

Effects of Docosahexaenoic Acid Supplementation on Cortical Network Integrity in Medication-Free Children with Attention-Deficit/Hyperactivity Disorder: A Preliminary Multimodal Neuroimaging Trial

Wade A. Weber, Max J. Tallman, Thomas J. Blom, Jennifer D. Schurdak, L. Rodrigo Patino and Robert K. McNamara*

Department of Psychiatry and Behavioral Neuroscience, Center for Imaging Research, University of Cincinnati College of Medicine, Cincinnati, OH, USA

Abstract: Children with attention deficit/hyperactivity disorder (ADHD) exhibit blood docosahexaenoic acid (DHA) deficits and cortical network pathology. This neuroimaging study investigated the effects of DHA supplementation on cortical attention network integrity in medication-free children with ADHD. Children (mean age 9.6 years, n=30) with ADHD were randomized to DHA (1,200 mg/d) or placebo for 10 weeks. Blood DHA levels and ADHD symptom severity ratings were obtained from all participants (n=30). Cortical network integrity was evaluated in a subset of patients (n=20) using functional magnetic resonance imaging (fMRI) and diffusion tensor imaging (DTI). Erythrocyte DHA levels increased significantly in patients receiving DHA (+60%, p<0.0001) but not placebo (-4%, p=0.77). There were no group differences in baseline-endpoint change in ADHD symptom severity scores, sustained attention performance, or voxelwise cortical activation patterns during performance of a sustained attention task. In the region-of-interest (ROI) analysis, patients treated with DHA but not placebo exhibited significant endpoint reductions in left amygdala activation. At study endpoint, but not at baseline, DHA-treated patients exhibited significantly greater event-related functional connectivity between the pregenual and subgenual anterior cingulate cortex and regions within the cortical attention network including the inferior parietal lobe and dorsolateral prefrontal cortex compared with placebo. Trends with large effect sizes for reductions in medial and radial diffusivity in the left corpus callosum were observed in DHA-treated patients. These preliminary findings suggest that DHA supplementation may be associated with subtle changes in cortical attention networks of medication-free children with ADHD which warrant additional investigation in a larger patient sample.

Keywords: Omega-3 fatty acids, Attention, Anterior cingulate cortex, Functional magnetic resonance imaging, Diffusion tensor imaging, Children.

1. INTRODUCTION

Attention deficit/hyperactivity disorder (ADHD) is one of the most common childhood psychiatric disorders [1]. ADHD is typically diagnosed in early childhood, a developmental period associated with rapid maturational changes in frontal gray and white matter [2]. Structural magnetic resonance imaging (MRI) studies indicate that ADHD children exhibit significantly smaller gray matter volumes in several structures including prefrontal regions [3] which appear to be normalized with psychostimulant treatment [4]. ADHD is also associated with reduced cerebral white matter volumes [5], and diffusion tensor imaging (DTI) studies indicate widespread reductions in white matter microstructural integrity [6]. Functional MRI (fMRI) studies further indicate reduced activation in prefrontal and anterior cingulate regions during performance of cognitive tasks [7-9], and abnormal resting-state connectivity within fronto-parietal and fronto-limbic

networks [10-14]. Despite these advances in our understanding of cortical pathology in ADHD, associated risk factors remain poorly understood.

The omega-3 polyunsaturated fatty acid (*n*-3 PUFA) docosahexaenoic acid (DHA, 22:6*n*-3) is the most abundant omega-3 fatty acid in the mammalian brain, and increases rapidly in the human frontal cortex during human perinatal development [15]. Breastmilk is an important postnatal source of DHA for the developing infant, and breastfeeding duration has been found to be inversely associated with ADHD risk [16-19]. Translational studies suggest that a deficit in brain DHA accrual during development is associated with impaired dopamine neurotransmission, as well as other neuropathological processes implicated in the pathophysiology and treatment of ADHD [20]. Importantly, meta-analyses indicate that ADHD youth exhibit significantly lower blood levels of DHA compared with healthy youth [21,22], and that supplementation with DHA containing formulations produce a modest but significant benefit over placebo for reducing ADHD symptoms [21-23]. While these associations support a potential link between early

*Address correspondence to this author at the Department of Psychiatry and Behavioral Neuroscience, University of Cincinnati College of Medicine, 260 Stetson Street, Cincinnati, OH 45219-0516, USA; Tel: 513-558-5601; Fax: 513-558-4805; E-mail: robert.mcnamara@uc.edu

deficits in cortical DHA accrual and neuropathological processes in ADHD, it is not known whether DHA supplementation initiated following the onset of symptoms can correct aberrant cortical circuit connectivity.

Emerging evidence from neuroimaging studies is providing important insight into the role of DHA in human brain structural and functional integrity [24]. For example, a placebo-controlled fMRI study found that DHA supplementation significantly increased prefrontal cortex activation during sustained attention in healthy children [25]. However, another placebo-controlled fMRI trial found that *n*-3 PUFA supplementation did not significantly alter functional cortical activation patterns in healthy children or ADHD patients [26]. In the latter study, the majority of ADHD patients were taking stimulant medications (i.e., methylphenidate) which was discontinued 24 h prior to scanning, and stimulant treatment or discontinuation has been shown to impact cortical activation patterns in ADHD patients [27-30]. Therefore, medication-naïve ADHD patients are better suited to investigate the effects of DHA supplementation on aberrant cortical circuit connectivity. The present randomized double-blind placebo-controlled trial investigated the effects of 10-week DHA supplementation in children who were either psychostimulant-naïve or were psychostimulant-free for at least one month prior to baseline. Changes in ADHD symptoms and indices of cortical attention network integrity were evaluated using multimodal neuroimaging techniques. Based on the evidence reviewed above, the overarching hypothesis was that 10-week DHA supplementation would increase cortical attention network integrity and improve associated impairments in attention.

2. MATERIALS AND METHODS

2.1. Participants

Participants were male or female, 6-15 years old, and met DSM-IV-TR criteria for ADHD (inattentive, hyperactive/impulsive, or combined type), as determined by the Kiddie Schedule for Affective Disorders and Schizophrenia for School-Age Children-Present and Lifetime version (KSADS-PL)[31]. Diagnostic instruments were administered by a trained psychiatrist or qualified clinician with established diagnostic reliability (κ and ICCs > 0.9). Patients were excluded from study participation if they had a history of a major medical (e.g., diabetes) or neurological illness (e.g., epilepsy); psychostimulant exposure within the 30 days prior to the baseline visit;

were greater than 1 year outside appropriate age/grade level; had a history of intolerance or hypersensitivity to omega-3 fatty acids or were taking omega-3 fatty acid supplements within the past 6 months; for females a positive pregnancy test; any history of a hematological disorder or concomitant use of anticoagulant medications; a personal history of substance abuse or a major mood, psychotic, or anxiety disorder; or an inability to swallow study capsules. Patients participating in the imaging component were additionally screened to ensure they were right-hand dominant using the Crovitz test for handedness [32], and could participate safely in an MRI scan. All patients completed the Omega-3 Dietary Intake Questionnaire at the screening visit [33]. This trial was approved by the University of Cincinnati Institutional Review Board, and was registered at clinicaltrials.gov as NCT01883817.

