Journal of Translational Medicine and Developmental Disorders, 2015, Vol. 2, No. 1, 1-9
Available online at http://pubs.sciepub.com/jtmdd/2/1/1

DOI:10.12691/jtmdd-2-1-1

Down-regulation of Signaling Mediator in Related to Increased Ratio of Docosahexaenoic Acid/Arachidonic Acid in Individuals with Autism Spectrum Disorders

Kunio Yui[1,*], George Imataka[2], Yohei Kawasaki[3], Tsutomu Yamada[3]

[1]Research Institute of Pervasive Developmental Disorders, Ashiya University, Rokurokusocho, Ashiya, Hyogo, Japan
[2]Department of Pediatrics, Dokkyo Medical University School of Medicine, 880 Kitakobayashi, Mibu, Tochigi, Japan
[3]Department of Drug Evaluation and Information, School of Pharmaceutical Science University of Shizuoka, Shizuoka, Japan
*Corresponding author: yui16@bell.ocn.ne.jp

Received September 10, 2015; Revised September 29, 2015; Accepted September 29, 2015

Abstract **Background and aim:** Autism spectrum disorder (ASD) is a highly prevalent neurodevelopmental disorder characterized by abnormal social interactions, communication deficits and stereotyped or repetitive behaviors. Converging lines of research indicate that the altered composition of polyunsaturated fatty acids (PUFAs) may contribute to the pathophysiology of ASD. **Methods:** We examined the relationships between the plasma ratios of omega-3PUFs/arachidonic acid (AA), plasma levels of 21 fatty acid fractions, and biomarkers of AA-related signaling mediators (ceruloplasmin, transferrin and superoxide dismutase) with the behavioral symptoms of 32 individuals with ASD (mean age, 13.5 ± 4.3 years old) and 20 age- and gender-matched normal controls (mean age, 13.2 ± 5.4 years old). Behavioral symptoms were assessed using the Aberrant Behavior Checklists (ABC). **Results:** Plasma levels of EPA, DPA and DHA, and the plasma ratios of docosahesaenoic acid (DHA)/AA were significantly higher while plasma levels of AA, 5,8,11,14-eicosatetraenoic acid and Cp were significantly lower in the 32 individuals with ASD compared with the 20 normal controls. The ABC scores were significantly increased in the ASD group compared to those of the control group. **Discussion and Conclusion:** High plasma DHA/AA ratio related to the increased plasma DHA levels and reduced plasma AA level may down-regulate mediators of AA signaling, such as Cp. Additionally, as 5,8,11,14-eicosatetraenoic acid is an arachidonate metabolite, the reduced plasma AA revels might have a pathophysiological factor in the reduced plasma Cp levels. Subsequently, reduced Cp levels may reduce the protective capacity of the brain against damage, which may cause in the pathophysiology underlying behavioral symptoms observed in individuals with ASD.

Keywords: *signaling mediators, ceruloplasmin, competitive interaction, arachidonic acid, docosahexaenoic acid*

Cite This Article: Kunio Yui, George Imataka, Yohei Kawasaki, and Tsutomu Yamada, "Down-regulation of Signaling Mediator in Related to Increased Ratio of Docosahexaenoic Acid/Arachidonic Acid in Individuals with Autism Spectrum Disorders." *Journal of Translational Medicine and Developmental Disorders*, vol. 2, no. 1 (2015): 1-9. doi: 10.12691/jtmdd-2-1-1.

1. Introduction

Autism spectrum disorder (ASD) comprises a group of pervasive developmental disorders characterized by social deficits, language impairment, and repetitive/restricted behaviors. Even though pathogenesis of ASD remains unclear, it appears to be associated with systemic metabolic abnormalities [1]. There is increasing evidence that altered metabolism of polyunsaturated fatty acids (PUFAs) may be involved in the pathophysiology of ASD [2]. PUFAs are essential components of cell membranes, where the majority of the cell signaling machinery resides [3]. The omega-3 PUFA docosahexaenoic acid (DHA, 22:6ω-3) and eicosapentaenoic acid (EPA, 20:5ω-3), and the omega-6 PUFA arachidonic acid (AA, 20:4ω-6) play important roles in the functions of the nervous system [4], neuroplasticity [5]], and signal transduction [6]. AA acts as a second messenger and regulates the activity of other signaling cascades [7]. Therefore, ASD may be attributed to altered PUFA composition and related signaling mediators. Previous studies have shown that there is a marked reduction in plasma DHA levels in various neuronal disorders [8] and ASD [9], with changes in plasma omega-3/omega-6 ratio [10]. Importantly, the balance between omega-3 and omega-6 PUFAs, which is critical for normal brain function and development, is achieved through both synergistic and antagonistic mechanisms [11]. The relationship between omega-3 PUFAs and AA is antagonistic at high EPA and DHA concentrations for maintaining homeostasis [12]. Both synergism and antagonism may aim at a balance between omega-3 and omega-6 PUFAs to maintain homeostasis [12]. Dietary omega-6 PUFAs lowering increases bioavailability of omega-3 PUFAs in human plasma [13]. This competitive interaction between omega-3 PUFAs and AA reflects increased plasma omega-3/omega-6 PUFA

ratios [12,15,16]. Omega-3 PUFAs may attenuate both tissue AA levels and eicosanoid formation [16]. The antagonism of AA-signaling has been reported [17,18], and this antagonism has important effects upon cell signaling in the CNS [17], modulating the activity of many neurotransmitters in affective disorders [17]. The omega-3 PUFAs are incorporated at existent of AA, and EPA and DHA inhibit AA metabolism, decreasing production of AA-derived eicosanoids [19,20]. Thus, reduced AA levels associated with the competitive interaction between omega-3 PUFAs and AA may downregulate AA-related signaling mediators. However, the relationship between this competitive interaction and related AA-related signaling mediators is unclear in neuropsychiatric disorders including ASD.

With respect to interactions between PUFAs and signaling mediators, ceruloplasmin (Cp) [21], superoxide dismutase (SOD) [22] and transferrin (Tf) [23] have been known to act as signaling mediators in ASD. Cp is an important copper signaling biomarker of neuronal function [24]. Cp has a natural neuroprotective mechanism [25,26], and may contribute to pathophysiological conditions in the central nervous systems [27]. SOD is a biomarker of copper signaling [28]. Tf is an iron signaling mediator [29]. Changes in the blood levels of SOD, TF and CP were shown to indicate altered antioxidant status [30-34] and copper dyshomeostasis [35,36] in ASD patients. As described above, the relationship between imbalance of omega-3/omega-6 PUFA ratios, which is indicative of the competitive interaction between increased omega-3 PUFAs and AA, and AA-related signaling mediators (i.e., Cp, Tf and SOD) remains unclear in ASD. Moreover, there is no information regarding the mechanisms that underlie alterations of these signaling mediators in psychiatric disorders including ASD.

