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Increased ω-3 polyunsaturated fatty acid/arachidonic acid ratios and upregulation of signaling mediator in individuals with autism spectrum disorders

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ABSTRACT

Aims: The investigation of links between the ratio of omega-3/omega-6 PUFAs and neuronal signaling is a research priority in autism spectrum disorders (ASD).
Main methods: We examine the relationships between the plasma ratios of docosahexaenoid acid (DHA)/arachidonic acid (AA) and eicopentaenoic acid (EPA)/AA and biomarkers of AA-related signaling mediators such as ceruloplasmin, transferrin and superoxide dismutase, in the behavioral symptoms of 28 individuals with ASD (mean age 13.5 ± 4.6 years) and 21 age- and gender-matched normal healthy controls (mean age 13.9 ± 5.7 years). Behavioral symptoms were assessed using the Aberrant Behavior Checklists (ABC). We conducted controlling for dietary intake and assessed the dietary intake of nutrients.
Key findings: There were no significant differences in intake of nutrients such as omega-3 and omega-6 PUFAs, saturated and unsaturated fatty acid, DHA, AA, iron and copper. Plasma EPA, DHA, and arachidic acid levels, and plasma DHA/AA and EPA/AA ratios were significantly higher, while plasma AA and adrenic acid were significantly lower in the 28 individuals with ASD than in the 21 normal controls. The ABC scores were significantly higher in the ASD group compared to the control group. The plasma ceruloplasmin levels in the ASD group were significantly reduced compared to those in the control group.
Significance: Increased plasma DHA/AA and EPA/AA ratios may be related to low plasma levels of ceruloplasmin which has neuroprotective properties. Reduced plasma ceruloplasmin levels may diminish the protective capacity against brain damage, and may contribute to the pathophysiology of behavioral symptoms in individuals with ASD.

1. Introduction

Studying alterations in the composition of PUFAs and related signal mediators may be a major strategy in understanding the pathophysiology of autism spectrum disorders (ASD) [1]. The omega-3 PUFAs docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA) and the omega-6 PUFA arachidonic acid (AA) are important in nervous system function [2], signal transduction [3], proper synapse formation [4] and neurotransmission [4]. Previous studies reported a marked reduction in plasma DHA levels [5,6] with changes in the plasma omega-3/omega-6 ratio [7]. The balance between omega-3 and omega-6 PUFAs is well known to be critical for normal brain function and development [8–10]. A recent clinical study reported that the relationship between omega-3 PUFAs and AA is antagonistic to maintaining homeostasis at high EPA and DHA concentrations. The incorporation of omega-3 PUFAs lowers the AA concentration [11,12], thereby down-regulating the synthesis of eicosanoids, the mediators of AA signaling in the cell membrane [12]. Dietary omega-3 PUFAs may attenuate tissue AA levels and eicosanoid formation [13]. The antagonism of AA signaling may have important effects upon cell signaling in the central nervous system [15,16], modifying biomedical and behavioral effects [15]. This competitive interaction between omega-3 PUFAs and AA reflects an increase in the plasma omega-3/AA ratios [8,9,11].

In respect to signaling mediators in ASD, alterations in ceruloplasmin (Cp) [17], superoxide dismutase (SOD) [18] and transferrin (Tf) [19] have been reported as pathophysiological factors. Cp is an important copper signaling biomarker of neuronal function [20] and a natural neuroprotective protein [21,22]. Cp reduces the synthesis of AA-derived eicosanoid mediators, such as leukotrienes [23] and cyclooxygenase-2 [24]. SOD is a biomarker of copper signaling [25]. AA [26] and essential PUFAs [27] increased the activity of SOD. Moreover, protecting SOD activity has been related to the excess production of the AA-derived

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eicosanoid family prostaglandin F2 [28]. Tf is an iron-signaling mediator [29]. The Tf receptor protein is elevated by DHA-enrichment [30]. Collectively, Cp, SOD and Tf are related to the functions of AA, DHA and PUFAs. Changes in the blood levels of SOD, TF and CP have been shown to indicate altered antioxidant status [31–33], vulnerability to oxidative stress [34,35], and copper dyshomeostasis [36,37] in ASD patients. However, the importance of the interaction between the altered composition of PUFAs and the AA-related signaling mediators (Cp, Tf and SOD) remains unclear.

We hypothesized that increased plasma DHA/AA and EPA/AA ratios are related to the down-regulation of AA-dependent signaling mediators in the behavioral symptoms of individuals with ASD. Thus, the levels of 23 fatty acid fractions and three AA-related signaling mediators in the plasma were determined. Plasma fatty acid levels were expressed as the percentage of total fatty acids and in μg/ml to examine the correlation among the fatty acids. Because there are multiple subfamilies of AA-derived eicosanoid signaling mediators [38] and these mediators are affected by multiple factors [39], we examined plasma Cp, SOD and Tf levels as known AA-related signaling mediators. We conducted controls for dietary intake and to assess the intake of nutrients.