2.2. Treatments

Patients were randomized to either algal DHA (DSM Nutritional Products, LLC) at a fixed DHA dose of 1,200 mg/day or placebo (corn/soy oil) in a double-blind manner for 10 weeks. The 1,200 mg/d dose of DHA was selected based on prior evidence that it significantly increased prefrontal cortex activation during sustained attention in healthy children [25]. Independent fatty acid analyses of placebo and DHA capsule oils confirmed that placebo capsules did not contain DHA, and that DHA capsules contained 40% DHA (~200 mg/capsule). The placebo oil was primarily composed of palmitic acid (10%), oleic acid (25%), and linoleic acid (56%). To maintain the blind, all patients took six capsules daily and placebo and DHA capsules were identical in color and size (550 mg capsules). Compliance was determined using returned capsule count and baseline-endpoint change in erythrocyte DHA levels.

2.3. ADHD Symptom Ratings

ADHD symptom ratings were obtained using the Attention-Deficit Hyperactivity Disorder Rating Scale (ADHD-RS-IV)[34] at baseline, interim visits (Weeks 2, 4, 6, 8), and at Week 10 (or termination). ADHD-RS total score as well as inattention and hyperactivity/impulsivity subscale scores were analyzed.

2.4. Safety and Tolerability Ratings

A physical examination and a complete medical and treatment history were performed at baseline, and vital signs and a structured side effect interview, the Side

Effects Form for Children and Adolescents (SEFCA) [35], were collected at all study visits. All patients received a blood draw on Week 2 for the Platelet Function Assay (PFA) to evaluate potential treatment effects on hemostasis [36]. Suicidality symptom ratings were monitored at each study visit using the Columbia Suicide Severity Rating Scale (C-SSRS) [37].

2.5. Gas Chromatography

At baseline and week 10, whole venous blood (6 ml) was collected into EDTA-coated BD Vacutainer tubes and centrifuged for 20 min (1,500x *g*, 4°C). Plasma and buffy coat were removed and erythrocytes washed 3x with 0.9% NaCl and then stored at -80°C. Total erythrocyte membrane fatty acid composition was determined with a Shimadzu GC-2014 (Shimadzu Scientific Instruments Inc., Columbia MD) using methods described previously [25,38]. All samples were processed by a technician blinded to group assignment. Fatty acid data are expressed as weight percent of total fatty acids (mg fatty acid/100 mg fatty acids).

2.6. fMRI Image Acquisition

fMRI scans were performed using a 4.0 Tesla Varian Unity INOVA Whole Body MRI/MRS system (Varian Inc., Palo Alto, CA). During the scan session, subjects recline in a supine position with their head in a radio-frequency coil. Non-ferromagnetic goggles are positioned to provide clear visualization of the stimuli, and padding was inserted around the subject's head to minimize movement. Headphones and a microphone were provided to allow communication with subjects during the scan acquisition. Following a three-plane gradient echo scan for alignment and brain localization, a shim procedure was performed to generate a homogeneous magnetic field. To provide anatomical localization for activation maps, a high-resolution, T1-weighted, 3-D brain scan was obtained using a modified driven equilibrium Fourier transform (MDEFT) sequence ($T_{MD}=1.1$ s, TR=13 ms, TE=6 ms, FOV=25.6 x 19.2 x 19.2 cm, matrix 256 x 192 x 96 pixels, flip angle=20 degrees). A midsagittal localizer scan was obtained to place 40 contiguous 4 mm axial slices that encompassed the entire brain. Subjects then performed a sustained attention task, the identical-pairs version of the continuous performance task (CPT-IP) as described previously [25], during an fMRI acquisition using a T2*-weighted gradient-echo echoplanar imaging (EPI) pulse sequence (TR/TE=2000/30 ms, FOV=25.6 x 25.6 cm, matrix 64 x 64 pixels, slice-thickness=4 mm, flip angle=75 degrees).

2.7. fMRI Image Processing

fMRI data were analyzed using Analysis of Functional NeuroImages (AFNI) (National Institutes of Health, Bethesda, Maryland; <http://afni.nimh.nih.gov/afni>). Structural and fMRI images were pre-processed using methods described previously [39]. Functional images were corrected for motion using a six-parameter rigid body transformation [40]. The blood oxygen level-dependent (BOLD) signal data were then converted to percent signal change. Activation was defined as the percent change in brain BOLD during the active attentional component (i.e., "press the button when the same number appears 2 times in a row") relative to the control component (watching the "1" flash repeatedly) of the CPT-IP task. "Attentional" blocks were concatenated and compared to concatenated "control" blocks. Individual voxelwise event-related activation maps were then created following standard AFNI procedures using an algorithm that compares the actual hemodynamic response to a canonical hemodynamic response function [41,42]. For fMRI data reported significant regions were at an adjusted $p=0.05$ using a voxelwise threshold of $p=0.005$ and a cluster-extent threshold of ≥ 37 contiguous voxels, to provide a false discovery rate of $p \leq 0.05$ (corrected)[43].

2.8. ROI Analysis

To specifically interrogate activation within prefrontal, ACC, limbic, and striatal subregions, a region of interest (ROI) mask was created using the Talairach-Tournoux Atlas (TTAtlas) in AFNI. ROI values represent percent change in activation during performance of "attentional" blocks relative to "control" blocks of the CPT-IP task. The *a priori* selected ROIs were bilateral DLPFC, orbitofrontal cortex (BA 10), subgenual ACC (BA 25), amygdala, and caudate.

2.9. Functional Connectivity Analysis

A region-of-interest (ROI) mask was created using the Talairach-Tournoux atlas in AFNI to create individual ROIs for the right and left pregenual and subgenual subregions of the ACC. An event-related ROI was applied to each fMRI activation map to obtain activation measurements within ROIs. To create the seed regions a mean time series for left or right ACC (anterior and subgenual) was calculated by averaging the time series for all voxels within the respective ROI. A mean times series for the entire brain was also calculated for each subject for use in global mean

correction in the final regression [44-46]. Regression analysis was then performed in AFNI between the mean ACC time series and the time series for all other individual voxels in the brain, resulting in activation maps for each participant. Global activation was included as a regressor in this analysis in order to remove any spurious brain-wide correlations. The *r*-values calculated from the regression results were then converted to z-scores using Fisher's Z-transformation in order to reduce skewness and normalize the values [47,48]. The individual activation maps of z-scores were combined across participants in each group to produce whole-brain composite maps. Regression analyses were performed using the 3dRegAna tool set in AFNI with group as the factor to produce activation maps.

2.10. DTI

DTI images were acquired using a spin-echo EPI pulse sequence. We acquired diffusion-weighted images in 30 distinct directions ($b = 1000.65 \text{ s/mm}^2$) and six images with no diffusion weighting ($b = 0 \text{ s/mm}^2$) with the following parameters: TR = 10 s, TE = 96.1 ms, FOV = 25.6 cm × 25.6 cm, matrix = 64 × 64, flip angle = 90 degrees, slice thickness = 4 mm. A corresponding multi-echo reference image was also acquired in order to reduce geometric distortions and Nyquist ghosting [49]. The reference scan was incorporated into image reconstruction using custom software. The DTI acquisitions were pre-processed using the methods described previously [50]. ROIs were the right and left genu and right and left forceps minor of the corpus callosum. These ROIs were selected based on prior evidence that omega-3 fatty acid supplementation increases indices of white matter microstructural integrity in these regions [51,52].