We hypothesized that higher plasma ratios of omega-3PUFAs/AA would correlate with a downregulation of AA-related signaling mediators in individuals with ASD. In investigating this hypothesis, we measured the plasma levels (%) of the compositions of PUFAs, monosaturated and saturated fatty acids, and DHA/AA and EPA/AA ratios. Since, multiple subfamilies of AA-derived eicosanoid signaling mediators exist [37] and since these mediators are affected by multiple factors [38], we examined plasma levels of Cp, SOD and Tf as known AA-related signaling mediators. Furthermore, because plasma fatty acid levels can be affected by prior dietary intake, we conducted controlling for dietary intake and assessed the consumption of nutrients using the self- administered diet history questionnaire system.

2. Methods

2.1. Subjects

A total of 52 young, physically healthy individuals who in 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, Ashiya University by the order of their submission to medical consultation in the Research Institute between January 2012 and July 2014. Diagnosis was performed based on DSM-IV-TR criteria, and additionally confirmed by the Autism Diagnostic Interview-Revised (ADI-R)[39]. Among the 52 individuals, 32 had an independent clinical diagnosis of ASD (22 males and 10 females, mean age: 13.5 ± 4.3 years old, range: 6-21 years old), and the remaining 20 were normal healthy controls (14 males and 6 females, mean age: 13.2 ± 5.4 years old, range: 5-21 years old). The ASD and control groups were matched for home environment, feeding habits, age, gender and full intelligent quotient (IQ) scores (Table 1). All 32 individuals with ASD were diagnosed by two psychiatrists specializing in ASD and developmental disorders. The 32 individuals with ASD 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 20 individuals in the control group were considered to be physically and mentally healthy based on initial physical and mental screening tests. Moreover, the 32 individuals with ASD and the 20 normal controls has not any gastrointestinal dysfunction including dysphagia, gastroparesis and bowel dysfunction. 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 52 participants. These 52 participants did not have any abnormalities on physical examinations and laboratory findings. 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 histories of neurological conditions, including seizure, movement disorders or a history of 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 [40] or the respective scale for adults (WAIS-R) [41] because subjects with high-functioning pervasive developmental disorders were required to have a total IQ of at least 70 [42]; (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 had remained stable for at least 3 months before and during the study).

This study complied with the Declaration of Helsinki and was performed with the approval of the ethics committee of the Fujimoto Medical Clinic in Kobe City, Japan. This ethics committee was registered with the Pharmaceuticals and Medical Devices Agency of Japan for registration of IRB information (http://www.info.pmda.go.jp/). Written informed consent was obtained from the participants and/or their parents.

2.2. Controlling for Dietary Intake and Assessment of Nutrient Intake

Because plasma fatty acid levels can be affected by prior dietary intake, many prior studies have used the 2010 dietary Guidelines for Americans [43]). In this study, all 52 participants received "Japanese Food Guide Spinning TOP" [44], which was characterized by favorable dietary intakes of foods and nutrients, based the "Dietary

Reference Intakes for Japanese. -2010-"[45]. All 52 individuals or their parents were provided a diet plan sample menus (KAWASAKI FOODMODEL) (http://item.rakuten.co.jp/foodmodel/751741), which was edited according to "Japanese Food Guide". Moreover, to assess daily food and nutrients intake, a semi-constructive questionnaire for assessment of dietary food and nutrient intake for Japanese (DHQ support center, Tokyo, Japan; http://www.ebnjapan.org/) was performed using the junior high school version (DHQ15) (DHQ support center, Tokyo, Japan). DHQ15 is a structured questionnaire that asks about the consumption of frequency and portion size of selected foods commonly consumed in Japan [46], and usual cooking methods. The validity of the junior high school version of the DHQ 15 has been verified [47]. Energy and selected nutrients for 150 various food and beverage items were calculated using a custom-designed computer algorism for DHQ based on the Standard Tables of Food Consumption in Japan (DHA support center). DHQ15 was administered during one month before this study in 17 individuals with ASD and 10 normal controls by the order of their submission to medical consultation during January 2014 and December 2014. Their mothers completed the DHQ15 at home and returned the questionnaires to the Fujimoto clinic at Kobe. If missing or unclear information was recorded on the sheet by the guardian, the researcher (KY) questioned the guardian by phone or e-mail.

2.3. Assessment of Behavioral Symptoms

The ABC was used to assess the behavioral symptoms of the 32 individuals with ASD and the 20 normal controls. The ABC is primarily intended to evaluate treatment responses in psychopharmacological and behavioral intervention trials for children and adolescents with mental retardation [48] and normal IQ levels [49]. 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 [50].

2.4. Assays of Plasma Levels of PUFAs, Cp, SOD and Tf

2.4.1. Methods of Blood Sampling

Whole-blood samples were collected in EDTA tubes by venipuncture and immediately placed on ice in a refrigerator. Plasma was obtained by centrifugation for 20 minutes at 3000 x g at room temperature (22 °C). To decrease the effects of circadian variation, the blood collection performed at 11:00-12:30 in a quiet laboratory room after supine rest for 20 minutes. The samples were frozen at –80°C until analysis. The plasma levels of PUFAs, Cp, Tf and SOD were measured by specialists at SRL, Inc (Tokyo, Japan).

2.4.2. Plasma Levels of PUFAs.

The fatty acid composition of the PUFA fraction from each patient's plasma was determined as previously described [51]. In summary, total lipids were extracted from the plasma according to [52]. After transmethylation with HCl-methanol, 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 level of each PUFA is expressed as the mean ± SD weight (percentage) of the total fatty acids.

2.4.3. Plasma Levels of Cp.

A Bering BN Ⅱ Nephelometer (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 Versa max 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

We calculated the ratios of DHA/AA and EPA/AA to evaluate the balance between omega-3 and omega-6 PUFAs. Because the data were not normally distributed, the non-parametric Mann-Whitney U test was used for multiple comparisons in order to find out the statistical differences in estimates of daily intake for nutrients, plasma levels of the 23 composition of fatty acids, plasma ratios of DHA/AA and EPA/AA, and the plasma levels of the three biomarkers (Cp, Tf and SOD), and the five subscale and total ABC scores between the 32 individuals with ASD and the 20 normal controls. Spearman's rank correlation coefficients (r) were used to determine the correlations between the plasma DHA/AA and EPA/AA ratios, plasma signaling levels of biomarkers (Cp, Tf and SOD), and the five subscale and total ABC scores in the entire population. In addition, Spearman's rank correlation coefficients were used to examine the relationship between dietary intake of main nutrients (protein, fat , omega-3 PUFA, omega 6-PUFAs, AA, EPA and DHA) affecting these plasma PUFA levels and ratios and plasma levels of EPA, DHA and AA, and plasma ratios of DHA/AA and EPA/AA.

