
Northumbria University
Newcastle, UK
NE1 8ST
Tel: +44 (0)191 243 7720

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Abbreviations: BIC, Bayesian Criterion; CI, confidence interval; COMPASS, Computerized Mental Performance Assessment System; deoxy-Hb, deoxygenated Hb; DHA,

docosahexaenoic acid; EI, Efficiency index; EMM, estimated marginal means; EPA, eicosapentaenoic acid; fMRI, functional magnetic resonance imaging; Hb, hemoglobin; LMM, linear mixed models; LTP, long-term potentiation; n-3 PUFAs, omega-3 polyunsaturated fatty acids; NIRS, near infrared spectroscopy; Ox%, oxygen saturation percentage; oxy-Hb, oxygenated hemoglobin; PFC, prefrontal cortex; PBS, phosphate-buffered saline; RVIP, rapid visual information processing; RT, reaction time; SMEDS, self-micro-emulsifying delivery system; t-Hb, total Hb; VAS, visual analogue scale.

Abstract

Background: Evidence regarding the effects of the omega-3 polyunsaturated fatty acids (n-3 PUFAs) docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA) on cognition is lacking.

Objective: We investigated whether supplementation with oils rich in EPA or DHA improves cognition, prefrontal cortex (PFC) hemoglobin (Hb) oxygenation and memory consolidation.

Design: Healthy adults (n = 310; age range 25–49 y) completed a 26-week randomized controlled trial in which they consumed either 900 mg DHA/d and 270 mg EPA/d (DHA-rich oil), 360 mg DHA/d and 900 mg EPA/d (EPA-rich oil) or 3000 mg/d refined olive oil (placebo). Cognitive performance and memory consolidation were assessed via computerized cognitive test battery. PFC Hb oxygenation was measured using near infrared spectroscopy (NIRS).

Results: Both global accuracy and speed improved with EPA-rich oil compared with placebo and DHA-rich oil (EPA vs placebo accuracy: EMM = 0.17 [95% CI = 0.09, 0.24] vs EMM = 0.03 [95% CI = -0.04, 0.11]; $p = 0.044$; EPA vs placebo speed: EMM = -0.15 [95% CI = -0.22, -0.07] vs EMM = 0.03 [95% CI = -0.05, 0.10]; $p = 0.003$). Accuracy of memory was improved with EPA compared with DHA (EMM = 0.66 [95% CI = 0.26, 1.06] vs EMM = -0.08 [95% CI = -0.49, 0.33]; $p = 0.034$). Both EPA- and DHA-rich oils showed trends towards reduced PFC oxygenated Hb (oxy-Hb) compared with placebo (placebo: EMM = 27.36 μM [95% CI = 25.73, 28.98]; DHA: EMM = 24.62 μM [95% CI = 22.75, 26.48]; $p = 0.060$; EPA: EMM = 24.97 μM [95% CI = 23.35, 26.59]; $p = 0.082$).

Conclusions: EPA supplementation improved global cognitive function and was superior to the oil enriched with DHA. Interpreted within a neural efficiency framework, reduced PFC oxygenated Hb suggests that n-3 PUFAs may be associated with increased efficiency.

Key words: Eicosapentaenoic acid, docosahexaenoic acid, n-3 polyunsaturated fatty acids, self-micro-emulsifying, cognition, memory

Introduction

Evidence from cross-sectional studies suggests that increased consumption of the bioactive omega-3 polyunsaturated fatty acids (n-3 PUFAs) docosahexaenoic acid (DHA; 22:6n-3) and eicosapentaenoic acid (EPA; 20:5n-3) is associated with better cognitive functioning across the lifespan (1, 2). In the UK, like many Western countries, consumption of these marine-derived long chain n-3 PUFAs is low (3). Systematic reviews of clinical trials investigating the effects of n-3 PUFA supplementation on cognition in healthy individuals have been largely inconclusive with methodological variations often cited as underpinning the inconsistency in the evidence (4-6). Methodological recommendations regarding dose, duration of intervention, outcome measures and populations from which study samples should be drawn have been proposed elsewhere (4, 5, 7, 8). Emerging evidence also highlights the importance of the formulation of the interventions themselves for optimal digestion and absorption to ensure maximal delivery of DHA and EPA to tissues (9, 10).

To date, most investigations of the cognitive effects of n-3 PUFAs have focused on DHA, due to its relative abundance in central nervous system tissue and important structural and signaling functions therein. However, recent evidence from short term trials indicates that treatment with EPA may be relevant to cognitive function in healthy adults (11, 12). Possibly underpinning these effects, emerging evidence suggests that EPA may improve neural efficiency (13), and increase prefrontal cortex (PFC) hemoglobin (Hb) oxygenation during the performance of cognitive tasks (14). Moreover, a pilot study that assessed the effects of 20 weeks' supplementation with 1600 mg DHA + 400 mg EPA daily found that the observed

improvement of neurovascular coupling to a cognitive test battery following the intervention was correlated with increases in erythrocyte levels of EPA, not DHA (15). Interestingly, with regards to modulation of PFC Hb oxygenation during cognitive tasks, results from a pilot study using continuous wave near infrared spectroscopy (NIRS) showed an effect of fish oil enriched with DHA but not EPA, and a dose-dependent increase in PFC Hb oxygenation following DHA-rich oil in a larger follow-up investigation using the same technique (16, 17). Direct comparisons of the effects of DHA and EPA enriched interventions in the literature are limited (12, 18), yet this approach would serve to address the aforementioned disparate findings, and further clarify the specific effects of each of these fatty acids on cognition.

