

# Low Doses of Long-chain Polyunsaturated Fatty Acids Affect Cognitive Function in Elderly Japanese Men: A Randomized Controlled Trial

Hisanori Tokuda<sup>1\*</sup>, Toshiaki Sueyasu<sup>1</sup>, Masanori Kontani<sup>1</sup>, Hiroshi Kawashima<sup>1</sup>, Hiroshi Shibata<sup>1</sup> and Yoshihiko Koga<sup>2</sup>

<sup>1</sup> Institute for Health Care Science, Suntory Wellness Ltd. 1-1-1 Wakayamadai, Shimamoto, Osaka 618-8503, Japan.

<sup>2</sup> Department of Neuropsychiatry, Kyorin University School of Medicine. 6-20-2 Shinkawa, Mitaka, Tokyo 181-8611, Japan.

**Abstract:** Several studies have reported that the supplementation of long-chain polyunsaturated fatty acids (LCPUFA), such as docosahexaenoic acid (DHA), eicosapentaenoic acid (EPA), and arachidonic acid (ARA) improve cognitive function in the elderly. However, the doses used in these studies were higher than general dietary LCPUFA intake levels. This randomized, double-blind, placebo-controlled trial evaluated the effects of low doses of LCPUFA supplementation corresponding to general dietary intake on cognitive function in non-demented elderly Japanese participants. Japanese men aged 55-64 years were enrolled and randomly allocated to the placebo or LCPUFA group. Participants received 4 weeks of supplementation with LCPUFA-containing oil (DHA, 300 mg/day; EPA, 100 mg/day; and ARA, 120 mg/day) or purified olive oil as placebo. Event-related potential P300, reflecting cognitive processes, was measured before and after supplementation. A total of 113 participants completed the supplementation period, and the per-protocol analysis included 69 participants. Changes in P300 latency were significantly different between the placebo group (+13.6 msec) and the LCPUFA group (-1.8 msec) after supplementation. Significant increases in DHA (+0.9%) and ARA (+0.6%) contents in plasma phospholipids were observed in the LCPUFA group; no changes were observed in the placebo group. Dietary DHA, EPA, and ARA intake were in the normal range for Japan participants and remained unchanged during the study. These results suggest that low doses of LCPUFA supplementation have the potential to improve cognitive function in elderly Japanese men.

**Key words:** event-related potentials, P300, cognitive function, arachidonic acid, docosahexaenoic acid, eicosapentaenoic acid, long-chain polyunsaturated fatty acid

## 1 INTRODUCTION

Age-related cognitive decline is a serious problem for the elderly. Long-chain polyunsaturated fatty acids (LCPUFA), such as docosahexaenoic acid (DHA) and arachidonic acid (ARA), are major components in the brain phospholipids. LCPUFA decreases with age in brains of animals<sup>1-3</sup> and humans<sup>4,5</sup>. Therefore supplementation with LCPUFA is expected to ameliorate the age-related cognitive decline associated with depleted LCPUFA levels. Supplementation with DHA or ARA was shown to ameliorate the decline of cognitive function in aged animals<sup>6,7</sup>. Various mechanisms have been shown to be associated with this effect in aged animals, such as improvement of synaptic plasticity, neurogenesis, and membrane fluidity<sup>1-3, 6, 8-10</sup>. In terms of human studies, several intervention trials of LCPUFA sup-

plementation have been conducted, and cognitive function in healthy elderly was shown to be improved by DHA/EPA or ARA supplementation<sup>11-14</sup>.

The doses of LCPUFA in these human trials ranged from 0.9-2.5 g/day of DHA/EPA<sup>11-13</sup> or 240 mg/day of ARA<sup>14</sup>, which are somewhat higher compared to general dietary intake levels. Dietary assessment studies revealed that the average dietary intake in various countries ranges from 0.1-0.6 g/day for DHA, 0.05-0.4 g/day for EPA, and 0.1-0.2 g/day for ARA<sup>15</sup>. Observation studies reported that dietary intake of LCPUFA, mainly DHA/EPA, was correlated with the reduction of risk of cognitive decline in non-demented participants<sup>16-21</sup>, and suggest the importance of dietary intake of LCPUFA. However, it is unclear whether low doses of LCPUFA that are close to general dietary intake

\*Correspondence to: Hisanori Tokuda, Institute for Health Care Science, Suntory Wellness Ltd. 1-1-1 Wakayamadai, Shimamoto, Osaka 618-8503, Japan.

E-mail: Hisanori\_Tokuda@suntory.co.jp

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levels affect cognitive function in the non-demented elderly.

Recently, neurophysiological methods have been developed to evaluate cognitive function. P300 is the major component of event-related potential (ERPs) measured using electroencephalograph. P300 component has a maximal positive waveform that appears approximately 300 msec after sensory stimulation given. P300 is considered a manifestation of central nervous system activity involved in the processing of new information when attention is engaged to update memory representation<sup>22</sup>. P300 has two components: latency and amplitude. Latency is considered to reflect cognitive processing speed, while amplitude is linked to the amount of attentional resources engaged in task completion. P300 latency is prolonged and P300 amplitude is generally decreased with aging<sup>23, 24</sup>, suggesting that P300 measurements can be used to evaluate cognitive function in healthy elderly participants. Studies have reported that the supplementation with 240 mg/day ARA-containing oil improved P300 latency and amplitude<sup>14</sup>, while sardine oil, including 491 mg/day of EPA and 251 mg/day of DHA, did not influence P300 components in elderly Japanese men<sup>25</sup>. Thus using P300 measurement is considered suitable for the evaluation of cognitive function in healthy elderly participants.

The purpose of this study was to evaluate the effects of low doses of LCPUFA supplementation, corresponding with dietary intake, on cognitive function in non-demented elderly participants. We examined the effect of 300 mg/day of DHA, 100 mg/day of EPA, and 120 mg/day of ARA on P300 components in elderly Japanese men. To detect the effects of low doses of LCPUFA, we carefully surveyed dietary LCPUFA intakes and the other lifestyle factors affecting P300 components, and removed these effects on P300 components.