2. Methods

2.1. Participants

A total of 49 young, physically healthy individuals who were from the Kansai area (Hyogo and Osaka Prefectures) of Japan participated in this study. They were recruited from medical care facilities of the Research Institute of Pervasive Developmental Disorders of Ashiya University by the order of their submission to medical consultation between January 2012 and July 2014. Diagnoses were performed based on the DSM-IV-TR criteria and were additionally confirmed by the Autism Diagnostic Interview-Revised (ADI-R) [40]. Among the 49 individuals, 28 had an independent clinical diagnosis of ASD (20 males and 8 females, mean age: 13.5 ± 4.7 years old, age range: 6–21 years old), and the remaining 21 were normal healthy controls (15 males and 6 females, mean age: 13.9 ± 5.7 years old, age range: 5–21 years old). The 28 ASD individuals and the 21 individuals in the control group were matched in respect to home environment, feeding habits, age, gender and full intelligent quotient (IQ) scores (Table 1). Two psychiatrists specializing in ASD and developmental disorders diagnosed all 28 individuals with ASD. These individuals had the core symptoms of the DSM-IV-R diagnostic criteria for ASD without any abnormal neurological symptoms (e.g., seizure or movement disorders). The 21 normal controls were considered to be physically and mentally healthy based on initial physical and mental screening tests. At the initial screening, physical (resting blood pressure and heart rate) and clinical laboratory examinations (hematology and plasma chemistry, including plasma fatty acids, cholesterol and triglycerides) were performed on all 49 participants. These 49 participants did not have any abnormalities in their physical examinations and laboratory findings. The weight and height of the 49 individuals were within average values for both 6–17 years old [41] and 6–22 years old (http://paro2day.blog122.fc2.com/blogentry9html). The IQs of the individuals were estimated using the Wechsler Intelligence Scale for children and adolescents aged 6–16 years old (WISC-III) [42] or the respective scale for adults (WAIS-R) [43] (Table 1). Comorbid psychiatric disorders were evaluated by the Structured Clinical Interview for DSM-IV Axis I Disorders (SCID). None of the ASD or control individuals had any history of neurological conditions, including seizure, movement disorders, head injury, Attention-Deficit Hyperactivity Disorder or learning disorders. The additional inclusion criteria were as follows: (a) the absence of any other medical or comorbid psychiatric disorders; (b) a baseline verbal or full IQ greater than 70 as calculated by the WISC-III [42] or the respective scale for adults (WAIS-R) [43] because subjects with high-functioning pervasive developmental disorders were required to have a total IQ of at least 70 [44]; (c) no treatment with antidepressants, anxiolytic medications or neuroleptics within the three months prior to the study (the treatment of ADHD symptoms with stimulants was allowed during this study, provided that the patient's dosage was stable for at least 3 months before and during the study).

The present work has been carried out in accordance with the Code of Ethics of the World Medical Association (Declaration of Helsinki) for experiments involving humans (http://www.wma.net/en/30publications/10policies/b3/index. html). This study was performed with the approval of the Ethics Committee of the Fujimoto Medical Clinic in Kobe City, Japan. This ethics committee is registered with the Pharmaceuticals and Medical Devices Agency of Japan to register the IRB information (http://www.info. pmda.go.jp/). Written informed consent was obtained from the participants and/or their parents.

Table 1
Subject characteristics and plasma levels of signaling mediators, and the ABC subscale scores in the 28 individuals with ASD and 21 normal controls.

Variables	ASD (n = 28)	Controls (n = 21)	U	p value	corr p value
Age (Year)					
Mean ± S.D	13.5 ± 4.7	13.9 ± 5.7	272.50	0.66	
Sex (male/female)	7/21	6/15	$\chi^2 = 0.00$	1.00	
Full IQ	94.7 ± 9.6	98.0 ± 6.6	70.00	0.47	
Scores of Autism Diagnostic Interview-Revised					
Domain A (social)	21.1 ± 7.0	N/A			
Domain B (communication)	13.7 ± 5.0	N/A			
Domain C (stereotyped)	5.8 ± 5.4	N/A			
Plasma biomarkers levels					
Cp (mg/dl)	23.92 ± 4.39	27.86 ± 5.12	163.00	0.007**	0.001*
Tf (mg/dl)	262.36 ± 42.54	268.24 ± 37.37	265.50	0.56	NS
SOD (U/ml)	3.36 ± 2.76	3.78 ± 2.94	229.00	0.18	NS
Subscale scores of the ABC					
Irritability	14.36 ± 9.45	0.67 ± 0.91	9.50	0.000***	
Social withdrawal	21.61 ± 9.37	0.86 ± 2.00	1.50	0.000***	
Stereotypy	5.79 ± 5.67	0.24 ± 0.62	68.50	0.000***	
Hyperactivity	19.04 ± 11.59	0.90 ± 2.10	10.00	0.000***	
Inappropriate speech	5.14 ± 4.46	0.29 ± 0.64	50.00	0.000***	
Total	65.96 ± 28.96	2.95 ± 5.11	1.00	0.000***	0.00**

Values are mean ± SD. corr p value, p value after the Bonferroni correction; ABC, Aberrant Behavior Cheklist; Cp, ceruloplasmin; Tf, transferrin, SOD, superoxide dismutase.
* $p < 0.05$ versus normal controls.
** $p < 0.01$ versus normal controls.
*** $p < 0.001$ from normal controls.