Therefore, the primary aim of the current trial was to build upon previous findings (19) by comparing the effects of treatments enriched with either EPA or DHA, designed for enhanced absorption, on cognition in healthy young adults who habitually consume low amounts of oily fish. As a secondary aim, PFC Hb oxygenation was measured using frequency domain NIRS. This technique allows for the quantification of changes in Hb parameters and therefore accurate comparisons of pre- and post-intervention assessments, a limitation of previous research (16, 17). Finally, an additional exploratory assessment of learning investigated overnight memory consolidation with the aim of building on the small number of n-3 PUFA trials that have employed learning-memory tasks to date (20-23).

Subjects and Methods

This paper describes results from three trials. Each trial answered individual research questions and the data from each trial were analyzed separately. All participants were enrolled into the cognitive function trial (NCT02763514) and sub samples from this total sample completed either the NIRS (NCT03158545) or learning-memory (NCT03592251) assessment components. The remaining participants took part in assessments of sleep (NCT03559361; data published elsewhere (24)).

All three trials reported here (NCT03158545, NCT03592251, and NCT02763514) were conducted at Northumbria University between October 2016 and November 2018 and adhered to the guidelines of the Declaration of Helsinki (2013). Ethical approval for the cognitive function trial (NCT02763514) was granted by the NHS REC (Yorkshire and The Humber – Bradford Leeds Research Ethics Committee) on the 12th September 2016; all three trials were granted approval from Northumbria University Ethics Committee (SUB067_Patan_230316, SUB087_Patan_080616, SUB808). Written informed consent was obtained from all participants.

Participants

Participant disposition through the trial is displayed in **Figure 1**. All participants enrolled onto the trials were aged between 25–49 years and had to pass a physical/lifestyle screening to demonstrate they were in good health. This age cohort was selected for the trial on the basis that it has been largely overlooked by previous studies that have tended to focus on children or older adults, yet a decline in cognitive performance measures is already apparent in early adulthood (25). Having good health was identified as being a non-smoker, free from prescription, herbal, illicit or recreational drugs (females taking the contraceptive pill were included), free from major illnesses, having a blood pressure lower than 159/99 mm Hg and a

BMI between 18.5 and 35 kg/m^2. Eligible participants consumed oily fish less than once per week, measured via the DHA food frequency questionnaire (26), and reported no habitual consumption of dietary supplements, including omega-3 supplements. Participants were recruited via posters, adverts placed on social media websites, or emails sent out to university staff and students, and were either students or staff attending/working at Northumbria University or individuals living in the Newcastle-upon-Tyne surrounding area. Participant baseline demographic data are provided in **Table 1**.

Three hundred and sixty-six males and females were recruited, with 337 being eligible for the study after the screening and training visit, of whom 310 completed all trial requirements. Of the 27 participants who were withdrawn from the trial; 15 participants were lost to follow up; five withdrew for personal reasons; two were withdrawn after randomization due to having a BMI above 35 kg/m^2; two withdrew due to gastrointestinal upset; two withdrew due to an unrelated illness; and one withdrew due to becoming pregnant.

Trial design

All trials employed randomized, placebo-controlled, double-blind, parallel group designs with participants being randomly assigned to one of three treatment groups (placebo oil, DHA-rich oil or EPA-rich oil) for a period of 26 weeks. All treatment capsules were supplied by BASF AS. Treatment was three approximately 1 g capsules daily. For the DHA-rich treatment, the amount of DHA was at least 300 mg and EPA at least 90 mg per capsule, providing a total of at least 900 mg DHA/d and 270 mg EPA/d (Accelon™ DHA EE capsules); for the EPA-rich treatment, the amount of DHA was at least 120 mg and EPA at least 300 mg per capsule, providing a total of at least 360 mg DHA/d and 900 mg EPA/d (Accelon™ EPA EE capsules). Each of the placebo capsules contained 1,000 mg refined olive oil. The active treatments contained oils derived from fish. The DHA- and EPA-rich

treatments also contained a self-micro-emulsifying delivery system (SMEDS), a proprietary mixture of surfactants and cosolvents that spontaneously emulsify the oils upon reaching the stomach and gastrointestinal tract resulting in a larger surface area of the contents upon dispersion in the gastrointestinal tract. These formulations successfully improve the bioavailability of the n-3 PUFAs (10). Participants were instructed to consume their capsules with a glass of water at night, prior to bedtime. A nighttime dosing regime was selected based on recent evidence for diurnal rhythms of DHA and EPA in humans, which suggest optimal times of dosing to maximize bioavailability (27).

A computer-generated randomization schedule was used to allocate participants to the treatments (www.randomization.com). Placebo and treatment capsules were identical in size and shape. Capsules were provided in opaque containers that were coded and distributed by researchers at Northumbria University according to the randomization schedule. To maintain blinding throughout the trial, a 3rd party within the university created the randomization schedule and the coded treatments prior to their delivery to the research team. Participants were sequentially allocated a randomization number and unblinding took place when all analyses were completed.