Moreover, multiple linear regression analysis was used to confirm the relationships between plasma DHA/AA and EPA/AA ratios, and the other main variables, adjusting for two subject groups, the three signaling biomarkers and the total ABC scores in order to confirm the relationship between the ratios of omega-3 PUFAs/AA and AA-related signaling mediators (Table 4). We conducted statistical analysis with SPSS version 18.0 (IBM Tokyo, 2009).

3. Results

3.1. Characteristics of Subjects

The mean total ABC score was 66.00 ± 29.22. An earlier study reported a total ABC score of 60.14 for children and adolescents with moderate to severe ASD [53]. Thus, our patients were suffering from moderate to severe ASD symptoms including restricted repetitive and stereotyped patterns of interests and behaviors. The age did not differ significantly between the two groups (p = 0.90). The ABC subscales scores for irritability (P = 0.000), social withdrawal (p = 0.000), stereotypic behavior p = 0.000), hyperactivity (p = 0.000) and inappropriate speech (p = 0.000) and total ABC scores (p = 0.000) were significantly higher in the ASD group than in the control group (Table 1).

Table 1. Subject characteristics, plasma levels of signaling mediators and the ABC subscale scores in the 32 individuals with ASD and 20 normal controls

Parameters	ASD (n=32)	Controls (n=20)	U	p value
Age (Year)	13.5 ± 4.7	13.9 ± 5.7	313.00	0.90
Sex (male/female)	10/22	6/14	χ^2=0.00	1.00
Scores of Autism Diagnostic Interview-Revised				
Domain A (social)	20.7 ± 6.9	N/A		
Domain B (communication)	13.9 ± 5.0	N/A		
Domain C (stereotyped)	7.0 ± 5.8	N/A		
Plasma biomarkers levels (%)				
Cp	24.90 ± 4.59	28.20 ± 4.97	209.50	0.037*
Tf	274.28 ± 47.77	270.50 ± 36.75	305.50	0.78
SOD	3.42 ± 2.58	3.84 ± 3.00	273.50	0.38
Scores of the ABC				
Irritability	13.66 ± 9.23	0.50 ± 0.76	9.50	0.000***
Social withdrawal	22.81 ± 9.23	0.85 ± 2.06	1.50	0.000***
Stereotypy	6.00 ± 5.72	0.15 ± 0.49	68.50	0.000***
Hyperactivity	18.59 ± 10.67	0.60 ±1.60	10.00	0.000***
Inappropriate speech	4.91 ± 4.19	0.20 ± 0.52	50.00	0.000***
Total	66.00 ± 29.22	2.30 ± 4.44	1.00	0.000***

Data are means ± SD. ABC, Aberrant Behavior Cheklist; Cp, ceruloplasmin; Tf, transferrin, SOD, superoxide dismutase.* p < 0.05, *** p < 0.001: individuals with ASD vs. controls, using Mann-Whitney-U test.

3.2. Assessment of Nutrient Intake

All 52 participants received "Japanese Food Guide Spinning TOP" [44]. As shown in the Table 2, there were no significant differences in weight, high, energy, and intake of cholesterol, protein, carbohydrate, fat, animal fat, saturated fatty acids and unsaturated fatty acids. Importantly, intake of omega-3 (p = 0.54) and omega-6 PUFAs (p = 0.26), AA (p = 0.46), EPA (p = 0.71), DHA (p =0.81), iron (p = 1.00) and cupper (p = 0.81) did not significantly differ between the ASD and control groups (Table 2).

Table 2. The intake of nutrients in the random subsamples of 17 individuals with ASD and 10 normal controls

	ASD (n = 17)	Control (n = 10)	U	p value
Age (years)	12.3 ± 4.1	13.3 ± 5.1	23.5	0.90
Heigh (cm)	146.0 ± 23.0	151.6 ±1 9.1	21.5	0.71
Weight (kg)	42.6± 23.6	45.6 ± 15.6	19.5	0.54
Energy (kcal)	2223.5 ± 563.3	2421.0 ± 503.3	19.0	0.54
Fat (g/day)	75.2 ± 28.5	87.3 ± 22.2	17.0	0.38
Saturated fatty acid (g/day)	25.8 ± 14.5	28.0 ± 10.2	20.0	0.62
Unsaturated fatty acid (g/day)	15.3 ± 4.2	18.4 ± 4.5	14.0	0.21
Omega-3 PUFAs (g/day)	2.8 ± 0.8	3.1 ± 0.5	19.0	0.54
Omega-6 PUFAs (g/day)	12.7 ± 3.7	15.6 ± 4.2	15.0	0.26
EPA (mg/day)	250.6 ± 168.1	197.8 ± 103.7	21.5	0.71
DPA (mg/day)	80.2 ± 47.0	72.3 ± 26.5	23.5	0.90
DHA (mg/day)	436.1 ± 237.0	380.8 ± 115.8	22.5	0.81
AA (mg/day)	172.6 ± 11.9	209.1 ± 75.0	18.5	0.34
Iron (mg/day)	9.7 ± 2.9	9.5 ± 3.0	22.0	1.00
Copper (g/day)	1.4 ± 0.4	1.3 ± 0.4	28.0	0.81
Zinc (mg/day)	10.3 ± 2.6	11.5 ± 3.3	16.0	0.32
Calcium (mg/day)	993.2 ± 633.1	876.9 ± 709.8	20.0	0.62
Protein (g/day)	84.2 ± 23.9	90.5 ± 24.1	19.0	0.54
Cholesterol (mg/day)	140.2 ± 195.8	95.6 ± 169.6	21.0	0.71
Carbohydrates (g/day)	298.9 ± 61.6	309.1 ± 59.7	24.0	1.00
Vitamin B6 (mg/day)	1.5 ± 0.5	1.4 ± 0.4	24.0	1.00
Vitamin D (mg/day)	10.6 ± 5.4	9.1 ± 2.4	21.5	0.71

Data are mean ± SD.

3.3. Plasma levels of PUFAs and Biomarkers

The Mann-Whitney U test revealed that plasma levels of EPA, DPA, DHA, and plasma DHA/AA and EPA/AA ratios were significantly higher while plasma levels of AA, 5,8,11,14-eicosatetraenoic acid and Cp were significantly lower in the 32 individuals with ASD than in the 20 normal controls (Table 3).