2.11. Statistical Analysis

Treatment group differences in demographic variables were evaluated using *t*-tests (2-tailed, $\alpha=0.05$) for continuous variables and Chi-square tests (2-tailed, $\alpha=0.05$) for dichotomous variables. Analyses were performed on the intent-to-treat (ITT) sample, which included all patients who received at least one dose of study medication. For ADHD symptom scores and vital signs obtained at weekly visits, a repeated measures model that included terms for group, time, and group-by-time interaction was used to examine group differences over the ten-week trial. Imaging (ROI) and fatty acid measures were evaluated using a two-factor ANOVA, with time (baseline, 10 weeks) and treatment (placebo, DHA) as independent variables.

For exploratory analyses, significance was defined by a voxelwise threshold of $p \leq 0.02$ and a voxel cluster-extent threshold of ≥ 37 contiguous voxels. For selected outcome measures, effect sizes were calculated using Cohen's *d*, with small, medium, and large effect sizes being equivalent to *d*-values of 0.30, 0.50, and 0.80, respectively. Statistical analyses were performed using the Statistical Analysis System (SAS Institute, Cary, NC, USA).

3. RESULTS

3.1. Subject Characteristics and Attrition

A CONSORT diagram illustrating the flow of subject recruitment and attrition is presented in Figure 1. A total of 31 patients were screened and 30 patients met entrance criteria and were randomized to treatment (placebo, $n=14$; DHA, $n=16$). A total of 19 patients completed the 10-week study (placebo, $n=10$; DHA, $n=9$). Five patients were lost to follow-up post-randomization (placebo, $n=3$; DHA, $n=2$). One patient randomized to placebo terminated study participation early (week 2) due to adverse events (excessive sweating, dilated pupils, and trouble sleeping). Five patients randomized to DHA terminated study participation early (all prior to week 2) due to: patient having trouble swallowing capsules ($n=1$), parental concerns regarding lack of efficacy for treating ADHD symptoms ($n=3$), or patient desiring to no longer participate in the study ($n=1$). For the imaging component, a total of 20 subjects met entrance criteria and were randomized to treatment (placebo, $n=10$; DHA, $n=10$). Eight patients (placebo, $n=4$; DHA, $n=4$) withdrew or were lost to follow-up prior to completing their endpoint scan, and twelve patients completed both baseline and endpoint scans (placebo, $n=6$; DHA, $n=6$). The majority of patients were male (77%), stimulant-naïve (83%), and diagnosed with combined type ADHD (70%). The two groups were demographically well-matched, and there were no significant treatment group differences in age ($p=0.7$), sex ($p=0.6$), and race ($p=0.5$) (Table 1).

3.2. DHA Intake and Erythrocyte Levels

At baseline, patients randomized to placebo or DHA had similar daily dietary DHA intake levels and erythrocyte DHA levels (Table 1). Based on patient body weights, the DHA group received a mean daily DHA dose of 31 mg/kg. Capsule counts at weekly visits found that there was a compliance rate of 90% for the placebo group and 91% for the DHA group ($p=0.89$).

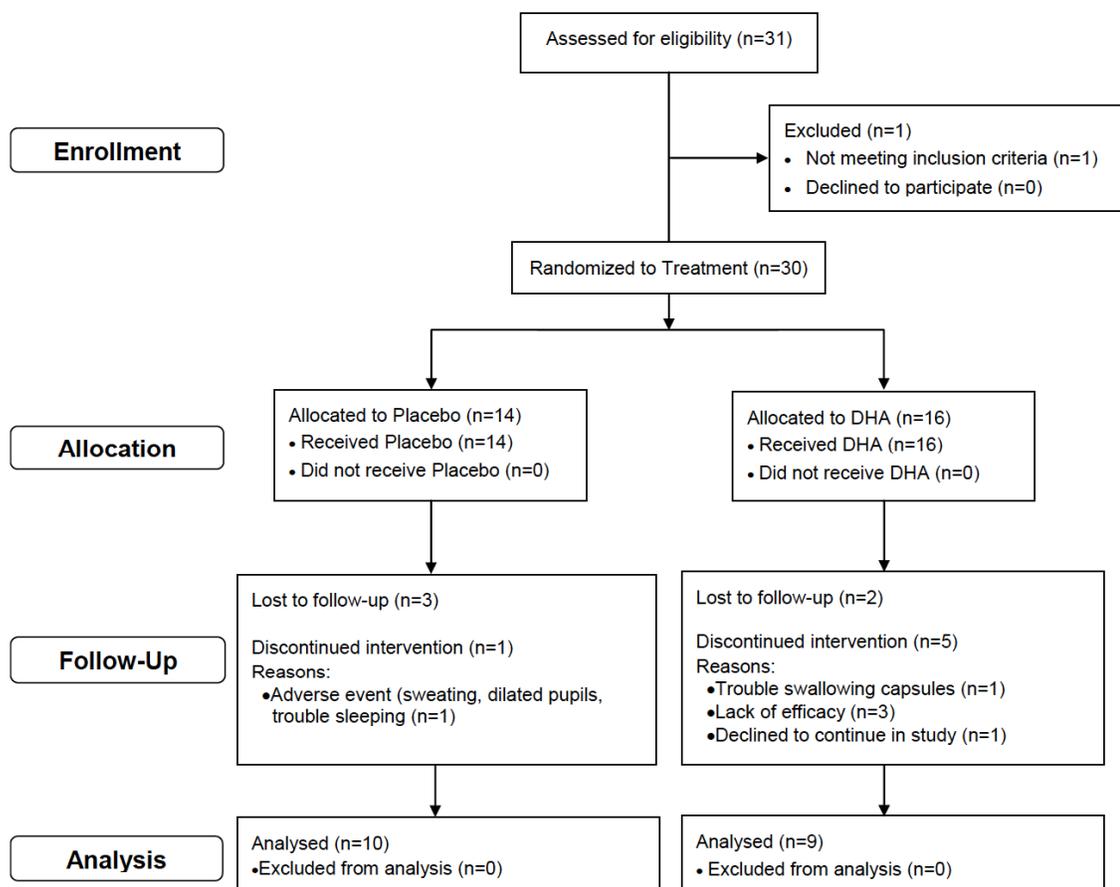


Figure 1: CONSORT diagram illustrating the flow of subject recruitment and attrition.