General demographic information and eligibility criteria were collected and assessed before participants were accepted onto the trials. Cognitive assessments, blood samples, NIRS measurements and measures of mood were obtained at baseline and after 26 weeks' supplementation. For the sub sample enrolled in the memory consolidation trial (NCT03592251), an additional assessment took place after 13 weeks. Participants were instructed to maintain their normal diets for the duration of the trials. Treatment compliance was determined via capsule count of leftover treatments returned by each participant.

The primary end points of the cognitive function study were accuracy of memory (cognitive function) and subjective mental fatigue (mood), and the primary endpoint of the NIRS study was PFC Hb oxygenation. Memory consolidation and associations between NIRS outcomes and n-3 index were exploratory outcomes, with all other outcomes being secondary endpoints.

Data were collected from September 2016 to May 2018.

Whole blood sample collection and assays

Blood samples (6 ml) were collected via venipuncture into ethylenediaminetetraacetic acid vacutainers. The samples were stored in an ice box or at 5°C and were processed within 4–8 hours of collection. Blood was centrifuged at 2000 rpm (913 x g) for 10 minutes at room temperature. Following this, the top layer of plasma was removed using a pastette and discarded. One ml of red blood cells (RBCs) were then collected from the bottom of the vacutainer, transferred into a 15 ml centrifuge tube and made up to 15 ml with phosphate-buffered saline (PBS). The mixture was then inverted and centrifuged at 1200 rpm (350 x g) for 10 minutes, at room temperature, with a low brake. The PBS was then removed and the washing process was repeated for a second time. After the second wash, the remaining RBCs were transferred into labelled 1.5 ml microtubes and immediately frozen at -80^{o}C until analysis. RBC fatty acid composition was then assessed by gas chromatography as described elsewhere (24, 28). The fatty acids are expressed as a weight percentage of total fatty acids present. The n-3 index was calculated as % EPA + % DHA.

Cognitive assessments

All cognitive tasks were delivered using the Computerized Mental Performance Assessment System (COMPASS – Northumbria University, UK - see: www.cognitivetesting.co.uk). This testing system can deliver a tailored collection of tasks, with fully randomized parallel

versions of each task administered during each assessment for each participant. The cognitive tasks used within the battery have previously been shown to be sensitive to a wide range of nutritional interventions, including n-3 PUFAs (19). The cognitive tasks that were completed included word recall, picture recognition, verbal fluency, simple reaction time, numeric working memory, Stroop, word recognition, serial subtract 3s and 7s, rapid visual information processing (RVIP), location learning, digit vigilance, and peg and ball. The cognitive demand battery was made up of 4 repetitions of the following tasks; serial subtract 3s, serial subtract 7s and RVIP. Outcomes from individual tasks were used to derive the following composite cognitive domain measures: accuracy of attention, speed of attention, accuracy of memory, speed of memory, global accuracy and global speed.

For all cognitive domains, Z scores were calculated by pooling baseline and 26 weeks' data as previously described (19, 22). Z scores were clustered into cognitive domains as follows:

(1) **Accuracy of Attention** = (Z-Stroop accuracy + Z-RVIP average accuracy) ÷ 2

(2) **Speed of Attention** = (Z-Stroop RT + Z-RVIP average RT + Z-SRT) ÷ 3

(3) **Accuracy of memory** = (Z-immediate word recall accuracy + Z-delayed word recall accuracy + Z-word recognition accuracy + Z-picture recognition accuracy) ÷ 4

(4) **Speed of Memory** = (Z-word recognition RT + Z-picture recognition RT) ÷ 2

(5) **Global Accuracy** = (Z-immediate word recall accuracy + Z-delayed word recall accuracy + Z-word recognition accuracy + Z-picture recognition accuracy + Z-verbal fluency accuracy + Z-Stroop accuracy + Z-NWM accuracy + Z-RVIP average accuracy) ÷ 8

(6) **Global Speed** = (Z-word recognition RT + Z-picture recognition RT + Z-Stroop RT + Z-SRT + Z-NMW RT + Z-RVIP average RT) ÷ 6

Individual task outcomes e.g. accuracy (percentage of correct responses made), reaction time (RT; msec), false alarms (number) were also assessed. The full battery of tasks took ~1 h to administer. See online supplementary material under "Supplementary method, section 1.2" for detailed descriptions of each individual task.

The cognitive testing was conducted under rigorously controlled conditions (see online supplementary material under "Supplementary method, section 1.4"). In brief, assessments at baseline and 26 weeks were completed at either 07:00, 08:30 or 10:00. Participants were scheduled to complete the week 26 assessment at the same time as their baseline assessment, although this was not possible for a small number of participants. Participants were instructed to avoid caffeinated food and beverages for 18 hours, alcohol for 24 hours and antihistamines for 48 hours prior to the baseline and 26 weeks assessments. Environmental factors such as noise and temperature were controlled to avoid any distraction during tests. During their screening visit, participants were instructed on the procedure for the administration of the cognitive battery and undertook a training session comprising a battery of shortened tasks where each 'mini task' was completed three times in succession to familiarize them with the procedure for each task and two full-length parallel versions of the test battery to mitigate potential learning effects. . Each participant had to meet minimum speed and accuracy requirements on each task before proceeding to the intervention phase.