Table 3. Plasma levels of fatty acid profiles of the 32 individuals with ASD and the 20 normal controls

Parameters	ASD (n=32)	Controls (n=20)	U	p value
Plasma PUFA levels (%)				
Omega-3 series				
C18:3ω3 (ALA)	0.74 ± 0.37	0.59 ± 0.20	234.00	0.11
C20:5ω3 (EPA)	1.36 ± 0.96	0.77 ± 0.40	160.00	0.003**
C22:5ω3 (DPA)	0.53 ± 0.17	0.45 ± 0.99	214.00	0046*
C22:6ω3 (DHA)	3.67 ± 1.40	2.96 ± 0.89	205.00	0.03*
Omega-6 series				
C18:2ω6 (LA)	29.08 ± 3.64	29.58 ± 3.66	316.50	0.95
C18:3ω6 (GLA)	0.32 ± 0.16	0.33 ± 0.03	300.50	0.71
C20:2ω6 (DGLA)	1.16 ± 0.40	1.36 ± 0.27	221.00	0.06
C20:2ω6	0.19 ± 0.04	0.19 ± 0.04	309.00	0.84
C20:4ω6 (ARA)	5.64 ± 1.36	6.77 ± 1.29	187.50	0.01*
Ratios of plasma levels of PUFAs				
DHA/AA	0.66±0.22	0.45 ±0.15	118.50	0.000***
EPA/AA	0.24 ± 0.16	0.12 ± 0.07	109.50	0.000***
Monounsaturated fatty acids				
C14:1ω5	0.02 ± 0.03	0.04 ± 0.04	194.00	0.07
C16:1ω7	1.17 ± 0.77	1.77 ± 0.69	208.00	0.82
C18:1ω9	20.44 ± 3.50	21.01 ± 2.79	260.00	0.26
C20:1ω9	0.16 ± 0.06	0.14 ± 0.03	274.50	0.39
C20:3ω9	0.08 ± 0.04	0.08 ± 0.02	191.50	0.020*
(5-8-11-14 eicosatetraenoic acid)				
C22:1ω9	0.04 ± 0.03	0.03 ± 0.02	292.50	0.59
C24:1ω9	1.17 ± 0.32	1.16 ± 0.25	305.50	0.79
Saturated fatty acids				
C12	0.14 ± 0.13	0.16 ± 0.25	282.50	0.48
C14	0.92 ± 0.44	0.83 ± 0.33	295.50	0.65
C16	23.27 ± 2.38	21.11 ± 5.09	270.00	0.43
C22	0.70 ± 0.17	0.67 ± 0.08	259.50	0.26
C24	0.60 ± 0.16	0.58 ± 0.09	260.00	0.26

Data are mean ± SD.

* p < 0.05. ** p < 0.01, *** p < 0.001: individuals with ASD vs. controls using Mann-Whitney-U test.

The Spearman's rank correlation coefficients indicated that plasma DHA/AA (Figure 1a) and EPA/AA (Figure 1b) ratios were significantly correlated with all five ABC subscale scores and total scores (all *r* values were greater than 0.32, $p < 0.05$, $p < 0.01$ or $p < 0.001$) for the whole population. Plasma levels of PUFAs (AA, EPA and DHA) and signaling biomarkers (Cp, Tf and SOD) were not significantly correlated with the ABC scores.

A multiple linear regression demonstrated that the plasma ratios of DHA/AA ratio ($R^2 = 0.324$, $P = 0.002$) and AA ($R^2 = 0.260$, $P = 0.01$) significantly associated with the variables adjusting for the two subject groups, the three signaling biomarkers and the total ABC scores (Table 4). Using a group as a dependent variable significantly contributed to the plasma DHA/AA ratio (unstandardized coefficients, $B = -0.229 \pm 0.091$, $\beta = -0.513$, and $p = 0.023$). These findings indicated that the plasma DHA/AA ratio allowed for the prediction of these variables in the ASD and control groups.

Table 4. Results of the multiple linear regression

Model	Model R2	Model P-value	Coefficients		
			B	Beta coefficiences	p value
DHA/AA	0.32	0.002**			
Cp			0.011 ± 0.006	0.249	0.077†
Tf			-0.001 ± 0.001	-0.235	0.079†
SOD			-0.012 ± 0.010	-0.145	0.248
ABC total score			0.001 ± 0.001	0.053	0.800
Group (1 = ASD,2 = control)			-0.229 ± 0.097	-0.513	0.023#

R2, R-squared values; Cp, ceruloplasmin; Tf, transferrin; SOD, superoxide dismutase; B = unstandardized coefficients; ABC, Aberrant Behavior Checklist.

** p < 0.01: significance of R square. #p < 0.05, ## p <0.01: significant contribution., †p < 0.07 or p < 0.08: a trend towards significance.