2.2. Controlling for dietary intake and assessment of nutrient intake

As plasma fatty acid levels may be confounded by prior dietary intake, many prior studies have used the "Dietary Approaches to Stop Hypertension (DASH)" to control for dietary intake [45,46]. In this study, all 49 participants received the "Japanese Food Guide" [47], which outlines the daily intake guide of nutrients and food based on the "Overview of Dietary Reference Intake for Japanese (2010)" [48]. All 49 individuals' parents were provided a sample of the diet meal plan and menu (KAWASAKI FOODMODEL) (http://item.rakuten.co.jp/foodmodel/751741/), which was edited according to the "Japanese Food Guide" [47]. Moreover, to assess the daily food and nutrient intake, a semi-constructive questionnaire for the Japanese (DHQ) was performed using the junior high school version (DHQ15) (DHQ Support Center, http://www.ebnjapan.org/). The DHQ15 consisted of 72 questions on the frequency of intake of 150 various food and beverage items and cooking methods. DHQ15 was administered one month before the study on randomly selected subsamples of 18 individuals with ASD and 10 normal controls by the order of their submission to medical consultation during January 2013 and June 2014. The food and beverage items and portion sizes in the questionnaire were derived primarily from the data in the Overview of Dietary Reference Intake for Japanese [49]. The DHQ15 sheets were checked by the researcher (KY). If there was any missing or unclear information recorded on the sheet by the guardian, the researcher (KY) questioned the guardian by phone or e-mail. The validity of the DHQ15 has been verified [50]. The estimated intake of nutrients was calculated using a dedicated program for the DHQ system (DHQ Support Center, Tokyo, Japan) [51,52].

2.3. Assessment of behavioral symptoms

The ABC was used to assess the behavioral symptoms of the 28 individuals with ASD and the 21 normal controls. The ABC is primarily intended to evaluate treatment responses in psychopharmacological and behavioral intervention trials for children and adolescents with mental retardation [53] and normal IQ levels [54]. The subscales are as follows: (1) irritability (15 items); (2) social withdrawal (16 items); (3) stereotypic behavior (seven items); (4) hyperactivity (16 items); and (5) inappropriate speech (four items). The ABC is a broad assessment that captures a wide variety of behavioral problems [55]. This test also appears to be capable of discriminating between several syndromes, such as disruptive behavior disorders and the behavioral symptoms of ASD [55].

2.4. Assays of plasma levels of PUFAs, Cp, SOD and Tf

2.4.1. Blood sampling procedures

Whole-blood samples were collected in EDTA tubes by venipuncture and immediately placed on ice in a refrigerator. After a clotting time of 20–25 min, the plasma was obtained by centrifugation for 20 min at 3000 $\times g$ at room temperature (22 °C). The plasma samples were prepared by spinning a tube of fresh blood containing an anticoagulant in a centrifuge until the blood cells fell to the bottom of the tube. To decrease the effects of circadian variation, the blood collection was performed at 11:00–12:30 pm in a quiet laboratory room after supine rest for 20 min. The samples were frozen at −80 °C until analysis. The specialists at SRL, Inc. (Tokyo, Japan) measured the plasma levels of PUFAs, Cp, Tf and SOD.

2.4.2. Plasma levels of PUFAs

The fatty acid composition of the PUFA fraction from the plasma of each patient was determined as previously described [56]. In summary, the total lipids were extracted from the plasma according to [57]. After transmethylation with HCl-methanol, the PUFA composition was analyzed using gas chromatography (GC2010 Shimadzu Co., Japan). A total of 24 long-chain fatty acids were identified. The intra- and inter-assay coefficients of AA were 110.14 μg/ml (standard deviation (SD), 3.87; coefficient of variation (CV), 5.28%) and 100.63 μg/ml (SD, 5.51; CV, 5.48%), respectively, and those of DHA were 73.87 μg/ml (SD, 2.30; CV, 3.11%) and 68.07 μg/ml (SD, 2.30; CV, 3.33%), respectively. The plasma levels of the fatty acids were expressed as both the mean ± SD weight (percentage) of the total fatty acids and the mean ± SD in μg/ml.

2.4.3. Plasma levels of Cp

A Bering BN IINephelometer (Siemens Healthcare Diagnostics K.K., USA) was utilized to estimate plasma CP levels. The assay sensitivity was 3.0 mg/dl. The intra- and inter-assay coefficients were 10.2 mg/dl and 10.1 mg/dl, respectively.

2.4.4. Plasma levels of SOD

Plasma SOD levels were estimated from the rate of decrease in nitrite produced by hydroxylamine and the superoxide anions based on the nitrite method, using a VersaMax instrument (Molecular Devices Co, Tokyo, Japan). Human plasma was assayed using an SOD Assay Kit (Takara Bio, Tokyo) according to the cytochrome c method. The plasma SOD levels are expressed as units per milliliter. The assay sensitivity was 0.3 U/ml. The intra-assay and inter-assay coefficients were 2.11 and 2.10 U/ml, respectively.

2.4.5. Plasma levels of Tf

A standard turbidimetric assay and an automated biochemical analyzer (JCA-BM8000 series, JEOL Ltd., Tokyo, Japan) were utilized to estimate plasma Tf levels. The intra- and inter-assay coefficients were 108.1 mg/dl and 107.4 mg/dl, respectively.