Table 1: Demographic and Clinical Characteristics

Variable ¹	All Patients (n=30)	Placebo (n=14)	DHA (n=16)	P-value ²
Age (yrs)	9.6 ± 2.3	9.7 ± 1.9	9.4 ± 2.6	0.75
Gender (% Female)	23	21	25	0.61
Race (n)				
Caucasian	16	7	9	0.48
African American	9	4	5	-
Mixed race	5	3	2	-
Height (cm)	139.6 ± 16.5	138.7 ± 18.4	140.4 ± 15.4	0.79
Weight (kg)	49.9 ± 13.8	44.7 ± 12.7	43.2 ± 15.2	0.78
BMI (kg/m ²)	19.6 ± 4.7	20.4 ± 5.6	18.9 ± 3.8	0.43
Heart rate, sitting (bpm)	79.2 ± 11.8	79.5 ± 11.8	78.9 ± 12.4	0.89
Blood Pressure, sitting (mmHg)				
Systolic	102.8 ± 14.7	102.8 ± 9.7	102.7 ± 18.9	0.97
Diastolic	63.7 ± 8.6	66.6 ± 8.2	61.6 ± 7.5	0.12
Temperature (Celsius)	97.9 ± 0.8	98.0 ± 0.7	97.9 ± 0.9	0.77
Dietary DHA intake (mg/d)	106.7 ± 70.8	120.0 ± 67.9	92.2 ± 73.6	0.32
Erythrocyte DHA (wt % TTL)	3.8 ± 1.0	3.9 ± 1.1	3.7 ± 0.9	0.64

(Table 1). Continued.

Variable ¹	All Patients (n=30)	Placebo (n=14)	DHA (n=16)	P-value ²
ADHD Type (n)				
Combined	21	11	10	0.02
Inattentive	9	3	6	-
Hyperactive/Impulsive	0	0	0	-
ADHD-RS				
Total score	37.1 ± 10.1	38.5 ± 9.7	35.8 ± 10.6	0.48
Inattention subscale	18.3 ± 5.3	18.6 ± 5.8	18.1 ± 4.9	0.80
Hyperactivity/Impulsivity subscale	18.8 ± 5.4	19.9 ± 4.5	17.8 ± 6.1	0.28
Prior stimulant exposure (n)				
Methylphenidate	3	2	1	-
Amphetamine	1	1	0	-
Atomoxetine	1	1	0	-

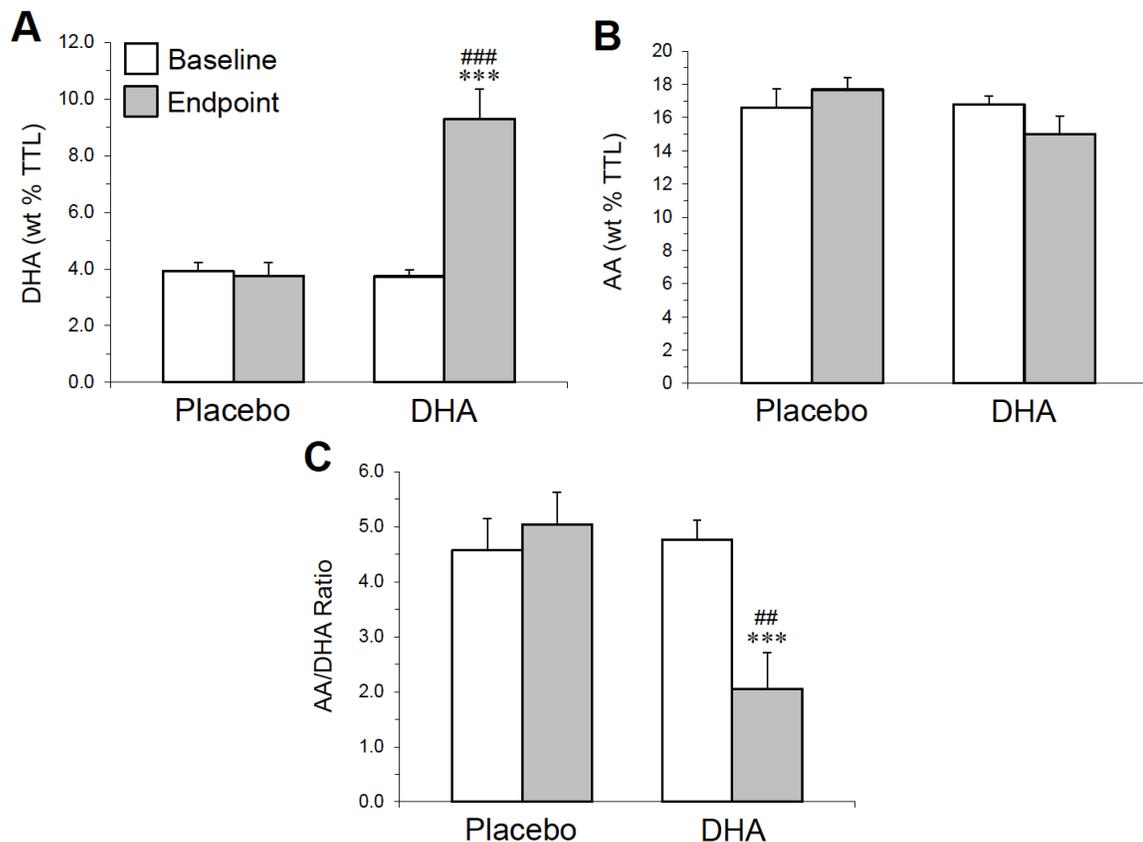
¹Values are group mean S.D, number, or percentage.²Two-tailed t-test or Chi-square (Placebo vs. DHA).

Figure 2: Erythrocyte membrane DHA (A), arachidonic acid (AA) (B) compositions (weight percent total fatty acid composition, wt % TTL), and the AA/DHA ratio (C) in ADHD patients treated with placebo or DHA (1,200 mg/d) at baseline and study endpoint (10 weeks). The time x treatment interaction was significant for DHA ($p \leq 0.0001$) and the AA/DHA ratio ($p = 0.007$). Values are group mean \pm S.E.M. *** $p \leq 0.0001$ vs. DHA baseline, ## $p \leq 0.001$, #### $p \leq 0.0001$ vs. placebo endpoint.

For erythrocyte DHA composition, the time by treatment interaction was significant ($p \leq 0.0001$), and DHA composition increased significantly between baseline and 10 weeks in subjects receiving DHA

(+60%, $p \leq 0.0001$) but not placebo (-4%, $p = 0.77$) (Figure 2A). At 10 weeks, mean erythrocyte DHA composition was 9.3% \pm 2.8% total fatty acids in the DHA group and 3.8% \pm 1.1% in the placebo group.

Erythrocyte arachidonic acid (AA, 20:4n-6) composition did not differ between treatment groups at baseline ($p=0.87$), and the time by treatment interaction was not significant ($p=0.16$) (Figure 2B). The AA/DHA ratio did not differ between treatment groups at baseline ($p=0.77$), and the time by treatment interaction was significant ($p=0.002$). The AA/DHA ratio decreased significantly from baseline in subjects receiving DHA (-57%, $p=0.0007$) but not placebo (+9%, $p=0.62$) (Figure 2C).

3.3. Safety and Tolerability

One patient randomized to placebo terminated study participation early (week 2) due to adverse events (excessive sweating, dilated pupils, and trouble sleeping). Otherwise there were no clinically significant treatment-emergent adverse events including suicidality reported, and no other patient discontinued treatment due to an adverse event. The time by treatment interaction was not significant for all vital signs. For the PFA test performed at week 2, there were no significant group differences for the collagen/epinephrine-induced platelet aggregation test

($p=0.46$) and collagen/adenosine triphosphate-induced platelet aggregation test ($p=0.95$).