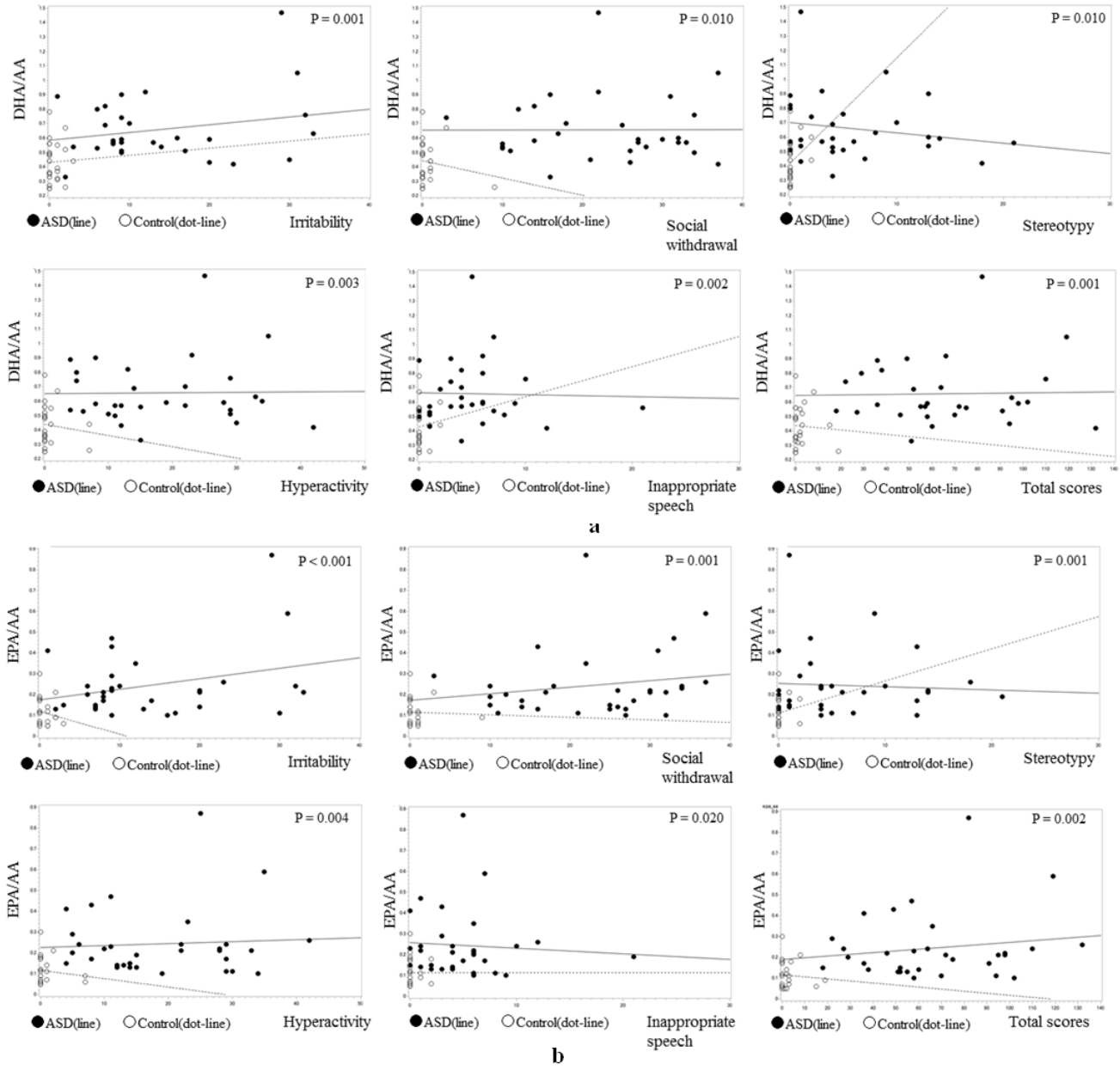

Figure 1. Correlation between plasma ratios of DHA/AA (Figure 1a) and EPA/AA (Figure 1b) and the ABC five subscale and total scores. Solid lines indicated Spearman's rank correlation coefficients in the 30 individuals with ASD and dotted lines indicated the 20 normal controls. Plasma DHA/AA and EPA/AA ratios were significantly correlated with all five ABC subscale scores and total scores (all r values were greater than 0.32, $p < 0.05$, $p < 0.01$ or $p < 0.001$) (Spearman's rank correlation coefficients)

3.4. The Relationship between Main Nutrients and Plasma Levels of EPA, DHA and AA, and Plasma DHA/AA and EPA/AA Ratios

Although plasma EPA levels were significantly negatively correlated to dietary intake of AA (r = -0.63. p = 0.015) in the random subsamples of 17 individuals with ASD and the 10 normal controls, there was no significant correlation between dietary intake of the main nutrients and the plasma variables (r = 0.02 -0.59, p = 0.192 - 0.969).

4. Discussion

Plasma PUFA levels have been shown to reflect changes in brain PUFA levels [54]. In this study, plasma levels of EPA, DPA, and DHA, and plasma DHA/AA and EPA/AA ratios were significantly higher, while plasma levels of AA, 5,8,11,14-eicosatetraenoic acid and Cp were significantly lower in the 32 individuals with ASD than in the 20 normal controls (Table 1). We conducted controlling for dietary intake in all 52 participants. Moreover, the assessment of daily nutrients revealed no significant differences in intake of fat, omega-3 and omega-6 PUFAs, AA, EPA, DHA, iron, copper, zinc, Vitamin B_6 and Vitamin D between the random subsamples of 17 individuals with ASD and the 10 normal controls. Although plasma EPA levels were significantly negatively correlated to intake of AA in the random subsamples of 17 individuals with ASD and the 10 normal controls, there was no significant correlation between the main nutrients and the plasma variables. Thus, dietary intake of nutrients may not directly affect the altered

composition of fatty acids detected in this study may not due to dietary food and nutrient intake.

Plasma DHA/AA and EPA/AA ratio were significantly correlated with all five of the ABC subscales and total scores. Notably, the multiple linear regression identified that plasma DHA/AA ratio was fitting models for distinguishing the ASD group from the control group and predicting the adjusted variables (Table 4).

Cp is an important copper signaling biomarker of neuronal function [55] with neuroprotective properties [25,26]. Cp is involved in the etiologies of several central nervous system diseases [56]. Collectively, the reduced plasma Cp levels may contribute to abnormal behavioral symptoms in the 32 individuals with ASD.

Considering that plasma 5,8,11,14-eicosatetraenoic acid is an omega-6 PUFA, known as a trivial name of AA [57], the reduced plasma concentrations of AA may constitute a pathophysiological factor that can lead to reduction in plasma Cp. As described above, Cp is closely related to AA-derived eicosanoid mediator [58]. The omega-3 PUFAs are incorporated at existent of AA, and EPA and DHA inhibit AA metabolism, decreasing production of AA-derived eicosanoids [16,19,20]. AA-derived eicosanoids levels is decreased by these omega-3 PUFAs [20]. The antagonism of AA-signaling has been proposed and plays important effects upon cell signaling in the CNS [17,18]. These previously reported strands therefore support the present finding that the reduced plasma AA levels were related to lowered plasma Cp levels.

In this study, increased plasma levels of omega-3 PUFAs such as DHA, DPA and EPA may be reflective of increased biosynthesis of these omega-3 PUFAs. In human cells, PUFAs are converted into elongated and desaturated forms by elongase and desaturase enzymes [59], particularly delta-5 and delta-5 desaturases. The activity of these enzymes is primarily regulated at the transcriptional level [59]. Moreover, PUFAs effect on the modulation of genes related to fatty acid oxidation [60]. Therefore, the biosynthesis of DHA, DPA and EPA may depend on the presence of transcriptional factors. However, effects of altered omega-3 and omega-6 PUFA biosynthesis in psychiatric disorders remains unclear. However, dietary intake of AA might affect the altered composition of fatty acids. Further studies are needed to study the effects of altered omega-3 and omega-6 PUFA biosynthesis and dietary intake of nutrients on plasma variables

Previous studies have shown altered PUFA compositions in autistic children aged 3-17 years old [9], 3-15years old [10] and 4-12 years old [61], and in those younger than 5 years of age [62]. In this study, an imbalance between plasma levels of omega-3 PUFAs and 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 [63]. By protecting the brain from oxidative stress, omega-3 PUFAs minimize brain damage and deterioration that occur with aging [64]. Therefore, the difference in the results between these previous studies and the present study might be due to an age-dependent metabolic mechanism.

This study had some limitations: a) the concentrations of eicosanoid family members (e.g., cleukotriene [64]) were not measured. However, AA-derived eicosanoid signaling mediators includes many prostaglandin families [37], and these mediators are affected by multiple factors [38]. The regulation of iron and copper homeostasis is essential for life and may be related to the pathophysiology of several neurodegenerative disorders [65]. We therefore measured Cp, Tf and SOD. Further studies should measure plasma levels of PUFA-related eicosanoid family members such as prosdtaglandin E2 [66] or 18 eicosanoids [67]; b) the higher number of male participants with ASD may have influenced the data presented here because ASD is strongly biased towards males with ratios of 4:1 (male : female) [68]; however, in this study, ASD and normal control groups were age- and gender-matched. Further studies should evaluate the effect of gender on PUFAs compositions.

