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Long chain polyunsaturated fatty acid supplementation in the first year of life affects brain function, structure, and metabolism at age nine years

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Abstract

The present study sought to determine whether supplementation of long-chain polyunsaturated fatty acids (LCPUFA) during the first year of life influenced brain function, structure and metabolism at 9 years of age. Newborns were randomly assigned to consume formula containing either no LCPUFA (control) or formula with 0.64% of total fatty acids as arachidonic acid (ARA; 20:4n6) and variable amounts of docosahexaenoic acid (DHA; 22:6n3) (0.32%, 0.64%, or 0.96% of total fatty acids) from birth to 12 mo. At age 9 years (± 0.6) 42 children enrolled in a follow up multimodal magnetic resonance imaging (MRI) study including functional (fMRI, Flanker task), resting state (rsMRI), anatomic, and proton magnetic resonance spectroscopy (¹H MRS). fMRI analysis using the Flanker task found that trials requiring greater inhibition elicited greater brain activation in LCPUFA-supplemented children in anterior cingulate cortex (ACC) and parietal regions. rsMRI analysis showed that children in the 0.64% group exhibited greater connectivity between prefrontal and parietal regions compared to all other groups. In addition, voxel-based analysis (VBM) revealed that the 0.32% and 0.64% groups had greater white matter volume in ACC and parietal regions compared to controls and the 0.96% group. Finally, ¹H MRS data analysis identified that N-acetylaspartate (NAA) and *myo*-inositol (mI) were higher in LCPUFA groups compared to the control group. LCPUFA supplementation during infancy has lasting effects on brain structure, function, and neurochemical concentrations in regions associated with

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attention (parietal) and inhibition (ACC), as well as neurochemicals associated with neuronal integrity (NAA) and brain cell signaling (mI).

Keywords

attention; brain imaging; long-chain polyunsaturated fatty acids; early life programming

1 | INTRODUCTION

The DIAMOND (DHA Intake And Measurement of Neural Development) study was designed to evaluate the effect of 3 different levels of long-chain polyunsaturated fatty acids (LCPUFA) supplementation on visual acuity and cognitive outcomes in the first year of life (Birch et al., 2010). The parent study, a randomized clinical trial (NCT00753818), began in 2002 and was completed in 2004. LCPUFAs began to be added to US infant formula beginning in 2002, making it possible for this study to measure a true formula-fed control group with no LCPUFA supplementation during infancy. This is no longer possible given most formula-fed infants in the US now receive LCPUFA.

Newborn infants (n=159) were randomized to one of four cows' milk-based term infant formula with the same nutrient amounts and ingredients except for LCPUFAs. Infants in the control group were fed formula without added LCPUFA. Infants in the LCPUFA supplemented groups were fed formulas with added arachidonic acid (0.64%; ARA) and one of 3 different levels of docosahexaenoic acid (0.32%, 0.64% or 0.96%; DHA) as a percent of total dietary fatty acids from birth to 12 months of age (Birch et al., 2010). For brevity, the four groups will hereafter be referred to as control, 0.32%, 0.64%, and 0.96%.

The parent study found that LCPUFA supplementation had a beneficial effect on visual, autonomic and attention development in the first year of life (Birch et al., 2010; Colombo et al., 2011). Additional long-term follow up studies found that early life supplementation had positive effects on several important measures of executive function during early childhood and on measures of IQ at the threshold of school age (Colombo et al., 2013). At age 5.5 years, children in the supplemented groups showed differences in brain electrophysiologic responses while performing a task requiring response inhibition (Liao et al., 2017). These studies established that manipulation of the diet in the first year of life had a long-term effect on cognitive and brain functional outcomes, especially for mechanisms related to attention, rule learning and inhibition.

Given the previously observed behavioral and cognitive benefits of LCPUFA supplementation in these children, we wanted to explore the associated functional, structural, and metabolic mechanisms underpinning these observations within the brain using a multi-modality brain imaging approach. Our first goal was to identify biological correlates associated with attention and cognitive control that we found previously to be improved with LCPUFA (Colombo et al., 2013). We chose the Eriksen Flanker task (Eriksen & Eriksen, 1974) because it has been shown to be a measure of conflict detection, attentional focusing, executive function, and inhibition that has been widely used in cognitive control literature (Bunge, Dudukovic, Thomason, Vaidya, & Gabrieli, 2002). Based on our earlier behavioral

findings, we hypothesized that LCPUFA supplementation would be associated with increased activation in inhibitory areas such as the anterior cingulate cortex (ACC) in supplemented compared to control children. This hypothesis is supported by evidence showing differences in functional MRI in 8–10 year old boys randomized to placebo or low (400 mg/day) vs. high (1200 mg/day) DHA for 8 weeks (McNamara et al., 2010). Boys in this study performed a sustained attention task (CPT-IP). Red blood cell DHA was positively correlated with functional cortical activity and inversely correlated with reaction time while performing the task.