NIRS assessment

The NIRS assessment was conducted in a sub sample of 78 participants, which took place within 1.5 hours of completing the cognitive assessment at baseline and 26 weeks. NIRS measures were taken at rest and during completion of serial subtraction tasks of varying difficulties [3s, 7s, 17s; confirmed via 'task difficulty' visual analogue scale (VAS)]. Cerebral hemodynamic response was measured using NIRS (OxiplexTS Frequency-Domain

Near-Infrared Tissue Oximeter; ISS Inc., Champaign, IL, USA). This system provides absolute measurements of the absorption of the near-infrared light emitted at two distinct wavelengths by the device, which allows for the quantification of oxygenated Hb (oxy-Hb) and deoxygenated Hb (deoxy-Hb) via their differing photon absorption properties. These values can then be used to determine total Hb (t-Hb; oxy-Hb + deoxy-Hb) and oxygen saturation percentage (Ox%; oxy-Hb/t-Hb × 100). This system can be used for quantifying changes in hemodynamic response during both acute (i.e. changes in response to cognitive tasks) and chronic (i.e. comparing pre- and 26 weeks' post-intervention time points) assessments.

Light was emitted at 691 and 830 nm by optical fibers glued in pairs to four prisms (eight fibers in total) separated from the collector bundle, which was also glued to a prism, by 2.0, 2.5, 3.0 or 3.5 cm. Each of the emitter and collector bundle prisms were embedded into a flexible polyurethane resin to form a sensor, with overall dimensions of 7.6 cm × 2.5 cm × 0.3 cm. Two identical sensors were attached to each side of the participants forehead and secured in place with a self-adhering bandage. The sensors were positioned so that the bottom edge was level with the top of the participants' eyebrows and the middle edge touching at the midline of the forehead. Data were collected at a rate of 5Hz and were measured in micromolar (μM). When used in this way, increases in oxy-Hb and t-Hb are usually interpreted as increased activation of the PFC (29).

Memory consolidation assessment

The memory consolidation assessment was an exploratory arm of the investigation conducted in a sub sample of 169 participants at baseline, 13 and 26 weeks' post intervention. A similar trial design to that used previously (30, 31) was employed to measure overnight memory consolidation and was adapted within the current trial to be administered via COMPASS on a

tablet computer. This involved completion of two learning tasks prior to going to sleep and completion of respective recall tasks the following morning. In addition, attention (simple reaction time, digit vigilance) and executive function (peg and ball) tasks and subjective morning alertness (VAS) was assessed upon waking, also via the tablet computer.

Statistical analysis

Statistical analyses were performed with IBM SPSS statistics software (version 25; IBM Corp). Data handling and cleaning procedures are described in full under "Supplementary method, section 1.5". Descriptive and comparison statistics (independent t test, 2 tailed or Chi-square test) of all baseline characteristics were based on all participants who provided data that could be analyzed [intention-to-treat population]. The general statistical approach was via linear mixed models (LMM). LMM have several advantages over using repeated measures ANOVA or ANCOVA including the ability to accommodate missing data points often encountered in longitudinal datasets and the ability to model nonlinear, individual characteristics (32). Therefore, data were analyzed using the MIXED procedure in SPSS, unless another analysis is stated. For each model, restricted maximum likelihood estimation methods were used and covariance matrix structure was chosen based on the structure that produced the lowest Schwarz's Bayesian Criterion (BIC), an indication of the best fitting model for the data (33). Changes within outcome variables during the treatment period were assessed via LMM that adjusted for respective baseline pre-intervention scores. For each of the models conducted throughout the analysis, age, years in education and baseline n-3 index were built into the model as covariates if they were identified as having a significant impact ($p < 0.05$) on the dependent variable, to control for the impact of these variables within the model. Participant was also entered as a random effect in each model if it lowered the BIC value, suggesting an improved model fit. Significant main or interaction effects of treatment ($p < 0.05$) were investigated further with Sidak corrected comparisons to account for multiple

group comparisons. Full descriptions of each LMM are outlined in section 1.6 of the supplementary method.

Using GPower 3.1.3, *a priori* sample sizes, based on ANOVA analysis, were calculated for each study separately, based on the range of small to medium effect size estimates seen previously (e.g. 19, 34), at 80% power and an alpha level of 0.05. In addition, an extra 10% was added to each estimate to allow for dropouts. The total sample size for the cognitive study was 336 participants (f = 0.18), 78 participants for the NIRS study (f = 0.32) and 168 participants for the memory consolidation study (f = 0.13). Additionally, a post-hoc power calculation for the mixed-effects model analysis that was applied to the cognitive data, computed using the online program RMASS (www.rmass.org/), at 80% power, an alpha level of 0.05, a small to medium effect size, and an attrition rate of 10%, confirmed a total sample size of 300 participants (35).

For the cognitive function, mood and memory consolidation assessments, outcome data from each task and VAS were analyzed. Additionally, the cognitive function data were also analyzed via the six cognitive domains described above.

NIRS data [Ox%, t-Hb, oxy-Hb and deoxy-Hb] were split into two distinct periods; resting and active. The resting period contained the averaged data from the 5-minute resting period before the cognitive tasks began and the active period consisted of the averaged data from all three repetitions of the serial 3s, serial 7s and serial 17s subtraction tasks. The cognitive and subjective task difficulty data collected during the NIRS assessments consisted of the average number of correct responses, accuracy % and task difficulty % from all three repetitions of the serial 3s, 7s and 17s tasks.