3.4. ADHD Symptom Ratings

Patients randomized to placebo or DHA had similar ADHD-RS total scores, as well as inattention and hyperactivity/impulsivity (H/I) subscale scores, at baseline (Table 1). Change in ADHD-RS total score, and inattention and hyperactivity/impulsivity subscale scores, over the 10 week trial are presented in Figure 3. Baseline-endpoint reductions in ADHD-RS total score (Placebo: -33%, $p=0.01$; DHA: -29%, $p=0.05$), H/I subscale score (Placebo: -28%, $p=0.04$; DHA: -33%, $p=0.05$), and inattention subscale score (Placebo: -40%, $p=0.01$; DHA: -26%, $p=0.07$) were observed in both treatment groups, and the time by treatment interaction term was not significant for the ADHD-RS total score ($p=0.98$), H/I subscale score ($p=0.94$), or inattention subscale score ($p=0.91$).

3.5. Sustained Attention Performance

Measures of CPT-IP performance are presented in Table 2. At baseline, there were no significant group

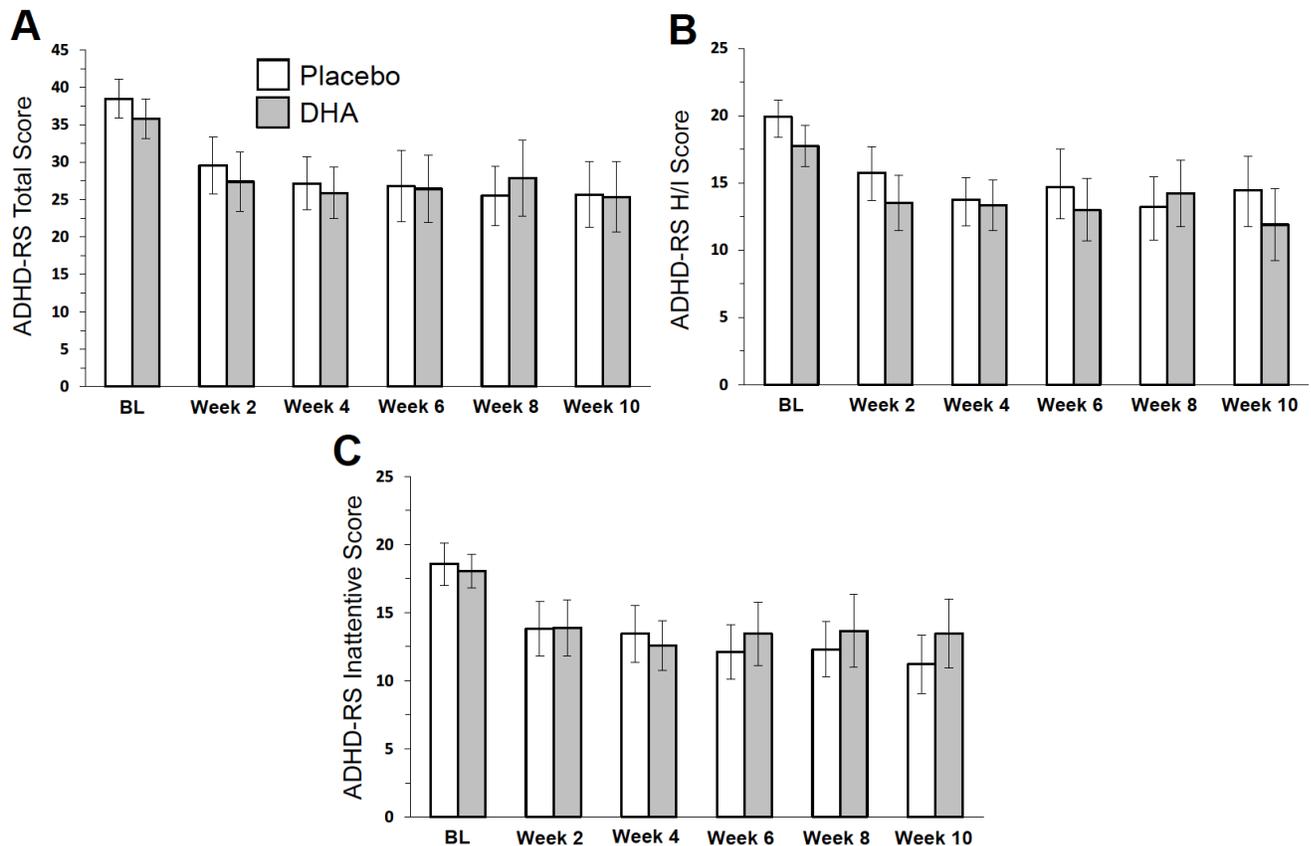


Figure 3: Overall ADHD symptom severity score (ADHD-RS total score) (A), hyperactivity/impulsivity (H/I) subscale score (B), and inattention subscale score (C) for ADHD patients treated with placebo or DHA over the 10 week trial. BL = baseline. Values are group mean \pm S.E.M.

Table 2: CPT-IP Task Performance

Variable ¹	Placebo		P-value ²	DHA		P-value ²	P-value ³
	Baseline	Endpoint		Baseline	Endpoint		
Percent correct	0.44 ± 0.09	0.40 ± 0.02	0.91	0.50 ± 0.09	0.46 ± 0.19	0.74	0.99
Discriminability	0.85 ± 0.03	0.85 ± 0.05	0.99	0.87 ± 0.03	0.86 ± 0.05	0.73	0.92
Commission errors	1.37 ± 1.10	0.81 ± 0.70	0.29	1.00 ± 1.60	0.91 ± 0.71	0.89	0.58
Reaction Time (msec)	617.2 ± 137.5	621.0 ± 179.2	0.96	539.0 ± 102.0	592.2 ± 140.0	0.43	0.65

¹Values are group mean ± S.D.

²Two-tailed *t*-test (baseline vs. endpoint).

³Two-way ANOVA – Time x Treatment Interaction.

differences for percent correct, commission errors, discriminability, or reaction time. At 10 weeks there were no significant group differences for any performance measure, and the time by treatment interaction was not significant for any performance measure.

3.6. fMRI

3.6.1. Voxelwise Analysis

There were no significant treatment group differences for baseline-endpoint change in voxelwise cortical activation patterns. Relative to baseline, patients treated with placebo exhibited significant reductions in the activation of several regions including the right cerebellar vermis, left caudate, left cingulate gyrus, and left inferior and middle frontal gyrus at study endpoint (Figure 4A). Patients treated with DHA exhibited significant reductions in the activation in the

left putamen and left medial frontal gyrus (Figure 4B). Neither group exhibited a baseline-endpoint increase in cortical activation.

3.6.2. ROI Analysis

A significant time by treatment interaction was observed for the left amygdala ($p=0.03$). Endpoint amygdala activation in DHA-treated patients was significantly lower than endpoint activation in placebo-treated patients ($p=0.01$), and there was a trend for a baseline-endpoint decrease in left amygdala activation in DHA-treated patients ($p=0.07$) (Figure 5A,B). Although the time by treatment interaction was not significant, endpoint activation in the left caudate of DHA-treated patients was significantly lower than endpoint activation in placebo-treated patients ($p=0.007$) (Figure 5C,D). The time by treatment interaction was not significant for the remaining ROIs.