2.5. Statistical analyses

Because the data were not normally distributed, the non-parametric Mann–Whitney U test for multiple comparisons was used to determine the significant differences in the plasma levels of PUFAs, the plasma ratios of DHA/AA and EPA/AA, and the plasma levels of the three biomarkers (Cp, Tf and SOD) as well as the five subscale and total ABC scores and the intake of nutrients between the ASD and the normal groups. Considering the potential false positive rate incurred by multiple comparisons of the most important variables, which were necessary to prove the relationship between the ratios of the omega-3 PUFAs/AA and the AA-related signaling mediators, we applied the Bonferroni correction method to adjust the p values for these important variables (e.g., the plasma DHA/AA and EPA/AA ratios, the levels of the three signaling mediators and the total ABC scores). p values $<0.05/6$ (0.0083) indicated statistical significance after the Bonferroni correction [58].

Spearman's rank correlation coefficients (r) were used to determine the correlations between the plasma DHA/AA and EPA/AA ratios, the plasma levels of the signaling biomarkers (Cp, Tf and SOD), and the five subscale and total ABC scores in the entire population. Moreover, multiple linear regression was used to confirm the relationship between the plasma DHA/AA and EPA/AA ratios and the other main variables, adjusting for the two subject groups, the three signaling biomarkers and the total ABC scores (Table 5). We conducted statistical analyses using SPSS version 18.0 (IBM Tokyo, 2009).

3. Results

3.1. Study population

The behavioral symptoms of the 28 young individuals with ASD included restricted, repetitive and stereotyped patterns of behavior, interests and activities ($n = 9$), irritability and crying ($n = 3$) and stereotyped behavior ($n = 16$). The mean total ABC score was 65.96 ± 28.96. An earlier study reported a total ABC score of 85.6 ± 27.3 for children and adolescents with ASD who were treated with neuroleptics [59]

(Table 1). Thus, our patients suffered from moderate ASD symptoms, including restricted repetitive and stereotyped patterns of interests and behaviors. The ages did not differ significantly between the two groups ($p = 0.66$).

3.2. Plasma levels of PUFAs and biomarkers

The Mann–Whitney U test revealed that plasma EPA, DHA, DPA and arachidic acid levels and the plasma DHA/AA and EPA/AA ratios were significantly higher, while the plasma AA, adrenic acid and Cp levels were significantly lower in the 28 individuals with ASD than in the 21 normal controls, as expressed as the mean ± SD weight (percentage) of the total fatty acids (Table 2). In the case of plasma fatty levels expressed as the mean ± SD in μg/ml, the significant difference in the plasma levels of DHA and arachidic acid of the two groups disappeared (Table 3).

There was no significant correlation between the plasma levels of PUFAs and plasma levels of Cp ($r = 0.08$–0.253, $p = 0.16$–0.64), and Tf and SOD($r = 0.02$–0.19, $p = 0.33$–0.92).

Even after the Bonferroni correction, the plasma ratios of DHA/AA ($p = 0.0002$) and EPA/AA ($p = 0.0012$), the level of Cp ($p = 0.001$), and the total ABC scores ($p = 0.000$) were significantly higher in the ASD group compared with the control group (Tables 1 and 2). The ABC subscale scores for irritability ($p = 0.000$), social withdrawal, stereotypic behavior, hyperactivity and inappropriate speech and the total ABC scores were significantly higher in the ASD group than in the control group (Table 1).

Table 2
Plasma levels of fatty acid fractions.

Variables	ASD (n = 28)	Controls (n = 21)	U	p value	corr p value
Plasma PUFA levels (%)					
Omega-3 series					
C18:3ω3 (ALA)	0.73 ± 0.38	0.59 ± 0.20	226.50	0.17	
C20:5ω3 (EPA)	1.33 ± 0.86	0.74 ± 0.41	142.50	0.002**	
C22:5ω3 (DPA)	0.54 ± 0.16	0.46 ± 0.10	169.50	0.01*	
C22:6ω3 (DHA)	3.59 ± 1.20	2.92 ± 0.93	189.00	0.03*	
Omega-6 series					
C18:2ω6 (LA)	29.49 ± 3.60	29.79 ± 3.46	290.50	0.94	
C18:3ω6 (GLA)	0.31 ± 0.14	0.33 ± 0.13	288.50	0.91	
C20:2ω6 (DGLA)	1.17 ± 0.42	1.32 ± 0.24	290.00	0.94	
C20:2ω6	0.19 ± 0.04	0.19 ± 0.04	259.00	0.48	
C20:4ω6 (ARA)	5.71 ± 1.25	6.62 ± 1.30	190.50	0.04*	
C22:4ω6 (adrenic acid)	0.20 ± 0.14	0.22 ± 0.07	193.00	0.04*	
Ratios of plasma levels of PUFAs					
DHA/AA	0.64 ± 0.23	0.44 ± 0.14	123.00	0.001**	0.0002*
EPA/AA	0.24 ± 0.16	0.12 ± 0.07	109.50	0.000***	0.000**
C14:1ω5	0.02 ± 0.03	0.05 ± 0.05	180.00	0.05	
C16:1ω7	1.18 ± 0.82	1.74 ± 0.69	291.00	0.95	
C18:1ω9	20.09 ± 3.37	21.16 ± 2.99	216.00	0.12	
C20:1ω9	0.16 ± 0.05	0.14 ± 0.03	247.00	0.34	
C20:3ω9	0.08 ± 0.04	0.08 ± 0.02	214.50	0.10	
C22:1ω9	0.04 ± 0.03	0.03 ± 0.02	261.50	0.49	
C24:1ω9	1.20 ± 0.32	1.12 ± 0.12	259.50	0.49	
Saturated fatty acids					
C12	0.14 ± 0.13	0.15 ± 0.24	284.50	0.85	
C14	0.92 ± 0.47	0.82 ± 0.33	274.50	0.69	
C16	23.18 ± 2.27	21.17 ± 4.97	255.00	0.43	
C18	7.81 ± 0.57	7.50 ± 0.52	201.00	0.06	
C20 (arachidic acid)	0.31 ± 0.51	0.27 ± 0.04	145.00	0.003**	
C22	0.71 ± 0.17	0.66 ± 0.09	226.00	0.17	
C24	0.61 ± 0.16	0.56 ± 0.09	216.50	0.12	