## 2 EXPERIMENTAL

### 2.1 Study design

A randomized, double-blind, placebo-controlled, parallel group intervention trial was designed to evaluate the effects of low doses of LCPUFA supplementation on cognitive function in non-demented elderly Japanese men. This study was performed from February 2012 to August 2012 at a medical facility in Chuo-ku, Tokyo, Japan. Participants were recruited in Tokyo and its environs. Physical (height and weight) and physiological (blood pressure and pulse rate) parameters, and blood and urine were sampled at the time of recruitment starting in February-March 2012. Four hundred and thirteen participants were screened, and 115 were randomly allocated to the placebo or LCPUFA group. Participants received capsules with purified olive oil (placebo) or LCPUFA-containing oil (including ARA, DHA,

and EPA) for 4 weeks during May-July 2012. The Mini-Mental State Examination (MMSE)<sup>47</sup> and Beck Depression Inventory-Second Edition (BDI-II)<sup>48</sup> were used for screening as described below. Blood and urine were sampled after an overnight fast, physical and physiological parameters were measured, and ERPs were recorded at the screening period, baseline, and 4 weeks after the supplementation period. Fatty acid contents in plasma phospholipids were measured at the same time. Dietary fatty acid intake and mood states were assessed at baseline and 4 weeks after the supplementation period. Blood hematological and biochemical parameters were also measured, and a study diary was distributed and collected. Participants were instructed not to change their daily living habits (such as diet, medicine, physical activity, alcohol, smoking, and sleep) from before the present study. Participants were requested to maintain their typical lifestyle in the 2 days before P300 measurements at baseline and at 4 weeks after the supplementation period. The required sample size (110 participants) was calculated based on our preliminary study. Ninety participants (45 participants in each group) in the per-protocol analysis would have 85% power at a 5% level of significance to detect differences in P300 latency changes between groups. The Ethics Committee on Human Experimentation of Suntory Holdings Ltd. approved the study protocol, which conformed to the principles set forth in the Declaration of Helsinki. Written informed consent was obtained from all participants.

### 2.2 Randomization and allocation

Enrolled participants were assigned in a 1:1 ratio based on a random number table to one of the two masked supplements: placebo or LCPUFA. Then, it was confirmed that 7 factors (ARA and DHA composition in plasma phospholipids, age, education, occupation, drinking, and smoking) were distributed almost equally. The randomization codes for these participants and the codes for masked supplements were held by 2 different persons who were not involved in this study. Information about these assignments was masked to researchers until all data were collected and analyzed.

### 2.3 Participants

Japanese male participants aged 55-64 years who were right-handed and had observable P300 were eligible to participate. Exclusion criteria were as follows: hearing loss; dementia or suspicion of dementia (MMSE score < 24); depression or suspicion of depression (BDI-II score > 16); a history of neurological disorder or serious disorders such as cardiovascular disease, diabetes, cancer, asthma, and clinically significant systemic diseases; an allergy to gelatin or olive oil; an irregular lifestyle such as shift work; a history of measurement of ERPs using an auditory oddball paradigm; consumption of drugs or supplements that affect

the efficacy evaluation such as lipid metabolism or brain function; and a higher ARA level in plasma phospholipids (ARA content higher than 10%). This criterion for ARA level was set to evaluate cognitive function properly without the effects of ARA level in plasma phospholipids, according to our previous study<sup>26)</sup>.

#### 2.4 Experimental supplement

Experimental supplements in this study were purified olive oil and LCPUFA-containing oil. Fatty acid compositions of these supplements are shown in **Table 1**. The LCPUFA group consumed 1,033 mg/day of LCPUFA-containing oil in 6 soft gelatin capsules, in which 300 mg of DHA, 100 mg of EPA, and 120 mg of ARA were included as free fatty acid equivalents. The dose of LCPUFA was designed to be in a range of that from normal daily diets in elderly Japanese<sup>27)</sup>. The same amount of purified olive oil was administered in the placebo group. Participants were asked to take 6 capsules at the same time after their first

meal for 4 weeks. We checked the compliance of the capsule intake by the study diary.

#### 2.5 Outcome assessments

The primary outcome in this study was the change in cognitive processing speed, assessed by P300 latency. The secondary outcomes were P300 amplitude, LCPUFA (DHA, EPA, and ARA) content in plasma phospholipids, dietary LCPUFA (DHA, EPA, and ARA) intake, and mood states assessed by the Profile of Mood Status (POMS). Safety was assessed based on side effects (supplement-related adverse effects); the incidence of adverse events in both groups was reported throughout the 4-week supplementation period, along with changes in physical, physiological, hematological, biochemical parameters, and urinalysis.

#### 2.6 P300 measurements

ERPs were measured using auditory discrimination tasks according to the evoked potential measurement guidelines

**Table 1** Fatty acid composition in experimental supplements.

FA		Placebo (Purified olive oil) (%)	LCPUFA containing oil (%)
Palmitic acid	16:0	13.6	6.4
Stearic acid	18:0	3.0	2.6
Arachidic acid	20:0	0.4	0.3
Behenic acid	22:0	—	0.9
Lignoceric acid	24:0	—	2.2
Palmitoleic acid	16:1	1.4	3.5
Oleic acid	18:1	68.1	11.7
Eicosenoic acid	20:1	0.2	1.4
Docosenoic acid	22:1	—	1.0
Tetracosenoic acid	24:1	—	0.6
Linoleic acid	18:2n-6	11.8	3.1
Eicosadienoic acid	20:2n-6	—	0.3
Dihomo- $\gamma$ -linolenic acid	20:3n-6	—	0.9
ARA	20:4n-6	—	12.2
Docosatetraenoic acid	22:4n-6	—	0.3
Docosapentaenoic acid	22:5n-6	—	1.3
$\alpha$ -Linolenic acid	18:3n-3	0.6	0.5
EPA	20:5n-3	—	9.7
Docosapentaenoic acid	22:5n-3	—	1.9
DHA	22:6n-3	—	29.8
Others		0.9	9.4
Total		100.0	100.0