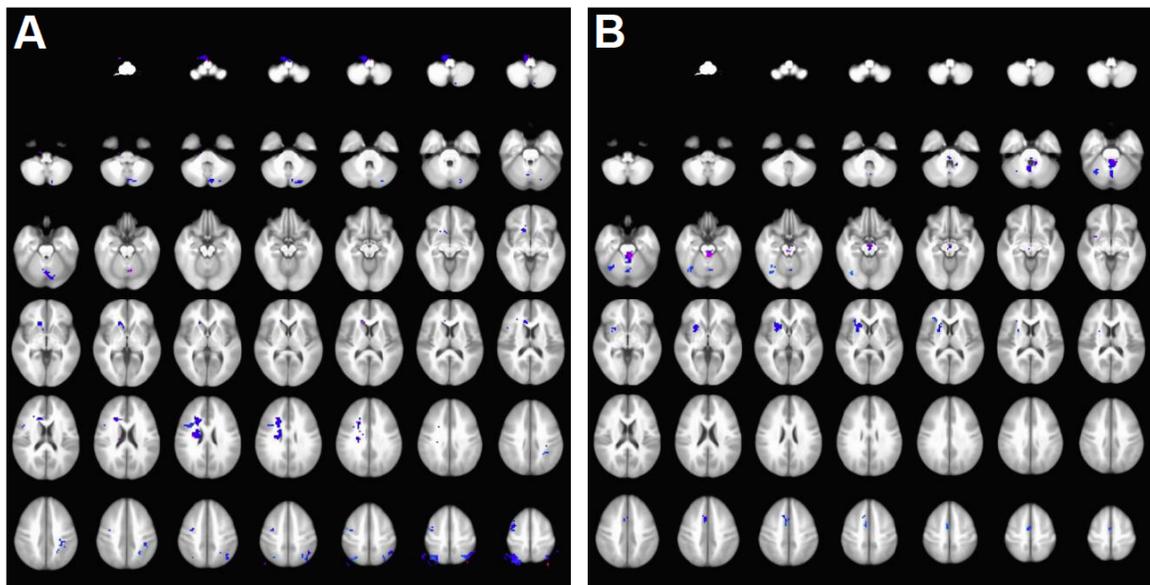


Figure 4: Statistical parametric maps illustrating baseline-endpoint reductions in regional activation during sustained attention in patients receiving placebo (A) or DHA (B). Images are overlaid on a T1-weighted anatomic image, and the color gradient (blue → purple) reflects increasing statistical significances in baseline-endpoint reduction in activation within each treatment group ($p \leq 0.05$ corrected).

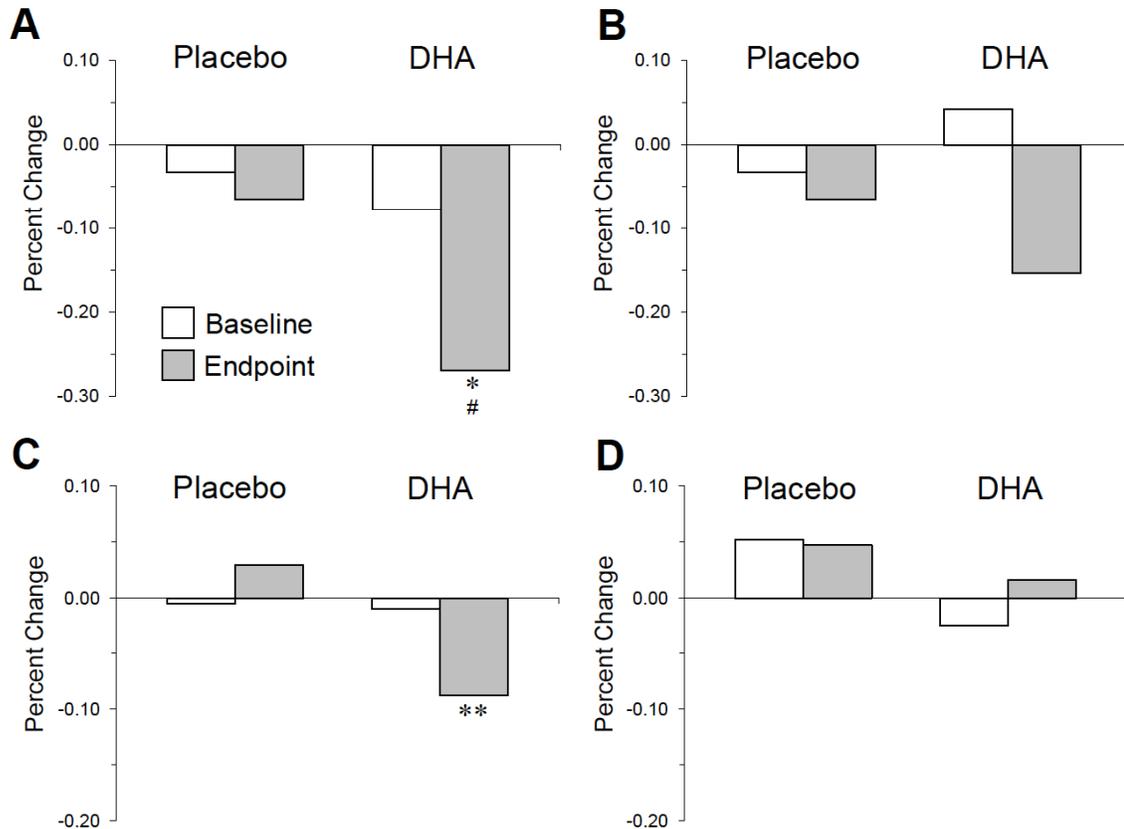


Figure 5: Mean activation in the left and right amygdala (A,B) and left and right caudate (C,D) during performance of “attentional” blocks relative to the “control” blocks at baseline and study endpoint (week 10) in ADHD patients treated with placebo or DHA. * $p=0.01$ vs. placebo endpoint, # $p=0.07$ vs. DHA baseline in (A), and ** $p=0.007$ vs. placebo endpoint in (C).

3.6.3. Functional Connectivity Analysis

At baseline patients randomized to DHA did not exhibit significantly greater connectivity between the pregenual ACC (seed) or subgenual ACC (seed) and any region compared with patients randomized to placebo. At study endpoint, patients treated with DHA exhibited significantly greater positive connectivity between the left pregenual ACC and the right inferior parietal lobe and right postcentral gyrus compared to placebo (Figure 6A), and significantly greater positive connectivity between the right pregenual ACC and the right inferior parietal lobe and right postcentral gyrus compared to placebo (Figure 6B). Patients treated with DHA also exhibited significantly greater positive connectivity between the left subgenual ACC and the right cingulate gyrus (anterior and posterior), right precentral gyrus, and DLPFC (BA 9) compared to placebo at study endpoint, and significantly greater negative connectivity between the right subgenual ACC and the bilateral cuneus and left precuneus compared to placebo.