Our second goal was to identify possible structural and metabolic mediators of these effects using functional connectivity, and structural and metabolic imaging acquisition and analysis. Resting state functional connectivity magnetic resonance imaging (rsfMRI), is a technique that examines how brain regions spontaneously function together. The method has been used to investigate the functional organization of the brain and identify distinct neural “networks” (correlated signals observed in spatially distinct brain regions). Studies have shown that as the brain matures, some regions become more tightly linked (Fair et al., 2008). We hypothesized that (i) LCPUFA supplementation would result in a more mature connectivity pattern, i.e., strengthening of long-range functional connections with development and (ii) that LCPUFA supplementation would result in greater functional connectivity between cognitive control regions and attention regions. This hypothesis was largely based on rsfMRI evidence from rhesus macaques with life-long LCPUFA dietary manipulation (Grayson, Kroenke, Neuringer, & Fair, 2014). Pregnant rhesus macaques were fed either an n-3 deficient diet, a high alpha-linoleic acid (ALA) diet (the essential fatty acid precursor of n-3 LCPUFA), or a diet rich in n-3 LCPUFA designed to mimic rhesus breast milk that included both DHA and eicosapentaenoic acid (EPA), ARA and ARA. Offspring were assigned to the same diet group as their mothers received during pregnancy and remained on the diets until they were 17–19 years of age. Functional connectivity was significantly decreased in the n-3 deficient group within the visual system and higher order cortical pathways. Connectivity in the group receiving the n-3 precursor, ALA, was better than the n-3 deficient group but reduced compared to the group receiving the n-3 enriched LCPUFA diet. A more recent study in 8–10 year old boys revealed reduced event-related functional connectivity in cortical attention networks of children with low-DHA status (Almeida, Jandacek, Weber, & McNamara, 2017). Despite differences in species, MRI methods and dietary manipulation between these two studies, they both provide evidence that LCPUFA biostatus plays a role in cortical network maturation.

Voxel-based morphometry (VBM), is a widely-used method of measuring quantities of gray and white matter regionally in the brain. We conducted VBM analyses to determine whether postnatal LCPUFA supplementation was associated with brain volume in various regions of the brain during development. We hypothesized that early life LCPUFA supplementation would be positively associated with regional gray and white matter volume, specifically in the medial frontal and ACC. Studies using VBM to investigate effects of LCPUFA have been limited to adults and have generally found increased grey matter volume (Bos, van Montfort, Oranje, Durston, & Smeets, 2016). One group in Norway tested the long-term effects of early life LCPUFA supplementation in very low birth weight children by measuring brain volume by MRI (Almaas et al., 2015) and diffusion tensor imaging (Almaas

et al., 2016). Infants were randomized to placebo or DHA plus ARA (0.86% and 0.91% total fatty acids respectively) for an average of 9 weeks after birth. At 8 years of age, there were no group differences in behavior, cognitive measures, white matter microstructure, segmental brain volumes, cerebral cortex volume, area or thickness. Like the study reported here, the investigators were interested in long-term outcomes of early life supplementation, however, there are differences in between this study and theirs; e.g., newborn weight (normal vs. very low birthweight), supplement duration (1 year vs. 9 weeks), and higher LCPUFA biostatus in Norway vs. Midwestern US that will limit making direct comparisons.

In vivo magnetic resonance spectroscopy (MRS) provides a unique tool to investigate neurochemical changes during brain development (Vigneron, 2006; Wang et al., 2016) and the effects of nutrients on the brain (Joncquel-Chevalier Curt et al., 2015; Koch et al., 2002; McNamara et al., 2013). The effect of low DHA intake on neurochemicals has been reported in healthy children with age between 8 – 10 years old (McNamara et al., 2013). McNamara et al., reported lower neurochemical concentrations, including N-acetylaspartate (NAA) and myo-inositol (mI) in children with low compared with high DHA intake. Although magnetic resonance spectroscopy (^1H MRS) has not been used to investigate the effects of LCPUFA supplementation in the developing human brain, MRS-based metabolic imaging provides unique quantitative biomarker evidence of specific cellular and molecular mechanisms by which LCPUFAs might improve cognitive function. Based on previous findings (McNamara et al., 2013), we hypothesized that LCPUFA supplementation would result in group differences in brain metabolites, indicating that LCPUFA supplementation might improve brain energetics, cell membrane integrity, and cerebral antioxidant capacity, each of which would likely contribute to neuronal integrity.

2 | METHOD

2.1 Subjects

This study was approved by the Human Subjects Committees at the University of Kansas Medical Center (protocol #13791; NCT02076048) and all applicable institutional and governmental regulations concerning the ethical use of human volunteers were followed and all subjects gave informed consent. Eighty-six possible participants and their families were solicited between April 2012 and February 2013 through use of a targeted letter. To be eligible to receive the letter, children must have previously completed the full 12 months of formula feeding in the parent trial (Birch et al., 2010) and could not have any serious health problems that would affect their cognition or ability to participate in brain scans. The formulas used in the parent trial had the same nutrient amounts and ingredients except for LCPUFAs. The content of other major fatty acids was similar between all 4 formulas. Forty-nine (57%) families expressed interest in the imaging follow up study and 42 were enrolled (control $n=11$, 0.32% $n=10$, 0.64% $n=8$, and 0.96% $n=13$). The remaining 7 families, while interested, could not commit to the two days of study procedures. Enrolled participants (17 male) were between the of ages 8 and 10 years (9 years $\pm$ 0.6)

2.2 | Study Visits

In line with data collected as part of the original DIAMOND studies from infancy through 6 years of life we collected measures of growth (height, weight and body composition), current dietary practices (24-hour diet recall), genetic markers that affect de novo DHA synthesis and methylation status, cardiovascular metrics (blood pressure, heart rate) and cognitive development (Wechsler Intelligence Scale for Children, 4th edition; WISC-IV) on the first day of study procedures. At that same visit, children were given an opportunity to tour the imaging center to determine whether they wanted to participate in the second day imaging arm of the study.

At the second study visit, MR imaging and magnetoencephalography (MEG) data were recorded. For brevity, only the results of the WISC-IV and MR imaging are presented here. MEG and other study outcome measures will be reported later. Four children were claustrophobic and could not complete the MR scan, leaving 38 active participants who underwent multimodal MR imaging including task-based fMRI (Flanker Task), resting state fMRI (rsfMRI), structural MRI, and ¹H MRS.

2.3 | Standardized IQ test (WISC-IV)

The WISC-IV is among the most widely-used and best-standardized tests of childhood intelligence available. It is valid for ages 6.0 through 16.9 years and can be administered in approximately 75 minutes. The WISC-IV has 10 core subtests that combine to form four different domain indices [Verbal Comprehension (VCI), Perceptual Reasoning (PRI), Processing Speed (PSI), and Working Memory (WMI)], as well as a Full Scale IQ (FSIQ).