FA, fatty acid; ARA, arachidonic acid; EPA, eicosapentaenoic acid; DHA, docosahexaenoic acid.

established by Evoked Potential Examination Standards Committee of the Japanese Society of Clinical Neurophysiology as described previously<sup>14</sup>). Auditory ERPs were obtained using an oddball paradigm. Infrequent (20%) target tones (2 kHz 60 dB sound pressure level) were presented randomly among frequent (80%) standard tones (1 kHz 60 dB sound pressure level). The tone duration was 100 msec, with a rise and fall time of 10 msec. The average interstimulus interval was 2 sec (range 1.6-2.4 sec) and total number of tones was 250 (target 50, standard 200). Participants were instructed to count silently the number of target tones, and to push a button quickly and accurately when they heard them. After P300 measurement, the participants were asked to report the number of target tones they counted, which we checked as the task performance. P300 measurements were performed by clinical laboratory technicians who were masked to treatment group.

The electroencephalogram (EEG) was recorded from electrodes (AP-C151-015, Digitex Lab. Co., Ltd. Mitaka, Japan) placed at midline frontal (Fz), vertex (Cz), and midline parietal (Pz), according to the international 10-20 system. The EEG was recorded and filtered from 0.05 Hz to 100 Hz, using linked electrodes at the earlobes as the reference. The electrooculogram (EOG) was recorded from electrodes placed above and below the eyelids. The EEG and EOG were sampled at a rate of 1 msec per point. The EEG was averaged off-line for a period of 1,000 msec, beginning 100 msec prior to the stimulus onset. The baseline was corrected to the mean value of the voltage during a period of 100 msec prestimulus. Trials were rejected if at any point during the averaging epoch the voltage exceeded  $\pm 100 \mu\text{V}$  in the EOG and EEG due to eye blinking and body movement. The first 20 artifact-free responses in 50 target stimuli were used; however the first 3 responses from measurement onset were excluded, and the 20 remaining measurements were averaged to obtain the individual ERP waveform. Both P300 latency and amplitude at Pz were measured as the most positive voltage sampled in the latency range of 250-450 ms after stimulus onset.

## 2.7 Fatty acid analysis

Blood were centrifuged at  $1,470 \times g$  for 10 min, and the plasma samples were stored at  $-80^\circ\text{C}$  before fatty acid analysis. Lipids extraction, purification, and fatty acid analysis were performed as described previously<sup>14, 28</sup>). In brief, lipids in plasma were extracted and purified by the method of Folch *et al.*<sup>29</sup>). Each lipid fraction was separated by thin-layer chromatography and incubated with an additional internal standard (pentadecanoic acid) in methanolic HCl at  $50^\circ\text{C}$  for 3 h for transmethylation of fatty acid residues. Fatty acid methyl esters were extracted with *n*-hexane and analyzed by capillary gas-liquid chromatography (Agilent 6890, Agilent Technologies, Santa Clara, CA, USA).

## 2.8 Dietary assessment and study diary

Dietary assessment was performed according to a previous study<sup>30</sup>). Dietary habits during the preceding month were assessed using the brief self-administered diet history questionnaire (BDHQ)<sup>31</sup>). Dietary intake including DHA, EPA and ARA was estimated using an ad hoc computer algorithm for the BDHQ based on the Standard Tables of Food Composition in Japan. Participants were asked to keep a record in the study diary throughout the study.

## 2.9 Statistical analysis

As defined by the protocol, the efficacy assessment was performed with the per-protocol analysis. Exclusion criteria for the per-protocol analysis were as follows: a change in dietary LCPUFA intake between baseline and 4 weeks after supplementation (3 times larger than the amount of LCPUFA supplementation); inability to obtain an EEG analysis (eg, a noise in EEG, a shortage of artifact-free responses, or an inappropriate task implementation); an event or action affecting the P300 components (such as an unexpected outside noise during ERP recording, a sleep state or sleepiness of the participant during ERP recording; or a change in smoking, exercise, medicine, disease, or incorrect fasting condition between baseline and 4 weeks of supplementation before ERP recording). Safety assessment was performed with the intention-to-treat (ITT) analysis. Data are shown as mean  $\pm$  standard error (SE). Baseline data between the groups were compared by unpaired student's *t*-test for quantitative variables or by chi-square test for qualitative variables. A change from baseline to 4 weeks after supplementation in each group was compared by paired student's *t*-test. Comparisons of changes between groups were performed by unpaired student's *t*-test. The incidence of adverse events between groups was compared by chi-square test. For all tests, *p*-values less than 0.05 were statistically significant.

## 3 RESULTS

### 3.1 Participant flow and characteristics

Figure 1 shows the participant flow diagram. A total of 413 participants were screened; 115 participants were enrolled and randomly allocated to the placebo ( $n=58$ ) or LCPUFA ( $n=57$ ) groups. One participant in each group withdrew consent before the supplementation. The ITT population (placebo,  $n=57$  and LCPUFA,  $n=56$ ) was used for the safety assessment. The per-protocol population (placebo,  $n=39$  and LCPUFA,  $n=30$ ) was used for the efficacy assessment. Participants were excluded from the per-protocol analysis for the following reasons: a change of dietary LCPUFA intake between before and after supplementation ( $n=12$ ); inability to achieve EEG analysis ( $n=6$ ); and an event or action affecting the P300 components