Based on ROI data indicating greater endpoint reductions in left amygdala activation in DHA-treated

patients, we performed exploratory analyses contrasting endpoint DHA and placebo groups using the left amygdala as the seed-region. Using a less stringent voxelwise threshold ($p \leq 0.02$), but not corrected $p \leq 0.05$, the DHA group exhibited greater positive connectivity between the left amygdala and the left caudate, left cingulate gyrus, left pre and post central gyrus, and right superior temporal gyrus, and greater negative connectivity with the left parahippocampal gyrus and hippocampus, right inferior frontal gyrus, left inferior and middle occipital gyrus relative to the placebo group.

3.7. DTI

There were no significant treatment group differences for baseline-endpoint change in FA, MD, AD, and RD in the right or left corpus callosum genu or forceps minor (Table 3). There were however notable trends with large effect sizes for a baseline-endpoint reduction in MD (-17%, $p=0.08$, $d = 1.0$) and RD (-26%, $p=0.09$, $d = 1.1$) in the left corpus callosum genu of patients receiving DHA, whereas little change in MD (-2%, $p=0.83$, $d = 0.12$) and RD (-9%, $p=0.59$, $d = 0.29$) was observed in patients receiving placebo. There was

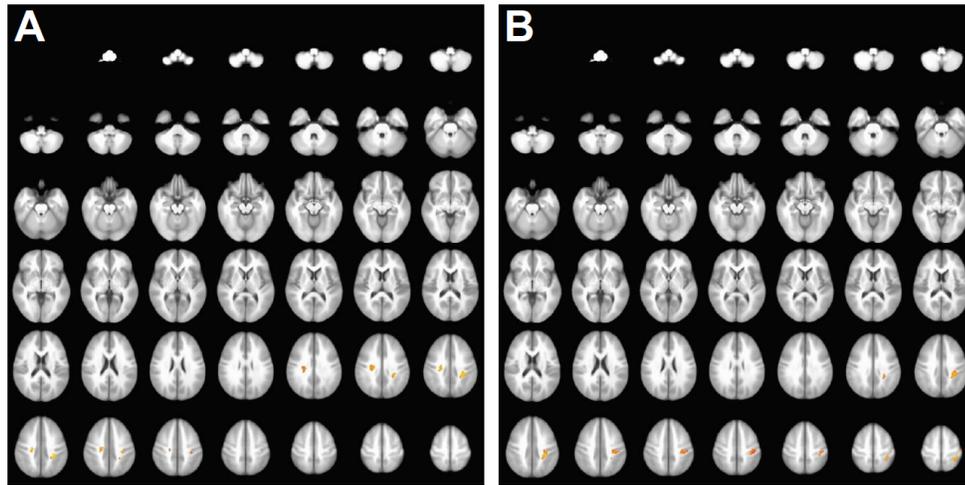


Figure 6. Regions exhibiting greater functional connectivity with the left (A) and right (B) pregenual ACC (seed-region) of patients receiving DHA compared with placebo while performing the CPT-IP task at study endpoint (all $p \leq 0.05$ corrected). Images are overlaid on a T1-weighted anatomic image. Color gradient (yellow → orange) reflects greater statistical significance of positive connectivity in the DHA group relative to the placebo group.

Table 3: Corpus Callosum White Matter Integrity

Variable ¹	Placebo		P-value ²	DHA		P-value ²	P-value ³
	Baseline	Endpoint		Baseline	Endpoint		
Left Genu							
FA	0.68 ± 0.14	0.71 ± 0.14	0.69	0.64 ± 0.13	0.73 ± 0.16	0.25	0.57
AD	1.52 ± 0.17	1.54 ± 0.09	0.79	1.41 ± 0.17	1.49 ± 0.19	0.43	0.64
MD	0.82 ± 0.14	0.81 ± 0.18	0.83	0.87 ± 0.17	0.73 ± 0.12	0.08	0.26
RD	0.50 ± 0.15	0.45 ± 0.18	0.59	0.50 ± 0.12	0.37 ± 0.15	0.09	0.45
Left Forceps Minor							
FA	0.45 ± 0.07	0.48 ± 0.06	0.44	0.43 ± 0.10	0.45 ± 0.09	0.68	0.67
AD	1.21 ± 0.05	1.18 ± 0.06	0.21	1.18 ± 0.05	1.18 ± 0.07	0.85	0.48
MD	0.79 ± 0.04	0.77 ± 0.05	0.47	0.80 ± 0.08	0.77 ± 0.09	0.53	0.81
RD	0.57 ± 0.06	0.55 ± 0.05	0.45	0.60 ± 0.10	0.57 ± 0.10	0.55	0.86
Right Genu							
FA	0.56 ± 0.17	0.58 ± 0.15	0.89	0.63 ± 0.15	0.66 ± 0.07	0.71	0.89
AD	1.44 ± 0.12	1.62 ± 0.23	0.06	1.36 ± 0.16	1.58 ± 0.36	0.13	0.86
MD	0.78 ± 0.12	0.88 ± 0.17	0.22	0.89 ± 0.19	0.93 ± 0.23	0.71	0.67
RD	0.50 ± 0.18	0.57 ± 0.18	0.48	0.56 ± 0.17	0.58 ± 0.21	0.84	0.72
Right Forceps Minor							
FA	0.43 ± 0.08	0.46 ± 0.09	0.33	0.41 ± 0.05	0.43 ± 0.07	0.49	0.93
AD	1.24 ± 0.05	1.24 ± 0.05	0.92	1.22 ± 0.05	1.24 ± 0.11	0.64	0.73
MD	0.82 ± 0.05	0.79 ± 0.05	0.24	0.84 ± 0.07	0.83 ± 0.08	0.93	0.55
RD	0.62 ± 0.08	0.57 ± 0.10	0.29	0.62 ± 0.06	0.61 ± 0.09	0.86	0.48

¹Values are group mean ± S.D.

²Two-tailed t-test (baseline vs. endpoint).

³Two-way ANOVA – Time x Treatment Interaction.

also a trend with a medium effect size for a baseline-endpoint increase in FA in the left corpus callosum

genu of patients receiving DHA (+13%, $p=0.25$, $d = 0.67$) but not placebo (+4%, $p=0.69$, $d = 0.22$). A trend

with a large effect size for a baseline-endpoint increase in AD in the right corpus callosum genu was observed for patients receiving placebo (+12%, $p=0.06$, $d = 1.1$).

4. DISCUSSION

The present pilot study evaluated the effects of 10-week DHA supplementation on cortical attention network integrity and attentional symptoms in medication-free children with ADHD. In the present study, baseline-endpoint change in measures of sustained attention performance, ADHD symptom severity, and voxelwise cortical activation patterns during performance of the sustained attention task did not differ between DHA- and placebo-treated patients. However, in the ROI analysis DHA-treated patients, but not placebo-treated patients, exhibited significantly greater baseline-endpoint reductions in left amygdala and left caudate activation. DHA-treated patients also exhibited significantly greater event-related functional connectivity between the bilateral pregenual ACC and inferior parietal lobe and right postcentral gyrus compared to placebo, and greater functional connectivity between the left subgenual ACC and right cingulate gyrus (anterior and posterior), right precentral gyrus, and DLPFC (BA 9) compared to placebo at study endpoint. The DTI analysis observed trends with large effect sizes for baseline-endpoint reductions in MD and RD in the left corpus callosum genu of DHA-treated patients, and a trend for a baseline-endpoint increase in AD right genu of placebo-treated patients. These preliminary findings suggest that short-term DHA supplementation may be associated with subtle changes in cortical attention networks of medication-free children with ADHD.