Statistical analyses: SPSS 17 was used for all statistical tests. Continuous demographic and cognitive variables were compared between the individual groups using ANOVA. Chi-square was used to compare categorical variables between groups.

2.4 | Imaging Data Acquisition

All MR scans were performed on a 3-T Siemens Skyra scanner (Siemens, Erlangen, Germany) fitted with a 20-channel head/neck coil. MR scans were performed in two sessions: one for the functional and structural MRI and the other for ¹H MRS, to achieve a manageable scan time per session for children. During the first MR session, following automated scout image acquisition and shimming procedures performed to optimize field homogeneity, a T1-weighted structural scan was completed (3D magnetization prepared rapid acquisition gradient echo (MP-RAGE) sequence, repetition time/echo time [TR/TE] = 2300/2.01 ms, flip angle = 9°, field of view [FOV] = 256 × 256 mm, 176 slices, slice thickness = 1.2 mm). This scan was used for VBM volumetric analysis of brain structure, slice localization for the functional scans, Talairach transformation, and co-registration with fMRI and rsfMRI data. Following structural scans, two gradient echo blood oxygen level dependent (BOLD) scans were acquired while participants completed the fMRI Flanker task in 50 contiguous oblique axial slices at a 40° angle (TR/TE = 2700/25 ms, flip angle = 90°, FOV = 232 mm, matrix = 80 × 80, slice thickness = 3 mm, in-plane resolution = 2.9 × 2.9 mm). All participants were positioned in the scanner so that the angle of the AC-PC plane was between 17° and 22° to the scanner coordinate space ensuring that the 40° slice

acquisition angle was constant for all subjects (Martin, Cox, Brooks, & Savage, 2014). Resting-state fMRI scans were acquired as gradient echo BOLD scans in 50 contiguous axial slices at an angle parallel to the AC-PC line (TR/TE = 3000/25 ms, slice thickness = 3 mm, in-plane resolution = 2.9×2.9 mm, 105 volumes). During the second session, a T1-weighted structural scan (3D MP-RAGE sequence, repetition time/echo time [TR/TE] = 2300/2.95 ms, flip angle = 9° , field of view [FOV] = 240×256 mm, 176 slices, slice thickness = 1.2 mm) was obtained for accurate placement of the ^1H MRS slab. Standard ^1H MRS data were acquired from an axial slab positioned superior to the corpus callosum (Figure 2B) using a point resolved spectroscopy (PRESS) localized ^1H MRS technique (TR/TE = 2000/30 ms, matrix size = 12×12 , field of view = 160 mm, slice thickness = 15 mm, volume of interest = $80 \times 80 \times 15$ mm).

2.4.1 | Flanker Task (fMRI)—The fMRI paradigm included an inhibition task known as the flanker task (Bunge et al., 2002; Eriksen & Eriksen, 1974) during which participants are presented with a row of arrows facing different directions and asked to respond with a button press in the direction the center arrow (target) is pointing while attempting to ignore the surrounding arrows (e.g., press the right button with the right thumb if the arrow pointed right and press the left button with the left thumb if the arrow pointed left). Children were given a single practice session of 10 trials prior to entering the scanner to assure that they understood the instructions. Three experimental conditions were presented: 1) congruent trials on which the center arrow pointed the same direction as the surrounding arrows; 2) control trials on which the center arrow was surrounded by diamonds; and 3) incongruent trials on which the center arrow pointed the opposite direction as the surrounding arrows (Figure 1D). Participants completed a total of two fMRI runs, each consisting of 25 trials per experimental condition. Trials were presented pseudo-randomly to prevent the same type of flanker from appearing more than three consecutive times. Visual stimuli were presented using E-Prime 2.0 software (Psychology Software Tools, Pittsburgh, PA). Stimulus presentation time was 800 ms, followed by a 300 ms blank screen, with a random duration inter-stimulus interval between 1600 ms and 14000 ms. Participants had up to 1600 ms to respond to each stimulus.

2.4.2 | Flanker Behavioral Data Analysis—The behavioral analysis included all 38 participants who completed the Flanker task. Mean reaction times and accuracy were computed for each participant for the Congruent and Incongruent conditions. ANOVA was performed in SPSS to determine interactions between Condition (Incongruent, Congruent trials) and Group (control, 0.32%, 0.64%, and 0.96%).

2.4.3 | fMRI Data Analysis—Nine of the 38 subjects were excluded from the fMRI analysis due to low accuracy on the Flanker task ($< 75\%$) indicating that they were not able to discriminate between the conditions. The fMRI analysis therefore included 29 participants (control $n=8$, 0.32% $n=8$, 0.64% $n=6$, and 0.96% $n=7$). Data pre-processing and statistical analysis were performed in Analysis of Functional Neuroimages software (AFNI; Medical College of Wisconsin (Cox, 1996, 2012; Pauli et al., 2016). The fMRI images were realigned to the third slice collected in each run, to correct for motion for each run for every subject. Timepoints during which participants moved more than 0.3 mm within a TR (2700

ms) were censored. No significant motion or censoring differences were found between groups ($[F(3,25)=1.98, p=0.14]$; control $M=.08$ $SD=0.03$, 0.32% $M=0.07$ $SD=0.02$, 0.64% $M=0.09$ $SD=0.02$, 0.96% $M=0.06$ $SD=0.01$). The images were spatially smoothed with a 4 mm FWHM Gaussian blur. Data were resampled to a $2.5 \times 2.5 \times 2.5$ mm resolution. Functional and anatomic images were aligned and normalized to Talairach and Tournoux's stereotaxic atlas (Talairach & Tournoux, 1988). Regressors representing the experimental conditions of interest (Congruent, Control and Incongruent) as well as a regressor representing incorrect trials were modeled with a hemodynamic response filter and entered into the multiple-regression analysis using a random-effects model.