Values are mean ± SD. corr p value, p value after the Bonferroni correction.
* $p < 0.05$ versus normal controls.
** $p < 0.01$ versus normal controls.
*** $p < 0.001$versus normal controls.

As shown in Table 4, the plasma DHA/AA and EPA/AA ratios were significantly correlated with all five ABC subscale scores and the total scores (all r values were greater than 0.36, $p < 0.05$, $p < 0.01$ or $p < 0.001$) for the whole population. In the case of the levels of plasma fatty acids expressed as the mean ± SD μg/ml, the Spearman correlation coefficients indicated similar results to the above described findings.

3.3. Assessment of nutrient intake

All 49 participants received a control for their dietary intake according to the "Japanese Food Guide" [47]. As shown in the Table 3, there were no significant differences in weight, height, energy intake, and intake of cholesterol, protein, carbohydrate, fat, animal fat, saturated fatty acids and unsaturated fatty acids. Importantly, the intake of omega-6 ($p = 0.19$) and omega-3 PUFAs ($p = 0.34$), AA ($p = 0.34$), EPA ($p = 0.96$), DHA ($p = 0.87$), iron ($p = 0.78$) and copper ($p = 1.00$) did not significantly differ between the ASD and control groups (Table 5).

3.4. Predictor variables

The multiple linear regression analysis demonstrated that the plasma ratios of DHA/AA ($R^2 = 0.336$, $p = 0.003$) and EPA/AA ($R^2 = 0.265$, $p = 0.018$) significantly contributed to the variables after adjusting for the two subject groups, the three signaling biomarkers and the total ABC scores (Table 5). The use of group assignments and the plasma Cp levels as the dependent variable significantly contributed to the plasma DHA/AA ratio (group, unstandardized coefficients, $B = -0.268 \pm 0.098$, $\beta = -0.621$, and $p = 0.009$; Cp, unstandardized coefficients, $B = 0.013 \pm 0.006$, $\beta = 0.295$, and $p = 0.046$). When the groups were used as the dependent variable, a trend toward the statistical significance of contribution to the plasma EPA/AA ratio was shown (group, unstandardized coefficients, $B = -0.122 \pm 0.098$, $\beta = -0.430$, and $p = 0.08$) (Table 6). In the case of the plasma fatty acid levels expressed as the mean ± SD in μg/ml, the multiple linear regression analysis indicated similar results to the above described findings. These findings indicated that the plasma ratios of DHA/AA as well as EPA/AA allowed for the prediction of these variables in the ASD and control groups.

4. Discussion

Plasma PUFA levels have been shown to reflect changes in brain PUFA levels [60]. In this study, the plasma EPA, DHA, DPA and arachidic acid levels and the plasma DHA/AA and EPA/AA ratios were significantly higher, while the plasma AA, adrenic acid and Cp levels were significantly lower in the 28 individuals with ASD than in the 21 normal controls (Table 1). In the case of the fatty acid levels in the plasma expressed as the mean ± SD in μg/ml, the significant difference in the plasma levels of DHA and arachidic acid between the two groups disappeared (Table 3). Plasma levels of DHA [61] expressed in μg/ml were different from those expressed as a percentage [61]. Similarly, plasma arachidic acid levels expressed in μg/ml were different from those expressed as a percentage [61,62]. Of reference, the time to reach peak concentration of plasma DHA levels expressed in μg/ml (about 40–100 h [63]) was later than those in a percentage (about 6 h [64]). Thus, low plasma DHA levels expressed in μg/ml might remain until the time of blood sampling, inducing disappearance of significant difference. Moreover, in the metabolism of EPA and DHA, there is limited conversion of EPA to DHA [65], potentially reflecting the lack of significant difference in the plasma DHA level between the two groups. Plasma AA levels expressed as μg/ml increased more rapidly than those expressed as percent after food intake [62]. Considering that arachidic acid is known to be formed by the hydrogenation of AA [66], high plasma levels of arachidic acid expressed in μg/ml might be refractory of rapid increase in plasma AA levels, inducing disappearance of significant difference of plasma arachidic acid.

Table 3
Plasma levels of fatty acid fractions.