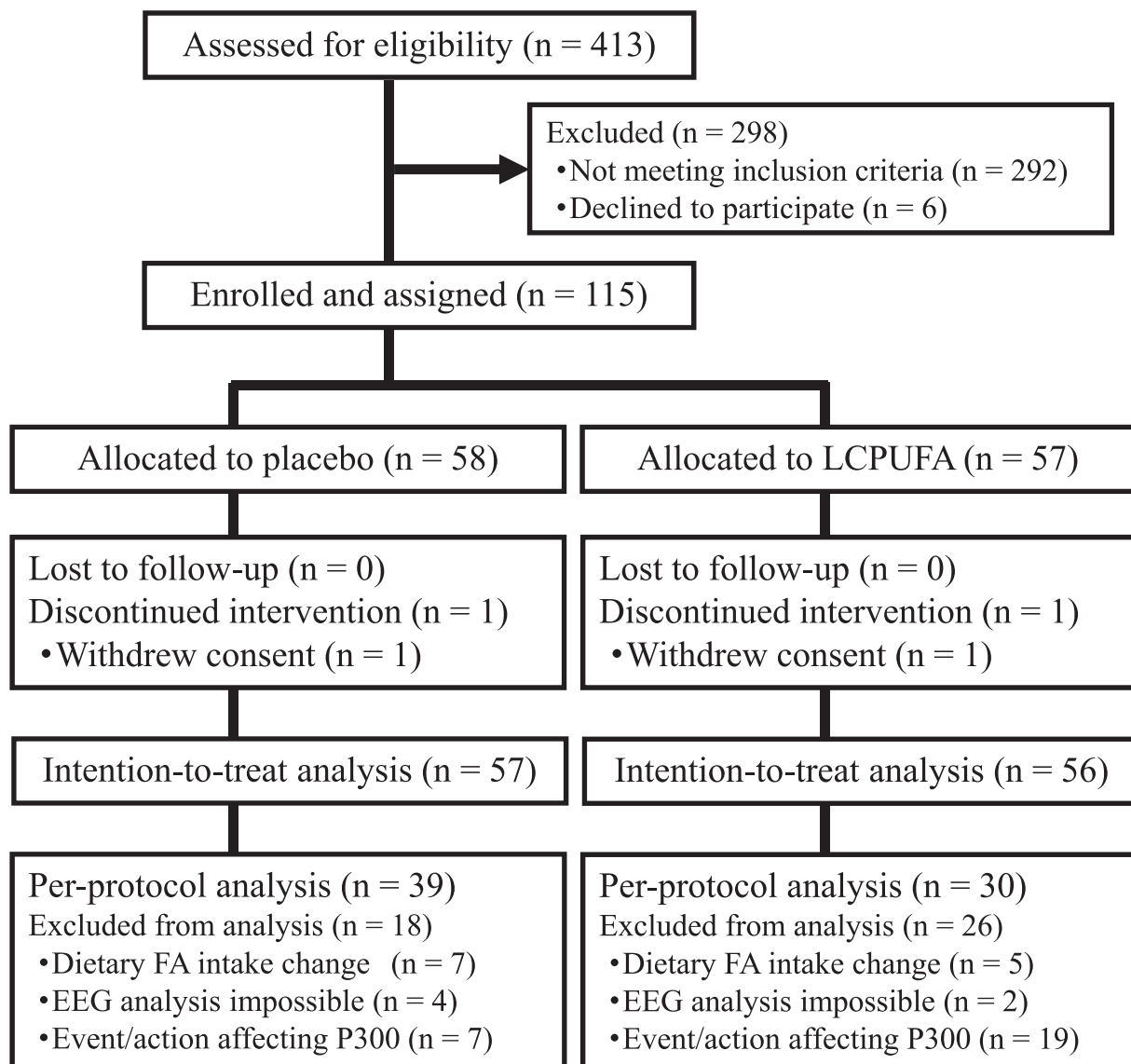


Fig. 1 Flow diagram of the study.

(n = 26). According to the study diary, mean capsule intake for the experimental period were 100.0% in the per-protocol population. Baseline characteristics of both groups in the per-protocol population are shown in Table 2. The placebo and LCPUFA groups were matched for age, height, bodyweight, body mass index, education, occupation, alcohol consumption, smoking status, LCPUFA (DHA, EPA, and ARA) composition in plasma phospholipids, and LCPUFA intake from daily diets.

### 3.2 P300 latency and amplitude

Table 3 shows P300 latency and P300 amplitude in both groups. The P300 latencies at baseline in the placebo and LCPUFA groups were  $331.9 \pm 6.1$  msec and  $339.9 \pm 6.1$  msec, respectively, with no significant difference between groups. P300 latency at 4 weeks in the placebo group was

prolonged significantly by 13.6 msec ( $p = 0.003$  vs at baseline), while that in the LCPUFA group was shortened by 1.8 msec compared with baseline, but changes were not significant. Changes in P300 latency after 4 weeks supplementation differed significantly between groups ( $p = 0.013$ ). With regard to the P300 amplitude, no significant differences were found between groups or between before and after supplementation. There were no significant differences in the task performance (the number of the target tones reported by the participants after P300 measurement) between the groups or between before and after supplementation (data not shown).

### 3.3 Fatty acid compositions in plasma phospholipids and fatty acid intakes from daily diets

LCPUFA composition in plasma phospholipids is shown

**Table 2** Baseline characteristics of participants.

	Placebo group (n = 39)	LCPUFA group (n = 30)	<i>p</i> value
Age (y)	59.5 ± 0.4	59.8 ± 0.5	0.706
Height (cm)	169.3 ± 0.9	168.9 ± 0.8	0.707
Body weight (kg)	66.7 ± 1.5	68.1 ± 1.5	0.514
BMI	23.2 ± 0.4	23.9 ± 0.5	0.328
Education			0.137
Junior high	0	0	
High	10	3	
College	5	2	
University	24	25	
Occupation			0.828
Full-time job	26	18	
Part-time job	7	7	
None	6	5	
Alcohol consumption			0.267
Daily	9	4	
1-6 days/week	20	15	
1-3 days/month	3	7	
None	7	4	
Smoking habit			0.530
> 19/day	4	1	
1-19/day	3	2	
None	32	27	
MMSE	29.6 ± 0.2	29.3 ± 0.2	0.249
BDI-II	4.3 ± 0.6	4.0 ± 0.7	0.791
FA composition in plasma PLs			
ARA (%)	8.8 ± 0.2	8.5 ± 0.2	0.343
EPA (%)	2.5 ± 0.2	2.6 ± 0.3	0.795
DHA (%)	7.0 ± 0.2	7.0 ± 0.3	0.949
Dietary FA intake			
ARA (mg/day)	156 ± 12	156 ± 11	0.988
EPA (mg/day)	329 ± 41	253 ± 29	0.159
DHA (mg/day)	543 ± 62	442 ± 45	0.219

Mean ± SE. There was no significant difference between the groups in baseline data (unpaired student's *t*-test or chi-square test).