Efficiency index (EI) was calculated by standardizing the raw active NIRS data (Ox%, t-Hb, oxy-Hb and deoxy-Hb) and the accuracy % for every task during each visit to a value of 0–1

(1= the highest accuracy % or highest respective NIRS value). Once these new standardized values had been calculated each participant's standardized NIRS value was then subtracted from their standardized accuracy % for each task. This then provided an EI score of between -1 and 1 for each NIRS parameter (Ox%, t-Hb, oxy-Hb and deoxy-Hb) with higher scores indicating greater neural efficiency. These EI calculations have been employed previously as a measure of neural efficiency within a similar paradigm (36).

The models used to analyze the cognitive function trial data consisted of treatment (DHA-rich, EPA-rich, Placebo). The models used to analyze the NIRS data consisted of treatment (DHA-rich, EPA-rich, Placebo), hemisphere (left, right), task (3s, 7s, 17s) and task randomization order (1 – 6). The models used to analyze the memory consolidation trial data consisted of treatment (DHA-rich, EPA-rich, Placebo) and visit (week 13 or week 26). Respective baseline values were also entered into every model as a covariate. Full descriptions of the fixed factors and covariates appearing in each respective model are reported in full under "Supplementary methods, section 1.6".

The primary focus of all analyses was placebo comparisons with both the DHA- and EPA-rich treatments. However, the design of each of the trials also offered the opportunity to compare the effects of the two active treatments. Therefore, comparisons between placebo and the active treatment groups, as well as comparisons between the two active groups are reported.

Exploratory NIRS Correlations

Pearson's bivariate correlations were conducted post hoc to further explore the relationship between n-3 index and NIRS outcomes. Correlations were therefore conducted between n-3 index and NIRS measures (Ox%, t-Hb, oxy-Hb and deoxy-Hb) during each of the subtraction

task (3s, 7s or 17s), for each hemisphere (left or right) and for each of the visits (baseline or week 26).

Results

Overall, of the 337 participants who were randomly assigned to treatments, 27 were lost to follow up or discontinued the intervention for various reasons (n = 11 in the placebo group; n = 8 in the DHA-rich group; n = 8 in the EPA-rich group) (**Figure 1**). The main cognitive function analysis was conducted in 310 participants (n = 101 in the placebo group; n = 105 in the DHA-rich group; n = 104 in the EPA-rich group) for whom baseline and end data were available. For the NIRS subsample, the final analysis was conducted in 75 participants (n = *25* in the placebo group; n = *25* in the DHA-rich group; n = 25 in the EPA-rich group) for whom baseline and end data were available. For the memory consolidation subsample, the final analysis was conducted in 155 participants for whom baseline and data from at least one of the post-intervention assessments (week 13 or 26) were available (n = *51* in the placebo group; n = *51* in the DHA-rich group; n = *53* in the EPA-rich group). Baseline characteristics of participants are summarized in **Table 1**. No significant differences between the treatment groups were identified for any of the baseline demographics.

For participants who completed the trial, compliance was high in all three groups (96.06% Placebo, 97.43% DHA-rich, 96.57% EPA-rich) with one way ANOVA identifying no significant differences for compliance percentage by treatment group [F (2, 306) = 1.39, p = 0.250]. A Chi-Square test was also conducted on the responses to the treatment guess questionnaire that was completed at the end of the final visit and revealed no significant differences in participants' ability to correctly identify whether they had been administered an active or placebo treatment between the three groups [χ^2 (2) = 1.52, p = 0.467]. Analysis of RBC fatty acid profiles further supports the compliance data (**Table 2**).

A similar number of adverse events was reported in each group (Placebo, *n* = 15; DHA, *n* = 20; EPA, *n*=17). Most of these were mild ailments e.g. headache, aches and pains, common cold. Ten participants reported gastrointestinal issues (Placebo, *n* = 3; DHA, *n* = 5; EPA, *n* = 2). One participant was withdrawn due to pregnancy. Adverse events resulting in participant withdrawal are shown in Figure 1.

Cognition

A significant effect of treatment for global accuracy was identified [F (2, 215) = 4.82, p = 0.009], with post hoc comparisons identifying significantly more accurate scores in the EPA-rich group (estimated marginal mean [EMM] = 0.17; 95% CI = 0.09, 0.24) compared to both the placebo (EMM = 0.03; 95% CI = -0.04, 0.11; p = 0.044) and DHA-rich groups (EMM = 0.01; 95% CI = -0.07, 0.09; p = 0.013) (**Figure 2A**).

Additionally, a significant effect of treatment for global speed was identified [F (2, 255) = 5.74, p = 0.004], with post hoc comparisons identifying significantly faster reaction times in the EPA-rich group (EMM = -0.15; 95% CI = -0.22, -0.07) compared to the placebo group (EMM = 0.03; 95% CI = -0.05, 0.10; p = 0.003) and a trend towards significantly faster reaction times compared to the DHA-rich groups (EMM = -0.03; 95% CI = -0.10, 0.05; p = 0.062) (**Figure 2B**).

A significant effect of treatment for accuracy of memory was identified [F (2, 290) = 3.28, p = 0.039], with post hoc comparisons showing significantly higher accuracy in the EPA-rich group (EMM = 0.66; 95% CI = 0.26, 1.06) compared to the DHA-rich group (EMM = -0.08, 95% CI = -0.49, 0.33; p = 0.034) (**Figure 2C**).