Statistical analyses: The primary data analysis focused on whole-brain voxelwise ANOVA to determine differences in brain activation during inhibition (Incongruent minus Congruent trials) across the four groups (control, 0.32%, 0.64%, and 0.96%). To correct for multiple comparisons, the spatial autocorrelation function (acf) option was used in AFNI's 3dFWHMx to estimate intrinsic smoothness and 3dClustSim to estimate probability of false positives (Cox, Chen, Glen, Reynolds, & Taylor, 2017; Eklund, Nichols, & Knutsson, 2016). This resulted in a cluster size of at least 2203 mm^3 ($p_{corrected} < .05$; $p_{voxelwise} < .05$).

2.4.4 | rsMRI Data Analysis—The rsMRI analysis included 38 participants (control $n=9$, 0.32% $n=10$, 0.64% $n=8$, 0.96% $n=11$). Data preprocessing and statistical analysis were conducted using AFNI (Cox, 1996, 2012; Pauli et al., 2016), and a modified version of the ANATICOR method, developed by Jo and colleagues, implemented in `afni_restproc.py` (Jo et al., 2013). The first four volumes of the functional scans were removed and a de-spiking interpolation algorithm (*i.e.*, 3dDespike) was used to remove any transient signal spikes from the data. The volumes were slice time corrected and co-registered to the first volume (which was registered to the anatomical scan). Several nuisance variables were measured [*i.e.*, linear trends and derivatives, six motion parameters (3 translations, 3 rotations), average ventricle signal, and average local white matter signal from a 15mm spherical neighborhood surrounding gray matter voxels (3dLocalstat)]. The nuisance variables' predicted time course was constructed and then subtracted from each resting-state voxel time course using multiple regression, yielding a residual time course for each voxel. The residual images were smoothed with a 6 mm FWHM Gaussian kernel, resampled to a $2 \times 2 \times 2$ mm grid, and spatially transformed to stereotaxic space conforming to the Talairach and Tournoux atlas (Talairach & Tournoux, 1988).

Further motion correction procedures (*i.e.*, scrubbing) were utilized to reduce false group differences due to uncontrolled subject motion (Caballero-Gaudes & Reynolds, 2017; Power, Barnes, Snyder, Schlaggar, & Petersen, 2012, 2013). The six motion parameters from the image registration process were used to construct a time series reflecting the Euclidean normalized derivative of the motion. The time series was censored one time point before and two time points after any time point where the derivative was greater than 0.3 (roughly 0.3 mm motion). We also censored any time point where more than 5% of brain voxels were considered outliers based on the magnitude of deviations in the voxel's time series from the overall mean (3dToutcount) (Cox, 1996, 2012; Cox et al., 2017). Time points censored by both methods were removed in the subsequent regression analysis. The groups did not differ

on the percentage of data censored due to motion censoring ($[F(3,34)=0.314, p=0.82]$; control $M=0.31$ $SD=0.34$, 0.32% $M=0.19$ $SD=0.27$, 0.64% $M=0.21$ $SD=0.22$, 0.96% $M=0.29$ $SD=0.32$)

Seed Region Selection and Identification. Based on our previously observed findings of differences in attention, cognitive control and visual discrimination in the same cohort, (Colombo et al., 2011; Colombo et al., 2013) we focused our analyses on functional brain networks that represent those cognitive domains. Seed regions were defined as 5 mm radii spheres in regions identified as network hubs (Whitfield-Gabrieli & Nieto-Castanon, 2012). Seeds were placed in regions associated with attention (Dorsal Attention Network – Inferior Parietal Lobule (Right [TAL: 54 –32 24] Left [–57 –31 18]); Ventral Attention Network – Posterior Superior Temporal Gyrus (Right [54 –32 24] Left [–57 –31 18])), visual processing (bilateral Primary Visual Cortex, Brodmann’s area (BA) 17 [+/-10 –88 5]), and cognitive control (ACC [1 0 40]). The seed location for primary visual cortex was defined from template masks within AFNI (Cox, 1996, 2012). At the subject-level, the four seed time series (bilateral IPL, bilateral PoSTG, bilateral BA17, and ACC) were constructed by calculating the average time series over the voxels within each of the seed regions. Using multiple regression, we produced for each seed a map of the correlations (r -values) between the seed time series and each voxel in the brain. These r -values were transformed to z -scores.

While the motion scrubbing procedure described above removes time points most affected by motion artifact, datasets with more motion may still contain residual effects. These effects may induce spurious correlations in an individual data set and increase noise in the overall sample, reducing power. Weighted regression is typically used when within-subject variances are heterogeneous, which results here due to censoring (Christensen, 2011). Usually, these within-subject variances must be estimated, or are erroneously assumed to be equal. In the case of imaging data, the within-subject variances are sometimes known. As fMRI data are often treated in a modified two-stage mixed effects model, where subject-level estimates are determined prior to inclusion in group analyses (Lazar, 2008, p. 69), those participants with more censoring (i.e. fewer useable volumes) have less reliable subject-level estimates than participants with complete datasets. Methods exist to estimate within-subject variance in a two-stage model (Beckmann, Jenkinson, & Smith, 2003; Chen, Saad, Nath, Beauchamp, & Cox, 2012). This can dramatically improve statistical power. However, if estimates are poor power could be reduced or the type I error rate could be inflated. In the case of correlation analysis - as is often applied in resting state MRI - within-subject variance is $\frac{1}{N_i - 3}$ after implementing Fisher’s variance stabilizing transformation,

where N_i is the number of observed volumes for subject i . Hence, there is no need to estimate within subject variance – it is observable. Our approach is to combine volume censoring based on participant motion with weighting of the remaining data based on the proportion of data that remains. In this way, the noise is adequately removed, and the participants with less reliable subject-level estimates contribute less to the group statistics. We implemented weighted regression as an additional conservative approach to minimize effects of motion, similar to previous work in resting state fMRI (Lepping et al., 2015). Z -

score maps for each dataset were weighted by a function of the participant's percentage of TRs remaining after censoring by the z -score at each voxel. This weighting function w was calculated as the participant's percentage of TRs remaining after censoring (T_{r}), divided by the sum of TRs remaining across all subjects, multiplied by the number of subjects (therefore, the sum of the weights across subjects = n).