BMI, body mass index; MMSE, Mini-Mental State Examination; BDI-II, Beck Depression Inventory-Second Edition; FA, fatty acid; PLs, phospholipids; ARA, arachidonic acid; EPA, eicosapentaenoic acid; DHA, docosahexaenoic acid.

in **Table 4**. DHA, EPA, or ARA content at baseline did not differ between groups. The DHA and ARA composition in the LCPUFA group was significantly increased from 7.0 ± 0.3% at baseline to 7.9 ± 0.3% at 4 weeks ( $p < 0.001$ ), and 8.5 ± 0.2% at baseline to 9.1 ± 0.2% at 4 weeks ( $p = 0.001$ ), respectively. The DHA and ARA contents in the placebo

group remained unchanged during supplementation. In addition, the change in the DHA content differed significantly between groups ( $p = 0.010$ ), and the change in the ARA content in the LCPUFA group also tended to be larger ( $p = 0.096$ ) than that in the placebo group. As for the EPA content in plasma, there were no significant difference

**Table 3** P300 latency (msec) and amplitude ( $\mu\text{V}$ ) in the placebo and LCPUFA groups at baseline and 4 weeks after supplementation.

P300	Placebo group (n = 39)			LCPUFA group (n = 30)		
	Baseline	4 weeks	$\Delta$	Baseline	4 weeks	$\Delta$
Latency	331.9 $\pm$ 6.1	345.5 $\pm$ 5.3 <sup>###</sup>	13.6 $\pm$ 4.2	339.9 $\pm$ 6.1	338.2 $\pm$ 5.7	-1.8 $\pm$ 4.1*
Amplitude	10.7 $\pm$ 0.7	10.2 $\pm$ 0.8	-0.5 $\pm$ 0.4	8.9 $\pm$ 0.5	9.2 $\pm$ 0.7	0.3 $\pm$ 0.6

Mean  $\pm$  SE. There was no significant difference between the groups in P300 latency and amplitude at baseline (unpaired student's t-test). \* $p$  < 0.05 vs. the placebo group (unpaired student's t-test). <sup>###</sup> $p$  < 0.01 vs. baseline (paired student's t-test).

**Table 4** Fatty acid composition (%) in plasma phospholipids in the placebo and LCPUFA groups during supplementation.

FA	Placebo group (n = 39)			LCPUFA group (n = 30)		
	Baseline	4 weeks	$\Delta$	Baseline	4 weeks	$\Delta$
PA	27.3 $\pm$ 0.2	27.4 $\pm$ 0.2	0.1 $\pm$ 0.1	27.5 $\pm$ 0.2	27.4 $\pm$ 0.2	0.0 $\pm$ 0.2
SA	14.4 $\pm$ 0.1	14.4 $\pm$ 0.1	0.1 $\pm$ 0.1	14.4 $\pm$ 0.2	14.5 $\pm$ 0.2	0.2 $\pm$ 0.1
OA	10.2 $\pm$ 0.2	10.7 $\pm$ 0.3	0.5 $\pm$ 0.3	10.5 $\pm$ 0.3	10.5 $\pm$ 0.3	0.0 $\pm$ 0.3
LA	21.2 $\pm$ 0.5	20.7 $\pm$ 0.5	-0.4 $\pm$ 0.3	20.6 $\pm$ 0.5	19.6 $\pm$ 0.6 <sup>###</sup>	-1.0 $\pm$ 0.4
ARA	8.8 $\pm$ 0.2	9.0 $\pm$ 0.2	0.2 $\pm$ 0.1	8.5 $\pm$ 0.2	9.1 $\pm$ 0.2 <sup>###</sup>	0.6 $\pm$ 0.2
EPA	2.5 $\pm$ 0.2	2.6 $\pm$ 0.2	0.0 $\pm$ 0.2	2.6 $\pm$ 0.3	2.8 $\pm$ 0.3	0.2 $\pm$ 0.2
DHA	7.0 $\pm$ 0.2	7.3 $\pm$ 0.2	0.2 $\pm$ 0.2	7.0 $\pm$ 0.3	7.9 $\pm$ 0.3 <sup>###</sup>	0.9 $\pm$ 0.2 <sup>**</sup>
EPA/ARA	0.29 $\pm$ 0.02	0.29 $\pm$ 0.02	0.00 $\pm$ 0.02	0.31 $\pm$ 0.04	0.32 $\pm$ 0.03	0.00 $\pm$ 0.02
DHA/ARA	0.81 $\pm$ 0.03	0.82 $\pm$ 0.04	0.01 $\pm$ 0.02	0.83 $\pm$ 0.04	0.88 $\pm$ 0.03	0.05 $\pm$ 0.02

Mean  $\pm$  SE. There was no significant difference between the groups in each fatty acid at baseline (unpaired student's t-test). <sup>\*\*</sup> $p$  < 0.01 vs. the placebo group (unpaired student's t-test). <sup>###</sup> $p$  < 0.01 vs. baseline (paired student's t-test).