Cognition: Exploratory Analysis

Learning and Recall

A significant main effect of treatment for word recognition was identified [F (2, 1306.00) = 4.42, p = 0.012], with post hoc comparisons identifying the DHA-rich (EMM = 851.67; 95% CI = 836.39, 866.94; p = 0.009) but not the EPA-rich (EMM = 869.23; 95% CI = 854.52, 883.94; p = 0.281) group as having significantly faster reaction times during the word learning trials compared to placebo (EMM = 885.18; 95% CI = 869.64, 900.72) (**Figure 3**). No other significant effects of treatment or significant interaction effects between treatment and visit were identified for any of the other outcomes from the learning or recall tasks.

Morning Cognitive Performance

There were no significant main effects of treatment or significant interactions between treatment and visit for any of the cognitive tasks.

Mood

There were no significant effects of treatment for subjective mood.

PFC Hb oxygenation

NIRS: Resting period

There were no significant main or interaction effects of treatment on PFC Hb oxygenation during the resting period.

NIRS: Active period

A significant interaction between treatment and hemisphere was identified for oxy-Hb, [F (2, 256.55) = 4.95, p = 0.008], with both the EPA (EMM = 24.97 μM; 95% CI = 23.35 μM, 26.59 μM; p = 0.082) and DHA (EMM = 24.62 μM; 95% CI = 22.75, 26.48; p = 0.060) groups showing a trend towards significantly lower oxy-Hb in the right hemisphere compared to the placebo group (EMM = 27.36 μM; 95% CI = 25.73, 28.98) during completion of the subtraction tasks. (**Figure 4**). A significant interaction between treatment and hemisphere

was also identified for t-Hb, [F (2, 251.24) = 3.65, p = 0.027]; however, post hoc comparisons revealed no significant group differences.

NIRS: Serial Subtraction and VAS Analysis

There were no significant main or interaction effects of treatment for the serial subtraction tasks. However, a significant effect of treatment was identified for ratings of subjective task difficulty [F (2, 56.99) = 3.91, p = 0.026], with the DHA-rich (EMM = 50.18; 95% CI = 45.58, 54.86) group rating the tasks more difficult than the placebo group (EMM = 42.01; 95 CI = 38.06, 45.95; p = 0.028).

NIRS: Efficiency Index

No significant main effect of treatment or interactions between treatment and any other factors were identified.

NIRS: Exploratory Analysis

Pearson's bivariate correlations identified significant negative correlations at both baseline and week 26 assessments, in the right hemisphere, between oxy-Hb and n-3 index during completion of the serial 3s (baseline, r = -0.27, p = 0.024; week 26, r = -0.25, p = 0.048), serial 7s (baseline, r = -0.28, p = 0.017; week 26, r = -0.25, p = 0.041) and serial 17s (baseline, r = -0.29, p = 0.013; week 26, r = -0.26, p = 0.038) tasks (**Figure 5**). Using Fisher's Z-transformation to compare baseline and week 26 correlations (37), no significant differences were observed for any of the subtraction tasks between time points (serial 3's: Fishers Z = -0.13, p = 0.448; serial 7's: Fishers Z = -0.20, p = 0.422; serial 17's: Fishers Z = -0.20, p = 0.422). No significant correlations were identified in the left hemisphere.

Discussion

The results of the current research reveal that 26 weeks' supplementation with EPA-rich oil improved global cognitive function in terms of both speed and accuracy, compared to placebo and DHA-rich oil. EPA-rich oil also significantly increased the accuracy of performing all the memory tasks compared to the DHA-rich oil. Compared to placebo, DHA-rich oil improved reaction time during word recognition compared to placebo during the learning phase of the overnight memory consolidation tasks. With regards the cerebral blood flow parameters, both the EPA- and DHA-rich oils showed trends towards lower oxy-Hb in the right PFC during completion of serial subtraction tasks. There were no significant effects of either treatment on mood, learning-memory recall tasks, waking cognitive performance or morning alertness.

To our knowledge, the current trial is the first to investigate and identify significant improvements in healthy young adults to both global accuracy and speed of cognitive function following supplementation with EPA enriched oil, compared to placebo. These results extend previous findings in healthy older adults, where observational data have revealed a positive relationship between EPA status and global cognitive function (38, 39), and beneficial effects of EPA-rich treatments on executive functions have also been observed (40). Collectively, these findings are interesting as they suggest that although EPA is stored in the brain in low amounts (41), it may still play an important role in higher order cognitive functions, as has been suggested previously (13). In contrast, and somewhat surprisingly, these beneficial effects were not seen following the DHA-rich oil, where improvements to cognition were limited to improved word recognition reaction times during the learning phase of the overnight memory consolidation tasks. In fact, direct comparisons between the active treatments revealed that global speed and accuracy, and accuracy of memory were improved following the EPA-rich oil in comparison to the DHA-rich oil. These results indicate that supplementation with EPA over DHA may be more beneficial in healthy young adults in terms of cognitive outcomes. It also demonstrates that the ratio of EPA and DHA in the

423 investigational treatments provided to participants could be an important consideration that
424 can greatly influence study outcomes. Given the emphasis on DHA in the literature to date, if
425 supplementation with DHA is indeed less relevant in healthy young populations, this may in
426 part explain the limited effects that have been reported previously (4, 6).