In conclusion, the present study reveals that a high plasma DHA/AA ratio related to the reduced plasma AA levels may down-regulate mediators of AA signaling, such as Cp, and dampen copper homeostasis. The plasma concentrations of AA related fatty acid fractions may constitute a pathophysiological factor that can lead to reduction in plasma Cp. Subsequently, copper dyshomeostasis may reduce the protective capacity of the brain against damage, which may result in the pathophysiology of behavioral symptoms observed in individuals with ASD.

Acknowledgements

Kunio Yui received a Grant-in-Aid for Scientific Research on Innovative Areas (Grant No 21200017) (2010-2012) and a Grant-in-Aid for Scientific Research (C) (2014-2016) from the Ministry of Education, Culture, Sports, Science and Technology, Japan.

Conflict of Interest

The authors have no conflict of interests to report.

References

[1] Frye, R.E., Delatorre, R., Taylor, H., Slattery, J., Melnyk, S., Chowdhury, N., James, S.J. “Redox metabolism abnormalities in autistic children associated with mitochondrial disease.” *Transl Psychiatry*, 3, e273, 2013.

[2] Wong, C., Crawford, D.A. *Lipid signaling in the pathology of autism spectrum disorders*. In: V.B. Patel, V.R. Preedy, C.R. Martin (Eds.), *Comprehensive guide to autism*, Springer Publishing, New York, 2014, 1259-1283, 2014.

[3] Holowka, D., Korzeniowski,.K., Bryant, K.L., Baird, B. “Polyunsaturated fatty acids inhibit stimulated coupling between the ER Ca_{2+} sensor STIM1 and the Ca_{2+} channel protein Orai1 in a process that correlates with inhibition of stimulated STIM1 oligomerization.” *Biochim Biophys*, 1841, 1210-1216, 2014.

[4] Vrablik, T.L., Watts, J.L. “Polyunsaturated fatty acid derived signaling in reproduction and development: insights from Caenorhabditis elegans and Drosophila melanogaster.” *Mol Reprod Dev*, 80, 244-259, 2013.

[5] R. Crupi, A. Marino, S. Cuzzocrea, n-3 fatty acids: role in neurogenesis and neuroplasticity. Curr. Med. Chem. 20 (2013) 2953-2963.

[6] Tamiji, J., Crawford, D.A. “The neurobiology of lipid metabolism in autism spectrum disorders.” Neurosignals, 18, 98-112, 2010.

[7] Kurlak, L.O., Stephenson, T.J. “Plausible explanations for effects of long chain polyunsaturated fatty acids (LCPUFA) on neonates.” *ArchDis Child Fetal Neonatal Ed*, 80, F148-154, 1999.

[8] Bazinet, R.P., Layé, S. "Polyunsaturated fatty acids and their metabolites in brain function and disease." *Nat Rev Neurosci*, 15, 771-785. 2014.

[9] Vancassel, S., Durand, G., Barthélémy, C. Lejeune, B., Martineau, J., Guilloteau, D., Andrès, C., Chalon, S. "Plasma fatty acid levels in autistic children." *Prostaglandins Leukot Essent Fatty Acids*, 65, 1-7. 2001.

[10] El-Anary, A.K., Bacha, A,G., Al-Ayahdi, L.Y. "Impaired plasma phospholipids and relative amounts of essential polyunsaturated fatty acids in autistic patients from Saudi Arabia." *Lipids Health Dis*, 10 63, 2011a. http://www.ncbi.nlm.nih. gov/pmc/articles/PMC3107801/.

[11] Simopoulos, A.P. "Evolutionary aspects of diet: the omega-6/omega-3 ratio and the brain." *Mol Neurobiol*, 44, 203-215, 2011.

[12] Luxwolda, M.F., Kuipers, R.S., Smit, E.N., Velzing-Aarts, F.V., Dijck-Brouwer, D.A., Muskiet, F.A. "The relation between the omega-3 index and arachidonic acid is bell shaped: synergistic at low EPA+DHA status and antagonistic at high EPA+DHA status." *Prostaglandins Leukot. Essent. Fatty Acids*, 8, 171-178, 2011.

[13] Taha, A.Y., Cheon, Y., Faurot, K.F., Macintosh,B., Majchrzak-Hong, S.F., Mann, J.D., Hibbeln, J.R., Ringel, A., Ramsden, C.E. "Dietary omega-6 fatty acid lowering increases bioavailability of omega-3 polyunsaturated fatty acids in human plasma lipid pools." *Prostaglandins Leukot Essent Fatty Acids*, 90, 151-157, 2014.

[14] Schmitz, G., Ecker, J. "The opposing effects of n-3 and n-6 fatty acid." *Prog Lipid Res*, 47, 147-155, 2008.

[15] Janssen, C.L., Kiliaan, A.J. "Long-chain polyunsaturated fatty acids (LCPUFA) from genesis to senescence: The influence of LCPUFA on neural development, aging, and neurodegeneration." *Prog Lipid Res*, 53, 1-17, 2014.

[16] Whelan J. "Antagonistic effects of dietary arachidonic acid and n-3 polyunsaturated fatty acids." *J Nutr*, 126, 1086S-10891S, 1996.

[17] Obajimi, O., Black, K.D., MacDonald, D.J., Boyle, R.M., Glen, I., Ross, B.M. "Differential effects of eicosapentaenoic and docosahexaenoic acids upon oxidant-stimulated release and uptake of arachidonic acid in human lymphoma U937 cells." *Pharmacol Res*, 52, 183-191, 2005.

[18] Mori, T.A., Chem, C.P., Beilin, L.J. "Omega-3 Fatty Acids and Inflammation." *Curr Atheroscler Rep*, 6, 461-467, 2004.

[19] Morbtaten, K., Haug, T.M., Kleiveland, C.R., Lea,T."Omega-3 and omega-6 PUFAs induce the same GPR120-mediated signaling events, but with different kinetics and intensity in Caco-2 cells." *Lipids Health Dis*, 12, 101, 2013.

[20] Calder, C.P. "Polyunsaturated fatty acids and inflammatory processes: new twists in an old tale." *Biochimie*, 91, 791-795, 2009.

[21] Sidhu, A., Miller, P.J., Hollenbach, A.D. "FOXO1 stimulates ceruloplasmin promoter activity in human hepatoma cells treated with IL-6. Biochem. Biophys." Res Commun, 404, 963-967, 2011.