FA, fatty acid; PA, palmitic acid; SA, stearic acid; OA, oleic acid; LA, linoleic acid; ARA, arachidonic acid; EPA, eicosapentaenoic acid; DHA, docosahexaenoic acid; EPA/ARA, ratio of EPA and ARA; DHA/ARA, ratio of DHA and ARA.

**Table 5** Dietary fatty acid intake (mg/day) in the placebo and LCPUFA groups during supplementation.

FA	Placebo group (n = 39)			LCPUFA group (n = 30)		
	Baseline	4 weeks	$\Delta$	Baseline	4 weeks	$\Delta$
LA	10,300 $\pm$ 568	9,900 $\pm$ 678	-391 $\pm$ 315	10,600 $\pm$ 520	10,000 $\pm$ 569	-588 $\pm$ 425
ALA	1,640 $\pm$ 96	1,580 $\pm$ 118	-55 $\pm$ 59	1,660 $\pm$ 88	1,560 $\pm$ 98	-101 $\pm$ 68
ARA	156 $\pm$ 12	147 $\pm$ 12	-9 $\pm$ 6	156 $\pm$ 11	148 $\pm$ 10	-8 $\pm$ 9
EPA	329 $\pm$ 41	316 $\pm$ 37	-3 $\pm$ 18	253 $\pm$ 29	267 $\pm$ 31	14 $\pm$ 24
DHA	543 $\pm$ 62	524 $\pm$ 59	-19 $\pm$ 27	442 $\pm$ 45	461 $\pm$ 49	19 $\pm$ 37

Mean  $\pm$  SE.

FA, fatty acid; LA, linoleic acid; ALA,  $\alpha$ -linolenic acid; ARA, arachidonic acid; EPA, eicosapentaenoic acid; DHA, docosahexaenoic acid.

between groups or between before and after supplementation.

Fatty acid intakes from daily diets are shown in **Table 5**. With regard to DHA, EPA, and ARA intakes, no significant

differences was found between groups or between before and after supplementation. Changes in these intakes did not differ between groups.

### 3.4 Safety

The safety assessment was performed with the ITT population. No side effects were observed. There was no significant difference ( $p = 0.636$ ) in the incidence of adverse events between the placebo group (17.5%) and the LCPUFA group (14.3%). For biochemical parameters, the change of alanine aminotransferase was significantly larger in the LCPUFA group (+1.7 IU/L) than in the placebo group (-0.2 IU/L). Changes of the other parameters in physical, physiological, blood hematological, biochemical parameters (Supplement Table 1) and urinalysis were not different between groups although minor changes, within a normal range, between before and after the supplementation in some parameters were observed.

## 4 DISCUSSION

It has been reported that supplementation with DHA/EPA or ARA improved cognitive function in the elderly<sup>11-14</sup>. However, it was unclear if low doses of LCPUFA supplementation corresponding to general dietary intake level can improve cognitive function. We performed a randomized, double-blind, placebo-controlled, parallel group intervention trial in elderly Japanese men and confirmed the efficacy of low doses of LCPUFA (300 mg/day of DHA, 100 mg/day of EPA, 120 mg/day of ARA) supplementation for 4 weeks on cognitive function using P300 components. This is the first report to show that low doses of LCPUFA supplementation corresponding to daily dietary intake have a positive effect on cognitive function in non-demented elderly. These findings suggest the potential of low doses of LCPUFA supplementation to improve cognitive function and also the importance of the amount and balance of low doses of dietary LCPUFA.

Mean amounts of DHA, EPA, and ARA intake from daily diets at baseline were 543 mg/day, 329 mg/day, and 156 mg/day respectively, and were consistent with a previous report for Japanese elderly (DHA, ca. 620 mg/day; EPA, ca. 350 mg/day; and ARA, ca. 170 mg/day)<sup>27</sup>. The LCPUFA intake of participants in the present study can be regarded as that of the typical Japanese elderly. It was also confirmed that the dose of LCPUFA supplementation in the present study was on-target and in a range of that from daily diets.

Significant increases in DHA (+0.9%) and ARA (+0.6%) contents in plasma phospholipids were observed in the LCPUFA group, although no significant changes were observed in the placebo group. The amounts of increases in DHA and ARA contents in plasma phospholipids are considered within a reasonable range, because the increase of DHA content was shown to be 3.2% with 900 mg/day of DHA supplementation<sup>11</sup> and the increase in ARA content was shown to be 0.7% and 2.5% with 80 mg/day and 240

mg/day of ARA supplementation, respectively<sup>30, 32</sup>. On the other hand, there were no significant changes in DHA, EPA, or ARA intake from daily diets between baseline and 4 weeks after supplementation in each group. These data suggest that the increases of plasma LCPUFA are caused by LCPUFA supplementation.

P300 has 2 components: latency and amplitude. The latency is considered to reflect cognitive processing speed, while the amplitude is linked to the amount of attentional resources engaged in task completion<sup>22</sup>. In the present study, changes in P300 latency were +13.6 msec in the placebo group and -1.8 msec in the LCPUFA group, and were significantly different. The difference in changes between groups was 15.4 msec, and was comparable to the effects of various treatments reported previously, such as exercise for 12 months (-11 to -23 msec)<sup>33</sup>, 120 mg/day of ginkgo extract for 8 weeks (-32 msec)<sup>34</sup>, and 240 mg/day of ARA for 4 weeks (-12 msec)<sup>14</sup> in healthy elderly participants. These data suggest that the difference in P300 latency in the present study was physiologically meaningful. P300 latency at 4 weeks in the placebo group was significantly prolonged by 13.6 msec compared with baseline. Since this increase was larger than age-related increase in P300 latency (ca. 1 msec/year)<sup>24</sup>, aging during 4 weeks in the present study could not cause the increase of P300 latency. It was considered that placebo oil (purified olive oil) was not the factor because we had confirmed that the olive oil did not change the P300 latency in Japanese elderly<sup>14</sup>. Another possibility is the repeated measurement of P300. It was reported that three times and more measurement of P300 increased the latency in the elderly<sup>34</sup>, although there were several reports that repeated measurement did not influence the latency in young adults<sup>49-51</sup>. In fact, we set the repeated measurement of P300 measurements at the screening, baseline and 4 weeks after the supplementation in the present study. Although an obvious reason was not defined, the repeated measurements might be one of the reasons for the increase of P300 latency in both groups.