Statistical Analyses: We implemented ANOVA comparing weighted z -scores for LCPUFA Supplementation Group (control, 0.32%, 0.64%, 0.96%). These F -statistic maps were corrected for multiple comparisons at $\alpha < 0.05$ using a voxel-wise threshold of $F > 4.411$; $p < 0.01$, combined with Monte Carlo simulations of minimum cluster size (2568mm^3) determined for the whole brain using the spatial autocorrelation function (acf) option in AFNI's 3dFWHMx to estimate intrinsic smoothness and 3dClustSim to estimate probability of false positives (Cox et al., 2017; Eklund et al., 2016; Forman et al., 1995). Subthreshold clusters in a priori regions were also examined if they passed a minimum cluster size of 1500mm^3 . The average weighted z -score across all voxels within a cluster was extracted for each subject. Post-hoc tests were assessed in SPSS with Tukey's HSD.

2.5 | MRI Volumetric Data Analysis

Data from 7 subjects were rejected due to excessive movement, visible as striping and ghosting artifact on the structural scan. Data analysis for the remaining 31 subjects (control $n=7$, 0.32% $n=8$, 0.64% $n=7$, 0.96% $n=9$) was performed using SPM8 software (Wellcome Department of Cognitive Neurology, London, UK) running under MATLAB 8.5 (MathWorks, Natick, MA, USA). Processing for voxel-based morphometry analysis was done by first creating a sample-specific DARTEL template (Diffeomorphic Anatomical Registration Through Exponentiated Lie algebra). DARTEL is a suite of tools for achieving more accurate inter-subject registration of brain images (Ashburner, 2007), increasing localization as well as sensitivity for VBM studies. We then used high-dimensional spatial normalization with DARTEL to normalize images to the DARTEL template, and the unified segmentation ("New Segment") model in SPM8 (Ashburner & Friston, 2005) with priors to output warped, modulated, segmented images. For this study, we were interested in gray matter volume differences between LCPUFA-supplemented groups, so we used nonlinear normalizations. These final images were smoothed with a 10 mm isotropic Gaussian kernel to accommodate inexact spatial normalization. Gray matter (GM), white matter (WM), and cerebrospinal fluid (CSF) segmentations were used to calculate total-intracranial volume (TICV). We divided whole gray and white matter by total-intracranial volume to represent normalized gray (nGMV), normalized white matter volumes (nWMV) as well as normalized whole brain volume (nWBV). We completed a Univariate analysis in SPSS testing for differences in brain volume (nGMV, nWMV, nWBV, and TICV) between all groups (control, 0.32%, 0.64%, and 0.96%), and proceeded to complete post-hoc analyses of mean differences in volume if there was an overall group difference present.

For the VBM analysis, a General Linear Model (GLM) full factorial analysis (ANOVA) with post hoc t tests was used to examine regional gray and white matter volume differences across the whole brain between LCPUFA-supplemented groups, including age and sex as confounding variables. Voxels are reported with reference to Talairach space within SPM8

(Honea et al., 2008). To avoid possible edge effects around the border between GM and WM and to include only relatively homogeneous voxels, we used an absolute threshold masking of 0.15. We had an a priori hypothesis that there would be white matter differences in the LCPUFA-supplemented group in the medial frontal and ACC, based on a previous study using fMRI (McNamara et al., 2010), thus after doing whole-brain voxel-wise statistics, we used a region-of-interest approach in this prefrontal region. For all analyses, results had a minimum cluster size of 100 voxels ($k > 100$) and were considered significant at $p < 0.05$ after correction for multiple comparisons (family wise error, FWE). Since this is the first study to investigate neuroanatomical differences between these unique samples, we also report data at $p < 0.001$ uncorrected for multiple comparisons at a cluster size greater than 100 voxels ($k > 100$).

Statistical Analyses: SPSS 17.0 was used for all statistical analysis. Imaging variables were compared between the individual groups using ANOVA. Chi-square was used to compare categorical variables between groups. For all analyses, results were considered significant at $p < 0.05$.

2.6 | MRS Neurochemical Data Analysis

Four children declined the second MR scanning session, therefore, ^1H MRS data were obtained from 34 subjects. A further six datasets were unusable due to motion artifact, visible by apparent lipid signal artifacts and diminished metabolite signals, therefore, 28 subjects were included in the MRS analysis: (control $n=5$, 0.32% $n=8$, 0.64% $n=7$, 0.96% $n=8$). Standard ^1H MRS data were acquired from an axial slab positioned superior to the corpus callosum (Figure 2B). ^1H MRS data processing included a spatial Fourier transformation with zero padding, resulting in the final matrix size of 16×16 voxels with nominal voxel size of $1 \times 1 \times 1.5$ cm. ^1H MRS data were processed using LCModel software (Provencher, 1993) and neurochemicals with Cramér–Rao lower bound of 30% or less were included in the data analysis. Quantified neurochemicals include creatine (Cr), N-acetylaspartate (NAA), Glu+Gln which is a sum of glutamate (Glu) and glutamine (Gln), myo-inositol (mI), and total choline (tCho). The final concentration of each metabolite in the fronto-parietal region was obtained by averaging neurochemical concentration values from voxels satisfied the quality criterion of the Cramér–Rao lower bound within the central 6×6 voxels. All neurochemical concentrations were reported in ratios to creatine.