Variables	ASD (n = 28)	Controls (n = 21)	U	p value	corr p value
Plasma PUFA levels (μg/ml)					
Omega-3 series					
C18:3ω3 (ALA)	18.45 ± 12.47	16.85 ± 6.69	280.50	0.78	
C20:5ω3 (EPA)	32.26 ± 17.87	20.75 ± 13.01	72.00	0.014*	
C22:5ω3 (DPA)	0.54 ± 0.16	0.46 ± 0.10	287.50	0.89	
C22:6ω3 (DHA)	84.76 ± 26.51	80.12 ± 28.59	275.00	0.70	
Omega-6 series					
C18:2ω6 (LA)	699.23 ± 150.00	795.24 ± 182.00	199.0	0.91	
C18:3ω6 (GLA)	7.28 ± 4.22	8.72 ± 3.82	219.50	0.13	
C20:2ω6 (DGLA)	36.23 ± 36.69	35.76 ± 9.42	192.50	0.04*	
C20:2ω6	4.73 ± 1.40	5.01 ± 0.90	259.00	0.48	
C20:4ω6 (ARA)	133.50 ± 32.00	180.41 ± 38.91	101.00	0.00***	
C22:4ω6 (adrenic acid)	4.23 ± 1.48	5.75 ± 1.72	155.00	0.05	
Ratios of plasma					
DHA/AA	0.65 ± 0.22	0.45 ± 0.15	117.00	0.00**	0.0002*
EPA/AA	0.25 ± 0.17	0.10 ± 0.07	81.00	0.00***	0.000***
C14:1ω5	0.46 ± 1.03	1.03 ± 1.10	164.50	0.04*	
C16:1ω7	40.62 ± 23.67	44.95 ± 17.41	219.00	0.13	
C18:1ω9	494.40 ± 222.53	536.97 ± 185.86	202.00	0.06	
C20:1ω9	4.00 ± 2.31	3.73 ± 0.94	251.00	0.38	
C20:3ω9	1.77 ± 0.93	2.22 ± 0.86	200.50	0.06	
C22:1ω9	1.60 ± 1.26	1.38 ± 0.89	272.50	0.65	
C24:1ω9	27.83 ± 5.52	30.23 ± 6.15	223.50	0.15	
Saturated fatty acids					
C12	3.68 ± 3.65	4.18 ± 7.08	289.00	092	
C14	12.13 ± 16.99	21.63 ± 9.88	265.50	0.57	
C16	563.77 ± 216.21	604.13 ± 127.61	197.00	0.05	
C18	188.85 ± 54.99	199.82 ± 37.65	21,050	0.09	
C20 (arachidic acid)	7.37 ± 1.98	7.08 ± 1.20	289.50	0.93	
C22	16.11 ± 5.99	17.67 ± 3.51	226.00	0.27	
C24	13.70 ± 5.13	15.16 ± 3.06	249.00	0.36	

Values are mean ± SD. corr p value, p value after the Bonferroni correction.
* $p < 0.05$ versus normal controls.
** $p < 0.01$ versus normal controls.

Only plasma Cp levels were significantly different between the two groups. Considering that Cp has no capacity to bind fatty acids [67] and that the serum Cp levels are not significantly correlated to the nutrient levels [68], no significant correlation between the plasma levels of PUFAs and plasma Cp levels in this study appears to be reasonable.

We conducted controls for the dietary intake of all 49 participants. Moreover, the assessment of daily nutrients revealed no significant differences in the intake of fat, omega-3 and omega-6 PUFAs, AA, EPA, DHA, iron and copper between the random subsamples of 18 individuals with ASD and the 10 healthy controls. Thus, the altered composition of PUFAs detected in this study may not be due to dietary food and nutrient intake.

Excessive omega-3 PUFAs significantly inhibit the conversion of omega-6 PUFAs as a result of substrate competition [8,10,11]. Both DHA and EPA compete for the enzymes and products of AA metabolism [8,13,14]. A concurrent increase in the dietary omega-3 PUFAs, a reduction in plasma AA levels [11,12], and the levels of AA-derived eicosanoids are decreased by these omega-3 PUFAs in a dose-dependent manner [14]. A previous clinical study of 1979 healthy male and female subjects reported an antagonistic relationship between omega-3 PUFAs and AA in the maintenance of homeostasis [9]. Drawing these facts together, it is reasonable to note that the present findings revealed, for the first time, that increased plasma DHA/AA and EPA/AA ratios, which are indicative of the competitive interaction between high plasma DHA and EPA levels and low plasma AA levels, may be correlated with reduced plasma levels of Cp, which is a neuroprotectant [21,22] involved in the etiology of central nervous system diseases [22,69], in the 28 young individuals with ASD. Plasma DHA/AA and EPA/AA ratios were significantly correlated with all five ABC subscales and the total ABC scores. Notably, the multiple linear regression analysis identified significant correlations between the plasma DHA/AA and EPA/AA ratios and the variables, after adjusting for the two subject groups, the three signaling biomarkers and the total ABC scores in the whole population (Table 4). These findings revealed that the plasma DHA/AA and EPA/AA ratios fit models that distinguished the ASD group from the control group and significantly predicted these important adjusted variables.