The efficacy of LCPUFA on P300 amplitude, a secondary outcome, was not observed in the present study. Supplementation of 240 mg/day of ARA was effective on P300 amplitude<sup>14</sup>, but no effects on P300 amplitude were observed with 491 mg/day of EPA and 251 mg/day of DHA or EPA (193 mg/day) and DHA (92 mg/day) containing phospholipids<sup>25</sup>. Although further studies are needed, the effect of LCPUFA on P300 amplitude might be limited.

Compared with previous studies, a low dose of DHA/EPA seemed to be not enough to improve cognitive function in non-demented elderly. While high doses (0.9-2.5 g/day) of DHA/EPA supplementation improved cognitive function in healthy elderly<sup>11-13</sup>, low doses (0.3-0.7 g/day) of DHA/EPA did not affect it<sup>25, 35-38</sup>. In terms of the P300 components, 491 mg/day of EPA and 251 mg/day of DHA did not



improve P300 components<sup>25</sup>). Although the dose of LCPUFA (300 mg/day of DHA, 100 mg/day of EPA, 120 mg/day of ARA) used in the present study was much lower than the effective doses reported previously, a significant effect was observed in P300 latency. It is difficult to draw a firm conclusion based on these contrasting results, but the various results may be due to differences in the dose and combination of DHA, EPA, and ARA or the supplementation period in each study. However, the present results suggest that the efficacy in low doses might be caused by the combination of DHA and ARA. In fact, plasma LCPUFA contents were increased in DHA and ARA, but not EPA in this study. The importance of ARA is consistent with the report indicating that ARA was effective on P300 components in healthy elderly<sup>14, 26</sup>. On the other hand, another study showed that EPA (193 mg/day) and DHA (92 mg/day) containing phospholipids supplementation for 12 weeks improved the P300 latency in elderly Japanese<sup>25</sup>. However, this efficacy was considered to be caused by the unique combination of EPA and phospholipids, because these doses of EPA and DHA were too low to increase blood EPA and DHA levels. Further studies are needed to clarify effects of low doses of individual, combination, or balances of LCPUFA, using DHA, EPA, and/or ARA.

It is interesting that how much of DHA/EPA and ARA intake is required to maintain cognitive function in the elderly. In this study, the efficacy of 300 mg/day of DHA, 100 mg/day of EPA and 120 mg/day of ARA supplementation on P300 latency was confirmed in elderly Japanese who took the average intake of LCPUFA from daily diets. In the previous studies, slightly larger amounts of LCPUFA supplementation (2.1 g/day of DHA/EPA<sup>13</sup> or 240 mg/day of ARA<sup>14</sup>) compared to those from the diets were effective for cognitive function in elderly Japanese. These results suggest that the amount of LCPUFA intake enough to maintain cognitive function may be larger than that from daily diets. In addition, because an age-related decrease of DHA and ARA levels in the brain was observed in human<sup>4, 5</sup>, a sufficient intake of these LCPUFA may be expected to compensate for the decrease in the elderly. However, the adequate amount of individual LCPUFA intakes or combination/balances of these LCPUFA to maintain cognitive function have not been revealed yet. Further studies are expected to clarify them.

Since P300 components are changed in patients of dementia or Alzheimer's disease<sup>40</sup>, the potential of LCPUFA against dementia is interesting. However, the potential remains poorly understood because the participants in the present study were non-demented elderly men. Further clinical, epidemiological and pharmacological approaches are expected to clarify its potential against dementia or Alzheimer's disease.

There were some limitations to this study. First, approximately 40% of the allocated participants were excluded

from the efficacy assessment. However, the strict exclusion criteria were essential to remove factors influencing the appropriate evaluation of effects of low doses of LCPUFA within a range of daily diet intake levels, and the exclusion criteria in the per-protocol analysis had been defined in the study protocol. Although the validity of these results was necessarily decreased compared to the intent-to-treat analysis, the reliability of the present results was considered to be maintained. Second, participants in this study were only Japanese men. With regard to gender, P300 latency was shown to not be affected by gender<sup>39, 40</sup>, and blood DHA, EPA, and ARA levels in elderly men and women have been shown to be similar<sup>27</sup>. These data suggest that gender might have only a small effect in the present study. However, differences in region or race should be considered, such as the amount of fatty acid intake from daily diets. The dietary intakes of DHA and EPA in Japanese are higher than those in Western countries<sup>15</sup>. Further studies are necessary to clarify the effect of LCPUFA supplementation on P300 components in other countries. Third, the present results were only from participants whose ARA contents in plasma were not high (< 10%). This exclusion criterion was set because it has been shown that elderly men with higher ARA levels might be difficult to show a response to ARA supplementation on the P300 evaluation<sup>26</sup>. However, ARA content of participants in the present study was 8.7% and was in the normal range of Japanese elderly (6.4%<sup>41</sup>, 7.3%<sup>42</sup>, 8.3%<sup>30, 43</sup>, and 10.4%<sup>44</sup> in serum/plasma phospholipids). These data suggest that the participants in the present study were not selected from a limited and special population but represent a general population of Japanese elderly men.

In terms of safety, changes in physical, physiological, blood hematological, and biochemical parameters were not significantly different between groups from before and after supplementation, except for alanine aminotransferase levels. Changes in this parameter were minor and within the normal range. No side effects were observed, and there was no significant difference in the incidence of adverse events between groups. In addition, no significant changes in the ratio of EPA and ARA (EPA/ARA) in plasma were observed in both groups in per-protocol population (Table 4) and ITT population (data not shown), although blood EPA/ARA was reported to correlate with the risk for cardiovascular disease<sup>45, 46</sup>. Thus, LCPUFA supplementation in the present study is considered safe under the conditions described here.