Statistical analyses: A separate ANOVA was conducted for each neurochemical comparing mean concentration across the central fronto-parietal region for the four groups (control, 0.32%, 0.64%, and 0.96%). Statistical significance levels for post-hoc tests were adjusted for multiple comparisons using the Dunnett’s method. For all analyses, results were considered significant at $p < 0.05$.

2.7 | Statistical Analysis

To summarize, a similar analysis strategy was used across all modalities: ANOVA (Group [fixed] X Participants [random]) comparing the four LCPUFA groups (control, 0.32%, 0.64%, and 0.96%), followed by post-hoc tests. Results are described as significant at $p < 0.05$ or are reported as trends.

3 | RESULTS

3.1 | Subject Characteristics

Group demographic variables for the 38 subjects that were included in the imaging analysis are presented in **Table 1**. There were no significant differences in age, sex, maternal education, income by zip code or ethnicity between the control group (n=9) and the LCPUFA supplemented groups (n=29). There were also no significant differences in WISC-IV measures between the two overall groups on FSIQ, VCI, PRI, WMI, and PSI.

3.2 | Flanker Results:

3.2.1 | Behavioral—No significant group differences or interactions with group were found for accuracy or reaction time. Overall, a main effect of condition was found for accuracy [$F(1,34)=29.61$, $p<0.001$] and reaction time [$F(1,34)=20.03$, $p<0.001$] with greatest accuracy and fastest reaction times on Congruent compared to Incongruent trials. No interactions or main effects of group were found ($p>0.10$).

3.2.2 | fMRI—A significant Group x Condition interaction was found in the ACC (TAL xyz = -4, 24, 16; 5391 mm³), bilateral inferior parietal (TAL xyz = 34, -46, 56; 9328 mm³ and TAL xyz = -36, -29, 36; 5187 mm³), left superior parietal (TAL xyz = -29, -46, 59; 2453), left fusiform gyrus (TAL xyz = -44, -51, -11; 3844 mm³) and cerebellum (TAL xyz = 9, -69, -19; 8172 mm³). Observation of the significant regions revealed greater activation for each LCPUFA supplemented group to incongruent compared to congruent trials. Less activation was found for the control group to incongruent compared to congruent trials in all regions (**Figures 1C and 2C**).

3.2.3 | rsMRI—Functional connectivity of the Dorsal Attention Network at age 9 was related to the level of LCPUFA supplementation during the first year of life. Children in the 0.64% group exhibited greater connectivity compared to all other groups between prefrontal and parietal regions of the Dorsal Attention Network. Clusters did not reach statistical significance threshold in the full interaction, therefore, trend clusters in the insula and DLPFC were probed with post hoc tests. (**Figure 1B, Table 2**).

Post-hoc tests revealed that connections between the inferior parietal cortex and distant regions of the left DLPFC were stronger in the 0.64% group compared to the control group, the 0.32% group and the 0.96% group. Connectivity between the IPL and insula was stronger in the control group compared to the 0.32% group and the 0.96% group, and stronger in the 0.64% group compared to the 0.32% group and the 0.96% group. Connectivity between the IPL and insula was not different between the control group and the 0.64% group. Neither was it different between the 0.32% group and the 0.96% group. Corresponding regions of the DLPFC and insula in the right hemisphere were also observed with weaker connectivity in the 0.96% group compared to the other groups, but these clusters were smaller and also failed to reach statistical significance.

For the ANOVA seeded in the bilateral posterior superior temporal gyrus (PoSTG) – Ventral Attention Network, there was a trend of early life LCPUFA supplementation. For the ANOVA seeded in the bilateral primary visual cortex – Visual Network, there were no

clusters that passed correction for whole brain comparisons, however, there were trends of LCPUFA supplementation level in medial prefrontal cortex and left prefrontal cortex. Finally, for the ANOVA seeded in the dorsal ACC – Executive Control Network, there was a trend of LCPUFA supplementation during the first year of life, although these clusters failed to pass correction for whole brain comparisons.

3.3 | MRI Volumetric Results:

When comparing all 4 groups in a Univariate analysis, there were significant differences in normalized gray matter volume (nGMV) ($p < 0.046$) and normalized white matter volume (nWMV) ($p = 0.014$). Post-hoc analyses of pair-wise group comparisons revealed that in the nGMV analysis, the 0.96% LCPUFA-supplemented group had greater gray matter than the 0.32%-supplemented group ($p = 0.036$) and the 0.64% supplemented group ($p = 0.011$). In the nWMV analysis, the 0.32% LCPUFA-supplemented group had greater white matter than the control group ($p=0.008$) as well as the 0.96% LCPUFA-supplemented group ($p=0.007$).

3.3.1 | VBM Results - Comparison of Gray and White Matter Volume Between Groups—In our gray matter voxel-based morphometry analysis comparing the 0.32%, 0.64%, and 0.96% groups independently to the control group, there were no significant differences between the four groups in the overall F-test, at a whole brain level, nor in the medial frontal/ACC region-of-interest.

In our white matter voxel-based morphometry analysis comparing control, 0.32%, 0.64%, and 0.96% there were differences between the 4 groups in the overall F-test in the left medial frontal gyrus in BA 10 ($p < 0.001$ uncorrected, peak voxel at $-4, 50, 12$; x, y, z; cluster size 230, $Z=4.23$). In the region-of-interest analysis in the overall F-test, the same region was significantly different between groups at a corrected level ($p < 0.05$ FWE corrected within the region). In the post-hoc between groups test we found that the 0.32% and 0.64% groups had more white matter volume in the region seen in the F-test, the left ACC ($p < .05$ FWE corrected in the 0.32% > control condition (**Figure 2A**); $p < 0.001$ uncorrected in the 0.64% > control condition). In addition, there was a trend for greater white matter volume in the 0.32% group compared to control in two clusters in the right posterior parietal cortex, and in the right posterior parietal cortex in the 0.64% group compared to control (see **Figure 1A, Table 3**).