427 Controlled trials published by our laboratory and others have sought to develop the
428 hypothesis that the effects of n-3 PUFAs on cognitive function in humans are at least in part
429 underpinned by their effects on cerebrovascular perfusion and/or neurovascular coupling.
430 Interestingly, whereas previous trials have demonstrated an increase in localized cerebral
431 blood flow (4, 17) or cerebrovascular reactivity (15) following n-3 PUFAs, the current data
432 reveal a trend towards decreased quantities of oxy-Hb in the right PFC during performance of
433 serial subtraction tasks following both active treatments, compared to placebo. As increases
434 in oxy-Hb are usually interpreted as augmented activation within the brain region where the
435 measurement is being made (16), the trend towards decreased quantities of oxy-Hb observed
436 here suggests that there was less activation in the PFC during completion of the serial
437 subtraction tasks. However, as this comparative reduction in activation had no associated cost
438 on performance during the serial subtraction tasks, these findings could be interpreted within
439 a neural efficiency framework. The neural efficiency hypothesis proposes that the inverse
440 relationship between total cortical activation and intelligence results from more focused
441 cortical activation and strengthened communication between brain regions (42). Interestingly,
442 the exploratory correlations between the n-3 index and NIRS parameters revealed inverse
443 associations, even prior to treatment, again suggestive of enhanced efficiency with increased
444 n-3 PUFA status. These findings are somewhat at odds with what has been reported
445 previously, as the only other trials to our knowledge that have directly compared the effects
446 of EPA and DHA enriched treatments on concurrent brain activity and cognitive function
447 found evidence of increased neural efficiency following oils enriched with EPA, but not

448 DHA (11, 12). This may be due to any number of factors including important differences in
449 the measurement techniques used (visual evoked potentials, fMRI) and treatment duration (4
450 weeks, 30 days). However, it is important to note that the current trial revealed statistical
451 trends only and the suggested effects should be verified with further investigation.

452 Taking the findings from the current NIRS assessments at face value, we found no evidence
453 of a difference between the treatments on these outcomes, which fits with previous evidence
454 that supports a role for both EPA and DHA in modulating cerebrovascular function (e.g. 15,
455 17). Comparing this with the observed differences on the cognitive measures, it follows that
456 other mechanisms unique to the actions of EPA must underpin these disparate effects.
457 Possibilities here include the displacement of membrane-bound arachidonic acid by EPA and
458 subsequent attenuation of the production of pro-inflammatory eicosanoids (43), its effects on
459 mitochondrial metabolism (11), or indeed another mechanism not currently fully understood.

460 Regarding memory consolidation, we observed no effects of EPA- or DHA-rich oil during
461 completion of the recall tasks, implemented as a proxy measure of overnight memory
462 consolidation. These findings seem to be at odds with previous *in vitro* and *in vivo* research,
463 which identifies positive effects of n-3 PUFAs on long-term potentiation (LTP) and synaptic
464 plasticity (44, 45), as well as on learning memory tasks in humans (20-23). We aimed to
465 gather learning-memory data as only a small number of trials have employed learning-
466 memory tasks likely to induce LTP and memory consolidation. Whilst we observed an
467 improvement in reaction time following the DHA-rich oil during the learning phase of the
468 word recognition task, the null recall results do appear to contradict the limited previous
469 findings (20, 21, 23). These disparate findings may be due to possible limitations of the novel
470 overnight recall protocol employed here such as the use of COMPASS in a non-laboratory
471 environment where participants might not have been free from distraction, or the learning

tasks themselves, which have not previously been used to assess learning over an extended period, for example.

The current trial addresses several limitations that have been present in previous controlled trials with regards sample size, supplementation period and dosage (4, 5, 7, 8). We also implemented standardized computerized cognitive testing, SMEDS formulated treatments to improve bioavailability, and a protocol that allowed for a direct comparison of the effects of oils rich in both EPA and DHA across a range of different outcomes. With regards cognition, our results suggest that at the doses provided, EPA is more beneficial than DHA in healthy, young adults. One consideration here is the nature of the participants included in the trial, who were indeed healthy and, on average, did not have a very low n-3 status (≤ 4%) at baseline (46). A recent systematic review recommends recruiting participants with an n-3 index of < 6% into trials of n-3 PUFAs (47), while another concluded that n-3 PUFA supplementation may only benefit those who are deficient in them to start with (6). Over a third of participants exceeded the recommended index of 6% at baseline, which may comprise a limitation of the trial. Taking baseline n-3 PUFA status of the trial participants together with the disparate findings for the two enriched oils, one could perhaps conclude that supplementation with DHA specifically is less relevant in healthy adults who do not have a very low n-3 PUFA status or that a higher dose of DHA must be administered in order to achieve an effect on cognition. This may be the reason why we found null results in the DHA group, compared to the positive effects reported by Stonehouse et al. (19), who assessed cognitive function following six months' daily supplementation with 1.16 g DHA + 0.17 g EPA using a similar battery of tasks and composite scores. Finally, as a recent systematic review has identified sex as an influencing factor on human n-3 PUFA levels (48), it may be useful for future trials to consider including sex as an independent variable as standard, as has been done previously (15, 19)

In conclusion, EPA-rich oil improved global cognitive function in healthy young adults who habitually consume low amounts of oily fish. The findings also provide further evidence that supplementation with both EPA and DHA may positively influence neural efficiency, although these results need further exploration. Overall, the findings highlight unique and overlapping functions of EPA and DHA, which future trials should seek to compare further with regards optimal health benefits.