Table 4
Spearman's correlation coefficient between the plasma ratios of DHA/AA and EPA/AA and signaling mediators, and the Aberrant Behavior Checklist scores in the 28 individuals with ASD and the 21 normal controls.

	Irritability		Social withdrawal		Stereotypy		Hyperactivity		Inappropriate speech		Total	
	r	p	r	p	r	p	r	p	r	p	r	p
DHA/AA	0.43	0.001**	0.45	0.001**	0.35	0.01*	0.41	0.002**	0.42	0.002**	0.45	0.001**
EPA/AA	0.45	0.001**	0.50	0.000**	0.42	0.002**	0.42	0.002**	0.36	0.009**	0.47	0.000**
Cp	−0.18	0.21	−0.13	0.37	−0.08	0.90	−0.17	0.23	−0.13	0.36	−0.16	0.24
Tf	−0.05	0.70	−0.02	0.89	0.13	0.36	0.02	0.88	0.05	0.73	−0.04	0.77
SOD***	−0.25	0.07	−0.12	0.39	−0.22	0.12	−0.21	0.14	−0.28	0.045*	−0.22	0.13

Cp, ceruloplasmin; Tf, transferrin; SOD, superoxide dismutase; r, Spearman correlation coefficient.
*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ were considered statistical significance.

Table 5
The intake of nutrients in the random subsamples of 18 of the 28 individuals with ASD and 10 of the 21 normal controls.

	ASD (n = 18)	Control (n = 10)	U	p value
Age (years)	11.8 ± 4.1	13.3 ± 5.1	24.0	0.69
High (cm)	142.9 ± 23.0	151.6 ± 1 9.1	22.5	0.54
Wight (kg)	40.0 ± 23.0	45.6 ± 15.6	20.5	0.38
Energy (kcal)	2153.2 ± 558.2	2421.0 ± 503.3	20.0	0.40
Fat (g/day)	72.0 ± 27.9	87.3 ± 22.2	18.0	0.28
Unsaturated fatty acid (g/day)	14.7 ± 4.2	18.4 ± 4.5	15.0	0.15
Saturated fatty acid (g/day)	24.4 ± 14.0	28.0 ± 10.2	21.0	0.46
Omega-3 PUFAs (g/day)	2.6 ± 0.9	3.1 ± 0.5	19.0	0.33
Omega-6 PUFAs (g/day)	12.3 ± 3.7	15.6 ± 4.2	16.0	0.19
EPA (mg/day)	226.8 ± 169.6	197.8 ± 103.7	27.5	0.96
DPA (mg/day)	73.3 ± 47.9	72.3 ± 26.5	25.5	0.78
DHA (mg/day)	400.8 ± 241.0	380.8 ± 115.8	26.5	0.87
AA (mg/day)	168.5 ± 15.9	209.1 ± 75.0	19.5	0.34
Iron (mg/day)	9.3 ± 2.9	9.5 ± 3.0	25.0	0.78
Copper (g/day)	1.3 ± 0.4	1.3 ± 0.4	28.0	1.00
Protein (g/day)	80.3 ± 24.7	90.5 ± 24.1	20.0	0.40
Animal protein (mg/day)	32.6 ± 8.6	32.3 ± 12.5	27.0	0.96
Cholesterol (mg/day)	124.9 ± 186.4	95.6 ± 169.6	22.0	0.54
Carbohydrates (g/day)	292.3 ± 60.0	309.1 ± 59.7	25.0	0.78
Vitamin B6 (mg/day)	1.4 ± 0.5	1.4 ± 0.4	25.0	0.78
Vitamin C (mg/day)	158.5 ± 79.2	121.4 ± 82.9	19.0	0.34
Vitamin D (mg/day)	10.0 ± 5.4	9.1 ± 2.4	27.5	0.96

Values are mean ± SD.

Thus, the lowered plasma Cp levels in association with the increased plasma DHA/AA and EPA/AA ratios may contribute to behavioral symptoms in the 28 individuals with ASD.

Interestingly, the plasma levels of arachidic acid were significantly higher in the ASD group than in the control group. Hydrogenation inhibits the release of prostacyclin, which is a prostanoid metabolized from endogenous AA [70]. This occurs by inhibiting the conversion of LA to AA or shunting the free AA released by the phospholipase A2 action to be metabolized by another pathway, leaving less AA available for phospholipase A2 synthesis [71,72]. In addition, hydrogenated fats increase the production of PGE_2-related tumor necrosis factor α (TNF-α) [73]. Collectively, increased plasma arachidic acid might reflect increased AA hydrogenation, which might inhibit the production of AA-related eicosanoids involving Cp.

The plasma adrenic levels were significantly lower in the ASD group. Adrenic acid is a quantitatively significant metabolic fate for AA [74], is formed by AA chain elongation or the elongation and desaturation of linoleic acid and can be converted to AA by β-oxidation [75]. The lowered plasma levels of adrenic acid may therefore reflect the lowered plasma AA levels.

Most studies of ASD have reported elevated serum Cp levels [37] or lower serum Cp levels [36] compared to the age-matched controls. Thus, previously reported findings on blood Cp levels in ASD were inconsistent [36,37]. Moreover, there is no data on the mechanisms underlying the alteration of Cp in ASD. The present study is the first to reveal that increased plasma DHA/AA and EPA/AA ratios, which is indicative of increased plasma DHA and EPA levels and decreased plasma AA levels, may be the underlying mechanisms of the reduced plasma Cp levels. Moreover, this is the first report that increased AA hydrogenation may have contributed to decreased plasma Cp levels.