3.4 | MRS Neurochemical Results:

LCPFUFA supplementation during the first year of life had significant effects on neurochemical concentrations. The ANOVAs indicated a significant difference between the four groups for NAA/Cr [$F(3,27)=3.085$, $p=0.046$], and a trend for a group difference for mI/Cr [$F(3,27)=2.768$, $p=0.064$]. Post hoc Dunnett's t revealed that the NAA to creatine ratio was significantly increased in the 0.64% group compared with the control group ($p = 0.03$, $n=7$ for 0.64% group and $n=5$ for controls) (**Figure 2B**). The mI to creatine ratio was significantly increased in the 0.96% group compared with the control group ($p = 0.04$, $n=8$ for 0.96% group and $n=5$ for controls). No group differences were found for Glu+Gln/Cr [$F(3,27)=0.343$, $p=0.795$], or tCho/Cr [$F(3,27)=0.767$, $p=.524$].

4 | DISCUSSION

Overall, our results support our previous studies on cognitive differences in children with LCPUFA supplementation, and point to a long-term beneficial effect of early life DHA-ARA supplementation specifically in the attention (**Figure 1**) and inhibition systems (**Figure 2**), as seen with structural, functional, and neurochemical neuroimaging 8 years after supplementation ended. These results are also consistent with results of ERPs to Go/No-Go stimuli in the same cohort when they were tested at 5.5 years of age (Liao et al., 2017). ERP responses of children in the LCPUFA-supplemented group showed the expected differences between Go and No-Go stimuli, while those in the control group did not, again reinforcing the hypothesis that attention and inhibition systems are vulnerable to early life LCPUFA insufficiency.

Our fMRI data using an inhibition task identified overall greater activation in the ACC and parietal regions during performance of the Flanker task in the LCPUFA supplementation group. We also found that a balance of DHA and ARA resulted in greater functional connectivity between regions of the dorsal (IPL) and ventral (DLPFC/Insula) attention networks. Although these two attention systems are involved in distinct attentional processes, coordination between them is critical for orienting and flexibly shifting attention (Vossel, Geng, & Fink, 2014). Our results in both task activation and resting state functional connectivity are similar to the recent report of Almeida, et al. who found low DHA biostatus associated with reduced event-related functional connectivity in cortical attention networks of 8–10 year old boys (Almeida et al., 2017). Almeida divided boys into ‘low-DHA’ and ‘high-DHA’ groups (n=18/group) based on a median split of RBC DHA at the time of imaging. Our sample of similar age included female children and our groups were determined by their early life randomization, not their current biostatus. Thus, these independent studies suggest that both early life LCPUFA supplementation and current DHA biostatus influence brain function in cortical attention networks in childhood.

While we did not find any significant regional differences in gray matter volume among the supplementation groups, we did find that white matter volume was both greater overall in the 0.32% and 0.64% groups, and it was locally greater in the ACC, supporting our hypothesis that LCPUFA supplementation would be associated with differential brain structure in regions supporting attention and inhibition. Decreased ACC volume has been reported in children with attention deficit hyperactivity disorder (ADHD) (Semrud-Clikeman, Fine, Bledsoe, & Zhu, 2017) and low LCPUFA status, in particular, DHA has been associated with childhood ADHD in several studies (Hawkey & Nigg, 2014).

Across a large area of the fronto-parietal cortex that includes the dACC and parietal regions highlighted in the other analyses, we observed increased NAA/Cr and mI/Cr in LCPUFA-supplemented children. Similarly, McNamara reported higher NAA and mI in the ACC in the same cohort of 8–10 year old boys described above with low vs. high DHA status (McNamara et al., 2013). It is interesting to observe similar neurochemical changes despite the differences in the two studies; i.e., current DHA status in boys vs. long-term effects in all children from early life supplementation. Further, the study of McNamara et al., (2013) focused on the ACC and measured absolute concentration of neurochemicals, whereas we

looked more broadly across the fronto-parietal area and measured concentration ratios with respect to creatine.

While there are parallels between our findings and those described in rhesus macaques with life-long dietary manipulation of LCPUFAs (Grayson et al., 2014), i.e., group differences in functional connectivity, direct comparisons should be approached with caution. Dietary manipulation in macaques was life-long, including fetal exposure to the experimental maternal diet. Nonetheless, we have evidence that LCPUFA supplementation from birth to 12 months has long-lasting effects in brain function using both electrophysiology and imaging modalities.

We were limited by the small sample size in each group and constrained by the ability of children to participate in lengthy imaging protocols that require them to hold still. Our results may not be generalizable as this was a low SES cohort as typical of parents who chose exclusive use of formula for infant feeding in the years the study was conducted. It is well known that lower cognitive outcomes are associated with lower SES. Children in this study would have been expected to have unusually poor LCPUFA status at birth and may have been more likely to benefit from postnatal supplementation.

The long-term follow up of this unique cohort concluding in multi-modal imaging is the primary strength of the current study. Results from this cohort, studied from 6 weeks to 9 years, brings us closer to understanding the beneficial effect of adequate dietary LCPUFA on brain development and executive function for mechanisms related to attention, rule learning and inhibition.