[22] Tsang, C.K., Liu, Y., Thoma, J., Zhang, Y., Zheng, X.F. "Superoxide dismutase 1 acts as a nuclear transcription factor to regulate oxidative stress resistance." *Nat Commun*, 5, 3446, 2014.

[23] Jian, J., Yang, Q., Huang, X. "Src regulates Tyr (20) phosphorylation of transferrin receptor-1 and potentiates breast cancer cell survival." *J Biol Chem*, 286, 35708-35715, 2011.

[24] Barbariga, M., Curnis, F., Spitaleri, A., Andolfo, A., Zucchell, C., Lazzaro, M., Magnani, G., Musco, G., Corti, A., Alessio, M. "Oxidation-induced structural changes of ceruloplasmin foster NGR motif deamidation that promotes integrin binding and signaling." *J Biol Chem*, 289, 3736-3748, 2014.

[25] Glezer, I., Chernomoretz, A., David, S., Plante, M.M., Rivest, S. "Genes involved in the balance between neuronal survival and death during inflammation." *PLoS One*, 2(3), e310, 2007.

[26] Ayton, S., Zhang, M., Roberts, B.R., Lam, L.Q., Lind, M., McLean, C., Bush, A.I., Frugier, T., Crack, P.J., Duce, J.A. "Ceruloplasmin and β-amyloid precursor protein confer neuroprotection in traumatic brain injury and lower neuronal iron." *Free Radic Biol Med*, 69, 331-337, 2014.

[27] Lazzaro, M., Bettegazzi, B., Barbariga, M., Codazzi, F., Zacchetti, D., Alessio, M. "Ceruloplasmin potentiates nitric oxide synthase activity and cytokine secretion in activated microglia." *J Neuroinflammation*, 11, 164, 2014.

[28] Fukai, T., Ushio-Fukai, M. "Superoxide dismutases: role in redox signaling, vascular function, and diseases." *Antioxid Redox Signal*, 15, 1583-1606, 2011.

[29] Moura, I.C., Hermine, O., Lacombe, C., Mayeux, P. "Erythropoiesis and transferrin receptors." *Curr Opin Hematol*, 193-198, 2015.

[30] Yorbik, O.Sayal, A., Akay, C., Akbiyik, D.I., Sohmen, T. "Investigation of antioxidant enzymes in children with autistic disorder." *Prostagland Leukot Essent Fatty Acids*, 67, 341-343, 2002.

[31] Söğüt, S., Zoroğlu, S.S., Ozyurt, H., Yilmaz, H.R., Ozuğurlu, F., Sivasli, E., Yetkin, O., Yanik, M., Tutkun, H., Savaş, H.A., Tarakçioğlu, M., Akyol, O. "Changes in nitric oxide levels and antioxidant enzyme activities may have a role in the pathophysiolgical mechanisms involved in autism." *Clin Chim Acta*, 331, 111-117, 2006.

[32] Zoroglu, S.S., Armutcu, F., Ozen, S., Gurel, A., Sivasli, E., Yetkin,O., Meram, I. "Increased oxidative stress and altered activities of erythrocyte free radical scavenging enzymes in autism." *Eur Arch Psychiatry Clin Neurosci*, 254, 143-147, 2004.

[33] Meguid, N.A., Dardir, A.A., Abdel-Raouf, E.R., Hashish, A. "Evaluation of oxidative stress in autism: defective antioxidant enzymes and increased lipid peroxidation." *Biol Trace Elem Res*, 143, 58-65, 2011.

[34] Parellada, M., Moreno, C., Mac-Dowell, K., Leza, J.C., Giraldez, M., Bailón, C., Castro, C., Miranda-Azpiazu, P., Fraguas, D., Arango, C. "Plasma antioxidant capacity is reduced in Asperger syndrome." *J Psychiat Res*, 46, 394-401, 2012.

[35] Chauhan, A., Chauhan, V., Brown, W.T., Cohen, I. "Oxidative stress in autism: increased lipid peroxidation and reduced serum levels of ceruloplasmin and transferrin-the antioxidant proteins." *Life Sci*, 75, 2539-2549, 2004.

[36] Tórsdóttir, G., Hreidarsson, S., Kristinsson, J., Snaedal, J., Jóhannesson, T. "Ceruloplasmin, superoxide dismutase and copper in autistic patients." *Basic Clin Pharmacol Toxicol*, 96, 146-148, 2005.

[37] F.H. Tessaro, F.H., T.S. Ayala, T.S., J.Q. Martins, J.Q. "Lipid mediators are critical in resolving inflammation: a review of the emerging roles of eicosanoids in diabetes mellitus." *Biomed Res Int*, 2015:568408. http://www.ncbi.nlm.nih. gov/ pmc/articles/PMC4383369/.

[38] Hata, A.N., Breyer, R.M. "Pharmacology and signaling of prostaglandin receptors: multiple roles in inflammation and immune modulation." *Pharmacol Ther*, 103, 147-166, 2004.

[39] Rutter, M., Le Couteur, A., Lord, C. "*ADI-R Autism Diagnostic Interview Revised Manual*." Western Psychological Services, Los Angeles, 2003, UAS.

[40] D. Wechsler, Wechsler Intelligence Scale for Children-Revised Manual. The Psychological Corporation, New York, NY, USA (1974).

[41] D. Wechsler. Wechsler Adult Intelligence Scale-Revised Manual. The Psychological Corporation, San Antonio, TX, USA (1981).

[42] Koyama, T., Kamio, Y., Inada, N., Kurita, H. "Sex differences in WISC-III profiles of children with high-functioning pervasive developmental disorders." *J* Autism *Dev Disord 39*, 135-141, 2009.

[43] Schroeder, N., Park, Y.H., Kang, M.S., Kim, Y., Ha, G.K., Kim, H.R., Yates, A.A., Caballero. B. "A Randomized Trial on the Effects of 2010 Dietary Guidelines for Americans and Korean Diet Patterns on Cardiovascular Risk Factors in Overweight and Obese Adults." *J Acad Nutr Diet*, 115, 1083-1092, 2015.

[44] Ministry of Health, Labour and Welfare, and Ministry of Agriculture, Forestry and Fishers, "Japanese Food Guide." Ministry of Health, Labour and Welfare, and Ministry of Agriculture, Forestry and Fishers, Tokyo, Japan, 2012.

[45] Ministry of Health, Labour and Welfare, "Dietary Reference Intakes for Japanese. -2010-." Ministry of Health, Labour and Welfare, Tokyo, Japan, 2010.

[46] Ministry of Health, Labour, and Welfare. "Overview of dietary reference Intake for Japanese (2015)." Ministry of Health, Labour, and Welfare, Tokyo, Japan, 2015.