Previous evidence indicates that ADHD patients exhibit reduced event-related activation in prefrontal and cingulate regions during performance of cognitive tasks [7-9], and that DHA supplementation significantly increased prefrontal activation during sustained attention in healthy children [25]. It was therefore predicted that DHA supplementation would increase prefrontal activation in children with ADHD. In the present study we did not observe significant group differences in baseline-endpoint change in voxelwise cortical activation patterns. This finding is consistent with another study that similarly found that 16-week supplementation with EPA+DHA (1,300 mg/d) did not significantly alter cortical activation patterns during performance of a Go/NoGo task in medication-withdrawn ADHD patients despite observing improvements in parent-related symptoms of

inattention [26]. Taken together, these findings suggest that reduced event-related activation in prefrontal and cingulate regions commonly observed in patients with ADHD are not reversible with short-term DHA supplementation.

An unanticipated finding from the fMRI ROI analysis was that DHA-treated patients exhibited significantly greater baseline-endpoint reductions in left amygdala activation during performance on the CPT-IP task. Although the CPT-IP task is emotion-neutral, previous fMRI studies have observed reductions in amygdala activation during performance of the CPT-IP task in healthy subjects [53] and that increasing attentional demands are associated with greater decreases in amygdala activation [54]. Therefore, the greater baseline-endpoint reduction in left amygdala activation observed in DHA-treated patients may reflect increased attention-mediated negative feedback on amygdala activation. It is also relevant that medication-free ADHD children exhibit left amygdala hyper-connectivity with the affective network compared with healthy controls [11], and higher parent ratings of emotional lability were associated with greater positive functional connectivity between the amygdala and rostral ACC in ADHD children [12]. These findings suggest that DHA supplementation may potentially reduce AMYGDALA activation in children with ADHD by altering connectivity within affective and frontal networks, and may be relevant to emotional dysregulation commonly observed in ADHD children [55].

We previously reported that low erythrocyte DHA levels were associated with reduced event-related functional connectivity between the ACC and prefrontal attention networks compared with high erythrocyte DHA levels in a cohort of typically developing children [56]. Moreover, a non-human primate study found that *n*-3 fatty acid insufficiency during perinatal development was associated with widespread reductions in resting-state functional connectivity in frontal cortical networks [57]. Based on these findings, it was predicted that DHA supplementation would increase functional connectivity within prefrontal attention networks in children with ADHD. In the present study we found DHA-treated patients exhibited significantly greater event-related functional connectivity between the bilateral pregenual ACC (seed) and inferior parietal lobe and right postcentral gyrus, and greater functional connectivity between the left subgenual ACC (seed) and right cingulate gyrus (anterior and posterior), right precentral gyrus, and DLPFC (BA 9) compared to placebo at study endpoint. In view of prior evidence

that psychostimulant treatment also increases connectivity among cingulate and parietal lobe networks [58,59], greater event-related functional connectivity observed in DHA-treated patients may represent an early normalization of connectivity deficits that warrants additional investigation.

Evidence from DTI studies indicate that ADHD is associated with widespread reductions in white matter microstructural integrity [6], and emerging translational evidence suggests that DHA may promote white matter microstructural integrity [24,51,52,60]. Although we did not find significant group differences in baseline-endpoint change in FA, MD, AD, or RD, we did observe trends with large effect sizes for decreases in MD and RD in the left corpus callosum genu in ADHD patients receiving DHA but not placebo. Animal studies suggest that an elevation in RD is a valid measurement of demyelination or dysmyelination, whereas a decrease in AD is more closely associated with axonal damage [61-63]. It is also notable that non-significant reductions in RD and MD were restricted to the left hemisphere, and a prior study found that DHA-deficient rats exhibit lateralized changes in RD and MD [60]. These preliminary findings encourage larger DTI studies to investigate whether DHA supplementation can reverse white matter integrity deficits in ADHD.

This imaging trial has two important limitations. First, the small number of subjects randomized to each treatment group completing the 10-week study may not be a representative sample of ADHD children, and was likely underpowered to detect small to moderate effect sizes. Therefore, the present findings should be considered preliminary until replicated in a larger cohort of ADHD patients. Second, the duration of DHA intervention was relatively short (10 weeks), and more robust changes in outcomes may have been observed following a longer period of DHA supplementation. Therefore, larger and longer controlled imaging studies are warranted to replicate and extend these findings. Strengths of this study include the well-characterized cohort of predominantly stimulant-naïve ADHD youth, the randomized double-blind placebo-controlled trial design, well-matched treatment groups, and use of different neuroimaging techniques to investigate cortical attention network integrity.

The results of the present preliminary randomized double-blind placebo-controlled trial suggest that 10-week DHA supplementation is associated with subtle changes in cortical attention networks in children with ADHD that are not accompanied by improvements in sustained performance or ADHD symptoms. The

results reveal greater reductions in amygdala and caudate activation, increases in event-related ACC functional connectivity within the cortical attention network, and trends for improvements in white matter microstructural integrity in children with ADHD receiving DHA supplementation. These findings provide preliminary targets for larger and longer controlled imaging studies which are necessary to better characterize the role of DHA in cortical attention network dysfunction in children with ADHD.

ACKNOWLEDGMENTS

This work was supported by an investigator-initiated trial grant from DSM Nutritional Products, LLC, the producer of the algal DHA used in this study. The authors thank Barbara Kaplan for her support of this project.

CONFLICT OF INTEREST STATEMENT

R.K.M. has received research support from NARSAD, Martek Biosciences Inc, Ortho-McNeil Janssen, AstraZeneca, Eli Lilly, Kyowa Hakko Bio Co., LTD, and the Inflammation Research Foundation (IRF), was a member of the IRF scientific advisory board, and served as a paid consultant for VAYA Pharma Inc., and Vifor Pharma Inc. DSM Nutritional Products, LLC did not have any role in the design, implementation, analysis or interpretation of the research. The other authors do not have any relevant financial disclosures.

CONTRIBUTORS

Dr. McNamara designed the study and wrote the manuscript. Mrs. Schurdak performed the gas chromatography, and Mr. Weber and Mr. Tallman performed the imaging analyses. Mr. Blom performed the statistical analyses. Dr. Patino participated in the clinical management of patients. All authors contributed to and have approved the final manuscript.

ROLE OF FUNDING SOURCE

Funding for this study was provided and investigator-initiated trial grant from DSM Nutritional Products, LLC. DSM Nutritional Products had no further role in study design; in the collection, analysis and interpretation of data; in the writing of the report; and in the decision to submit the paper for publication.

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Received on 03-11-2017

Accepted on 21-11-2017

Published on 04-01-2018

[DOI: https://doi.org/10.6000/1929-5634.2017.06.04.3](https://doi.org/10.6000/1929-5634.2017.06.04.3)