In the ASD group, the increased plasma levels of the omega-3 PUFA fractions, such as EPA, DPA and DHA, may reflect an increased biosynthesis of these omega-3 PUFAs, while the reduced plasma levels of AA and adrenic acid may reflect the reduced biosynthesis of AA and AA-formed adrenic acid. In human cells, PUFAs are converted into their elongated and desaturated forms by elongase and desaturase enzymes [76], particularly delta-5 and delta-5 desaturases. The activity of these enzymes is primarily regulated at the transcriptional level [76,77]. Therefore, alterations in fatty acid biosynthesis may depend on the presence of transcriptional factors. In addition, the existence of DHA synthesis from EPA through a Sprecht-independent pathway in some mammals has been reported [78]. Further studies of the altered biosynthesis of omega-3 and omega-6 PUFAs and saturated fatty acids in psychiatric disorders are needed.

Eicosanoids, which are signaling mediators, are primarily derived from the enzymatic oxygenation of AA [79–81]. Thus, the increased plasma DHA/AA and EPA/AA ratios may be related to the down-regulation of AA-related eicosanoid signaling mediators, as indicated by the lowered plasma Cp levels in the ASD group.

Previous studies have shown altered PUFA compositions and have not mentioned the competitive interaction between omega-6 and omega-6 PUFAs in autistic children aged 3–17 years old [6], 3–15 years old [7] and 4–12 years old [82], as well as those in children younger than 5 years of age [83]. In this study, an imbalance between the plasma levels of omega-3 PUFAs and the plasma AA levels was found in ASD individuals with an average age of 13.5. At higher ages, omega-3 PUFAs may protect against neurodegeneration [84]. By protecting the brain from oxidative stress, omega-3 PUFAs minimize brain damage and deterioration that occurs with aging [85]. Therefore, the difference in the results between these previous studies and the present study might be due to an age-dependent metabolic mechanism.

Our study had some limitations: a) the concentrations of eicosanoid family members (e.g., cyclooxygenase [86]) were not measured, and we examined only three biomarkers of copper and iron signaling mediators. As AA-derived eicosanoid signaling mediators include many prostaglandin families [38] and these mediators are affected by multiple factors [39], studying AA-derived eicosanoids may be very complex. In this study, we examined plasma copper and iron signaling mediators. The regulation of iron and copper homeostasis is essential for life and may be related to the pathophysiology of several neurodegenerative disorders [87]. Therefore, we measured Cp, Tf and SOD levels. Further studies should measure plasma levels of PUFA-related eicosanoid family members and other useful biomarkers, such as 20-hydroxyeicosatetraenoic acid [88], the plasma levels of 18 eicosanoids [89], or the urinary eicosanoid levels (e.g., tetranor-Prostaglandin E metabolite [90]). b) Even though the high number of male participants with ASD may affect the study results, this disorder is most prevalent in males, with a male to female ratio of 4 to 1 [91]. In this study, ASD and normal control groups were age- and gender-matched. Further studies should evaluate the effect of gender on the composition of PUFAs. c) The small sample size in this study affects our ability to generalize our results to entire ASD populations. Future studies should investigate the increased biosynthesis of PUFAs using large sample sizes.

Table 6
Results of the multiple linear regression.

Model	Model R2	Model p-value	Coefficients B	Beta coefficients	p value
DHA/AA	0.34	0.003**			
Cp			0.013 ± 0.006	0.295	0.046
Tf			−0.001 ± 0.001	−0.274	0.047#
SOD			−0.003 ± 0.010	−0.133	0.299
ABC total score			−0.001 ± 0.001	−0.463	0.645
Group (1 = ASD, 2 = control)			−0.268 ± 0.098	−0.621	0.009##
EPA/AA	0.265	0.018*			
Cp			0.006 ± 0.004	0.226	0.142
Tf			−0.001 ± 0.000	−0.251	0.083
SOD			−0.005 ± 0.007	−0.091	0.499
ABC total score			0.000 ± 0.001	0.071	0.759
Group (1 = ASD, 2 = control)			−0.122 ± 0.068	−0.430	0.080

R2 = R-squared values; Cp, ceruloplasmin; Tf, transferrin; SOD, superoxide dismutase; B = unstandardized coefficients; ABC, Aberrant Behavior Checklist.
*p < 0.05, **p < 0.01, significance of R square. #p < 0.05, significant; ##p < 0.01, significant contribution.

In conclusion, high plasma DHA/AA and EPA/AA ratios, which were indicative of increased plasma DHA and EPA levels and decreased plasma AA levels, were correlated with decreased plasma levels of Cp, which is a neuroprotectant involved in the pathogenesis of neuronal disorders. It is plausible that increased AA hydrogenation might have contributed to reduced plasma Cp levels. It is plausible that increased plasma DHA/AA and EPA/AA ratios may be risk factors in the development of behavioral symptoms in young individuals with ASD.

Conflict of interest statement

All authors must disclose any financial and personal relationships with other people or organizations that could inappropriately influence our work.

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