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Author Contributions: The study was conceived and designed by PAJ, MJP, DOK, CH, SOH. MJP, JK, JF collected the data. MJP, PAJ and PCC analyzed the data. All authors contributed to preparing the 1st draft and gave final approval for publication of the manuscript.

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Tables

Table 1. Baseline characteristics for the 310 subjects who completed all aspects of the cognitive function study[1].

Variable	Treatment	Mean	SD	X^2 *or F*
Males/Females (n/n)	Placebo	35/66	-	2.53
	DHA-rich	26/79	-	
	EPA-rich	33/71	-	
Age (years)	Placebo	36.63	7.33	1.25
	DHA-rich	35.15	8.01	
	EPA-rich	35.14	7.88	
Systolic BP (mmHg)	Placebo	123.91	12.83	1.09
	DHA-rich	122.90	12.67	
	EPA-rich	121.36	11.95	
Diastolic BP (mmHg)	Placebo	81.36	9.69	1.53
	DHA-rich	81.40	10.58	
	EPA-rich	79.28	9.54	
Heart Rate (BPM)	Placebo	71.94	11.17	0.07
	DHA-rich	71.35	11.17	
	EPA-rich	71.78	11.92	
Weight (kg)	Placebo	73.36	13.58	0.09
	DHA-rich	74.09	15.93	
	EPA-rich	73.30	14.41	
Height (cm)	Placebo	168.88	9.63	0.17
	DHA-rich	168.86	8.97	
	EPA-rich	169.52	9.09	
BMI (kg/m^2)	Placebo	25.68	3.95	0.25
	DHA-rich	25.85	4.31	
	EPA-rich	25.44	4.25	
Years in Education	Placebo	16.82	2.49	0.39
	DHA-rich	16.90	2.59	
	EPA-rich	16.61	2.29	
Fruit & Vegetable (self-reported no. of portions per day)	Placebo	3.80	1.77	0.17
	DHA-rich	3.89	2.05	
	EPA-rich	3.95	1.67	
Alcohol (Self-reported units per day)	Placebo	1.25	0.87	1.15
	DHA-rich	1.14	0.83	
	EPA-rich	1.33	1.00	

[1] Baseline difference were assessed using separate one-way ANOVAs or Chi-Square tests; respective F and X^2 values from these analyses are presented. No F or X^2 values were significant at the $p < 0.05$ level. BP, blood pressure; BPM, beats per minute; RBC, red blood cell; SD, standard deviation.

Table 2. Red blood cell fatty acid outcomes by treatment group[1].

Variable	Baseline (n = 285)	Week 26 (n = 272)	Change[2] (n = 263)	*F*-value[3]
% of EPA in RBC				
Placebo	0.88 ± 0.32	0.85 ± 0.36	-0.03 ± 0.36	152.38[4]
DHA-rich	0.86 ± 0.35	2.07 ± 0.82	1.21 ± 0.72	
EPA-rich	0.87 ± 0.42	2.68 ± 1.05	1.87 ± 0.98	
% of DHA in RBC				
Placebo	4.98 ± 1.09	4.73 ± 1.03	-0.19 ± 0.95	111.24[4]
DHA-rich	4.81 ± 1.19	7.43 ± 1.57	2.61 ± 1.56	
EPA-rich	5.00 ± 1.26	6.03 ± 1.05	1.04 ± 1.16	
n-3 index (EPA + DHA)				
Placebo	5.86 ± 1.28	5.58 ± 1.21	-0.21 ± 1.12	134.75[4]
DHA-rich	5.68 ± 1.41	9.50 ± 2.25	3.81 ± 2.04	
EPA-rich	5.87 ± 1.52	8.71 ± 1.80	2.91 ± 1.84	

[1] Means ± SD of the raw bloods data are presented for DHA, EPA and n-3 index at Baseline, Week 26 and change values.

[2] Change values are only calculated for those participants who successfully provided data at both Baseline and Week 26.

[3] F-values for change data from one-way ANOVA are presented.

[4] $p < 0.001$

RBC, red blood cell; SD, standard deviation.

Figure legends

Figure 1. CONSORT flow diagram.

* Data from the sleep sub sample are published elsewhere (24).

Figure 2. Estimated marginal means (+ SE) from LMM analysis for post-dose (A) global accuracy (average standardized % accuracy scores across all cognitive tasks), (B) global speed (average standardized reaction time across all cognitive tasks), (C) accuracy of memory (average standardized % accuracy across all the memory tasks).

* $p < 0.05$

Figure 3. Estimated marginal means (+ SE) from LMM analysis for word recognition RT during the learning trials.

* $p < 0.05$; msec, milliseconds; RT, reaction time.

Figure 4. Estimated marginal means (+ SE) from LMM analysis for post-dose quantities (µM) of oxy-Hb, in the right PFC, during the serial subtraction tasks.

In the right hemisphere, placebo vs DHA-rich ($p = 0.060$); placebo vs EPA-rich ($p = 0.082$).

Figure 5. Pearson's bivariate correlations during the serial subtraction tasks between oxy-Hb quantities (µM) in the right PFC and n-3 index at Baseline (n = 78) and Week 26 (n = 75).