[47] Okuda, M., Sasaki, S., Bando, N., Hashimoto, M., Kunitsugu, I., Sugiyama, S., Terao, J., Hobara, T. "Carotenoid, tocopherol, and fatty acid biomarkers and dietary intake estimated by using a brief self-administered diet history questionnaire for older Japanese children and adolescents." *J Nutr Sci Vitaminol (Tokyo)*, 55, 231-241, 2009.

[48] Rojahn, J., Aman, M.G., Matson, J.L., Mayville, E. "The Aberrant Behavior Checklist and the Behavior Problems Inventory: convergent and divergent validity." Res Dev Disabil, 24 (2003) 391-404, 2003.

[49] Hollander, E., Chaplin, W., Soorya, L., Wasserman, S., Novotny, S., Rusoff, J., Feirsen, N., Pepa, L., Anagnostou, E. "Divalproex sodium vs. placebo for the treatment of irritability in children and

adolescents with autism spectrum disorders." *Neuropsychopharmacology*, 35, 990-998, 2009.

[50] Karabekiroglu, K., Aman, M.G., "Validity of the aberrant behavior checklist in a clinical sample of toddlers." *Child Psychiatry Hum Dev*, 40, 99-110, 2009.

[51] Hamazaki, H., Itomura, M., Hamazaki, T., Sawazaki, S. "Effects of cooking plant oils on recurrent aphthous stomatitis: a randomized, placebo-controlled, double-blind trial." *Nutrition*, 22, 534-536, 2006.

[52] Bligh, E.G., Dyer, W.J. "A rapid method of total lipid extraction and purification." *Can J Med Sci*, 37, 911-917, 1959.

[53] Singh, K., Connors, S.L., Macklin, E.A., Smith, K.D., Fahey, J.W., Talalay, P., Zimmerman, A,W. "Sulforaphane treatment of autism spectrum disorder (ASD)." *Proc Natl Acad Sci U S A*, 111, 15550-15555, 2014.

[54] Sublette, M.E., Bosetti, F., DeMar, J.C., Ma, K., Bell, J.M., Fagin-Jones, S., Russ, M.J., Rapoport, S.I. "Plasma free polyunsaturated fatty acid levels are associated with symptom severity in acute mania." *Bipolar Disord*, 9, 759-765, 2007.

[55] Barbariga, M., Curnis, F., Spitaleri, A., Andolfo, A., Zucchelli, C., Lazzaro, M., Magnani, G., Musco, G., Corti, A., M. Alessio, M. "Oxidation-induced structural changes of ceruloplasmin foster NGR motif deamidation that promotes integrin binding and signaling." *J Biol Chem*, 289, 3736-3748, 2014.

[56] Squitti, R., Ghidoni, R., Siott, M., Ventriglia, M., Benussi, L., Paterlini, A., Magri, M. Binetti, G., Cassetta, E., Caprara, D., Vernieri, F., Rossini, P.M., Pasqualetti, P. Value of serum Nonceruloplasmin copper for prediction of mild cognitive impairment conversion to Alzheimer disease. *Ann Neurol*, 75, 574-580, 2014.

[57] Dalvi, S., Nguyen, H.H., On, N., Mitchell, R.W., Aukema, H.M., Miller, D.W., Hatch, G.M. "Exogenous arachidonic acid mediates permeability of human brain microvessel endothelial cells through prostaglandin E_2 activation of EP_3 and EP_4 receptors." *J Neurochem*, 2015.

[58] Sokov, A.V., Golenkina, E.A., Kostevich, V.A., Vasilyev, V.B., Sud'ina, G.F. "Interaction of ceruloplasmin and 5-lipoxygenase." *Biochemistry (Mosc)*, 75, 1464-1469, 2010.

[59] Seegmiller. A.C. "Abnormal unsaturated fatty acid metabolism in cystic fibrosis: biochemical mechanisms and clinical implications." Int J Mol Sci, 15, 16083-16099, 2014.

[60] Jacometo, C.B., Schmitt, E., Pfeifer, L.F., Schneider, A., Bado, F., da Rosa, F.T., Halfen, S., Del Pino, F.A., Loor, J.J., Corrêa, M.N., Dionello, N.J. "Linoleic and α-linolenic fatty acid consumption over three generations exert cumulative regulation of hepatic expression of genes related to lipid metabolism." *Genes Nutr*, 9, 405, 2014.

[61] El-Ansary, A.K., Bacha, A.G., Al-Ayahdi. L.Y. "Plasma fatty acids as diagnostic markers in autistic patients from Saudi Arabia." *Lipids Health Dis*, 10, 62, 2011b.

[62] Al-Farsi, Y.M., Waly, M.I., Deth, R.C., Al-Sharbati, M.M., Al-Shafaee, M., Al-Farsi, O., Al-Khaduri, M.M., Al-Adawi, S., Hodgson, N.W., Gupta, I., Ouhtit, A, "Impact of nutrition on serum levels of docosahexaenoic acid among Omani children with autism." *Nutrition*, 29, 1142-1146, 2013.

[63] Karr, J.E., Alexander, R.G. Winningham, R.G. "Omega-3 polyunsaturated fatty acids and cognition throughout the lifespan: a review." *Nutr Neurosci*, 14, 216-225, 2011.

[64] Innis SM. Dietary (n-3) fatty acids and brain development. *J Nutr*, 137, 855-859, 2007.

[65] Adel, S., Kakularam, K.R., Horn, T., Reddanna, P., Kuhn, H., Heydeck, D. "Leukotriene signaling in the extinct human subspecies Homo denisovan and Homo neanderthalensis. Structural and functional comparison with Homo sapiens." *Arch Biochem Biophys*, 565, 17-24, 2015.

[66] Zheng, W., Monnot, A.D. "Regulation of brain iron and copper homeostasis by brain barrier systems: implication in neurodegenerative diseases." *Pharmacol Ther*, 133,177-188, 2012.

[67] J.H. Chen C.J. Perry, Y.C. Tsui, M.M. Staron I.A. Parish, C.X. Dominguez, D.W. Rosenberg, S.M. Kaech, Prostaglandin E2 and programmed cell death 1 signaling coordinately impair CTL function and survival during chronic viral infection. Nat. Med. 21(2015) 327- 334

[68] Rago, B., Fu, C. "Development of a high-throughput ultra performance liquid chromatography-mass spectrometry assay to profile 18 eicosanoids as exploratory biomarkers for atherosclerotic diseases." *J Chromatogr B Analyt Technol Biomed Life Sci*, 936, 25-32, 2013.

[69] Baron-Cohen, S., Lombardo, M.V., Auyeung, B., Ashwin, E., Chakrabarti, B., Knickmeyer, R. "Why are autism spectrum conditions more prevalent in males?" PLoS Biol. 9, e1001081, 2012.