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Abbreviations:

LCPUFA	long-chain polyunsaturated fatty acids
ARA	arachidonic acid
DHA	docosahexaenoic acid
MRI	magnetic resonance imaging
fMRI	functional MRI
rsMRI	resting state MRI

¹H MRS	proton magnetic resonance spectroscopy
ACC	anterior cingulate cortex
VBM	voxel-based morphometry
Cr	creatine
Glu	glutamate
Gln	glutamine
tCho	total choline
GPC	glycerophosphoryl-choline
PCho	phosphorylcholine
mI	myo-Inositol
NAA	N-acetylaspartate
ALA	alpha-linoleic acid
EPA	eicosapentaenoic acid
VCI	Verbal Comprehension
PRI	Perceptual Reasoning
PSI	Processing Speed
WMI	Working Memory
FSIQ	Full Scale IQ
MP-RAGE	magnetization prepared rapid acquisition gradient echo
BOLD	blood oxygen level dependent
PRESS	point resolved spectroscopy
IPL	inferior parietal lobule
PoSTG	posterior superior temporal gyrus
GM	gray matter
WM	white matter
CSF	cerebrospinal fluid
TICV	total-intracranial volume
nGMV	normalized gray matter volume
nWMV	normalized white matter volume

nWBV	normalized whole brain volume
FWE	family wise error

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Figure 1: Attention System: Long-term effects of LCPUFA supplementation as observed by multi-modality imaging in brain structure (A), connectivity (B), and function (C).

(A) *Structural*: There was a trend for greater white matter volume in the 0.32% group and the 0.64% group compared to control in the right posterior parietal cortex. (B) *Functional*: Connections between the inferior parietal cortex and distant regions of the left DLPFC were stronger in the 0.64% group. Connectivity between the IPL and insula was stronger in the control group compared to the 0.32% group and the 0.96% group, and stronger in the 0.64% group compared to the 0.32% group and the 0.96% group. (C) *Functional*: Activation maps showing a group effect for incongruent compared to congruent trials. The parietal regions in the LCPUFA-supplemented groups show greater activation to incongruent trials compared to congruent trials compared to control. (D) Example of the Flanker task.

Figure 2: Inhibition System: Long-term effects of LCPUFA supplementation as observed by multi-modality imaging in brain structure (A), metabolism (B) and function (C).

(A) Structural: The 0.32% and 0.64% groups had more white matter volume in the left ACC

(B) Metabolic: The NAA:creatinine ratio was greater in the 0.64% group compared to control. The mI:creatinine ratio was greater in the 0.96 group compared to control.

(C) Functional: There was greater activation for the LCPUFA-supplemented groups to incongruent compared to congruent trials in the ACC. The control group showed less activation to incongruent compared to congruent trials.

Table 1.

Demographic Characteristics of the Study Subjects

	Control	All LCPUFA Groups	0.32% DHA	0.64% DHA	0.96% DHA	<i>p</i>
	n=9	n=29	n=10	n=8	n=11	All groups
Age	8.9 (.64)	9.0 (.54)	9.0 (.42)	9.1 (.61)	9.0 (.64)	0.855
Sex m/f	2/7	10/19	4/6	1/7	5/6	0.388
Maternal education (y)	12.5 (2.1)	11.8 (1.5)	12.2 (1.8)	11.0 (1.2)	11.9 (1.2)	.261
Income, by zip code (US\$)	\$36,632 (\$11,990)	\$32,958 (\$10637)	\$31,907 (\$9,626)	\$39,856 (\$14,411)	\$28,895 (\$5,542)	.129
Ethnicity (%)						.430
African American	18	39	13	8	18	
Other	0	3	3	0	0	
White	5	35	11	13	11	
Cognitive performance (WISC-IV)	9	29	10	8	11	
FSIQ	92.7 (8.7)	94.3 (12.9)	96.2 (14.2)	95.4 (15.4)	91.8 (10.2)	0.833
VCI	93.0 (10.1)	93.2 (12.3)	97.6 (11.8)	92.6 (11.8)	89.6 (12.8)	0.487
PRI	95.6 (9.8)	100.2 (14.1)	99.0 (13)	106.4 (19.9)	96.9 (8.9)	0.347
WMI	93.7 (8.1)	92.3 (12.6)	91.7 (12.3)	91.5 (13.9)	93.5 (13.1)	0.978
PSI	94.8 (9.0)	95.1 (12.7)	97.5 (11.3)	91.6 (14.8)	95.4 (12.9)	0.789

Table 2.

Functional Connectivity Analysis of the Dorsal Attention Network, Clusters

Region	Brodmann Area (BA)	X	Y	Z	mm ³
Left Insula/ Planum Polare	22/13	45	1	-4	2424
Left Fusiform Gyrus	18	21	-95	-12	2168
Left DLPFC	10/46	39	41	14	1744
Left Insula/ Precentral Gyrus	13/4	47	-3	14	1432
Left Precuneus	31	13	-65	24	1112
Right Insula	22	-49	-5	-2	736
Right Cerebellum	N/A	-27	-51	-20	560
Right DLPFC	10/46	-33	29	20	488

Table 3.

VBM Analysis in White Matter

		Peak p(FWE-corr)	Peak T	Peak equivZ	cluster (k)	Peak p(unc)	x,y,z (mm)	Regions
F-Test across 4 groups	Whole Brain	n.s.		4.23	230	<.001	-4, 50, 12	Left medial frontal gyrus (BA 10)
		n.s.	5.34	4.32	402	<.001	-4 48 13	Left Medial Frontal Gyrus (BA 10), Left Anterior Cingulate
Control vs. 0.32% DHA	Whole Brain	n.s.	4.4	3.75	131	<.001	39 -30 24	Parietal-Temporal junction
		n.s.	4.39	3.74	347	<.001	29 -58 32	Posterior Parietal white matter
	Region of Interest	<.05					-4 48 13	Left Medial Frontal Gyrus (BA 10), Left Anterior Cingulate
Control vs. 0.64% DHA	Whole Brain	n.s.	5.15	4.21	179	<.001	34 -50 25	Inferior Parietal White Matter
Control vs. 0.96% DHA	Whole Brain	n.s.				n.s.		