## 5 CONCLUSION

In conclusion, the efficacy of the LCPUFA (300 mg/day of DHA, 100 mg/day of EPA, and 120 mg/day of ARA) supplementation on P300 latency in elderly Japanese men was

confirmed under these study conditions. These results suggest that low doses of LCPUFA supplementation, corresponding to general dietary intake levels, have the potential to improve cognitive function in elderly Japanese men.

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#### CONFLICT OF INTEREST

H. T., T. S., M. K., H. K. and H. S. belong to Suntory Wellness Ltd., which markets health food products that include LCPUFA. Y. K. declares no conflict of interest regarding this study.

#### AUTHOR CONTRIBUTIONS

H. T. participated in the study design, acquired the data, analyzed the data and drafted the manuscript. T. S. participated in acquired the data, analyzed the data. M. K. participated in the study design. H. K. participated in the study design, analyzed the data and drafted the manuscript. H. S. participated in the study design and helped to interpret the findings. Y. K. participated in the study design, analyzed the data, drafted and reviewed the manuscript.

#### Supplement Table

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#### References

- 1) McGahon, B.; Clements, M. P.; Lynch, M. A. The ability of aged rats to sustain long-term potentiation is restored when the age-related decrease in membrane arachidonic acid concentration is reversed. *Neuroscience* **81**, 9-16 (1997).
- 2) McGahon, B. M.; Martin, D. S.; Horrobin, D. F.; Lynch, M. A. Age-related changes in synaptic function: analysis of the effect of dietary supplementation with omega-3 fatty acids. *Neuroscience* **94**, 305-314 (1999).
- 3) Tokuda, H.; Kontani, M.; Kawashima, H.; Kiso, Y.; Shibata, H.; Osumi, N. Differential effect of arachidonic acid and docosahexaenoic acid on age-related decreases in hippocampal neurogenesis. *Neurosci. Res.* **88**, 58-66 (2014).
- 4) Söderberg, M.; Edlund, C.; Kristensson, K.; Dallner, G. Fatty acid composition of brain phospholipids in aging and in Alzheimer's disease. *Lipids* **26**, 421-425 (1991).
- 5) McNamara, R. K.; Liu, Y.; Jandacek, R.; Rider, T.; Tso, P. The aging human orbitofrontal cortex: decreasing polyunsaturated fatty acid composition and associated increases in lipogenic gene expression and stearoyl-CoA desaturase activity. *Prostaglandins Leukot. Essent. Fatty Acids* **78**, 293-304 (2008).
- 6) Kotani, S.; Nakazawa, H.; Tokimasa, T.; Akimoto, K.; Kawashima, H.; Toyoda-Ono, Y.; Kiso, Y.; Okaichi, H.; Sakakibara, M. Synaptic plasticity preserved with arachidonic acid diet in aged rats. *Neurosci. Res.* **46**, 453-461 (2003).
- 7) Gamoh, S.; Hashimoto, M.; Hossain, S.; Masumura, S. Chronic administration of docosahexaenoic acid improves the performance of radial arm maze task in aged rats. *Clin. Exp. Pharmacol. Physiol.* **28**, 266-270 (2001).
- 8) Kawakita, E.; Hashimoto, M.; Shido, O. Docosahexaenoic acid promotes neurogenesis *in vitro* and *in vivo*. *Neuroscience* **139**, 991-997 (2006).
- 9) Fukaya, T.; Gondaira, T.; Kashiya, Y.; Kotani, S.; Ishikura, Y.; Fujikawa, S.; Kiso, Y.; Sakakibara, M. Arachidonic acid preserves hippocampal neuron membrane fluidity in senescent rats. *Neurobiol. Aging* **28**, 1179-1186 (2007).
- 10) Suzuki, H.; Park, S. J.; Tamura, M.; Ando, S. Effect of the long-term feeding of dietary lipids on the learning ability, fatty acid composition of brain stem phospholipids and synaptic membrane fluidity in adult mice: a comparison of sardine oil diet with palm oil diet. *Mech. Ageing Dev.* **101**, 119-128 (1998).
- 11) Yurko-Mauro, K.; McCarthy, D.; Rom, D.; Nelson, E. B.; Ryan, A. S.; Blackwell, A.; Salem, N. Jr.; Stedman, M.; MIDAS Investigators. Beneficial effects of docosahexaenoic acid on cognition in age-related cognitive decline. *Alzheimers Dement.* **6**, 456-464 (2010).
- 12) Nilsson, A.; Radeborg, K.; Salo, I.; Bjorck, I. Effects of supplementation with n-3 polyunsaturated fatty acids on cognitive performance and cardiometabolic risk markers in healthy 51 to 72 years old subjects: a randomized controlled cross-over study. *Nutr. J.* **11**, 99 (2012).
- 13) Hashimoto, M.; Yamashita, K.; Kato, S.; Tamai, T.; Tanabe, Y.; Mitarai, M.; Matsumoto, I.; Ohno, M. Beneficial effects of daily dietary omega-3 polyunsaturated fatty acid supplementation on age-related cognitive decline in elderly Japanese with very mild dementia: a

- 2-year randomized, double-blind. *J. Aging Res. Clin. Practice* **1**, 193-201 (2012).
- 14) Ishikura, Y.; Ikeda, G.; Akimoto, K.; Hata, M.; Kusumoto, A.; Kidokoro, A.; Kontani, M.; Kawashima, H.; Kiso, Y.; Koga, Y. Arachidonic acid supplementation decreases P300 latency and increases P300 amplitude of event-related potentials in healthy elderly men. *Neuropsychobiology* **60**, 73-79 (2009).
  - 15) Elmadfa, I.; Kornsteiner, M. Fats and fatty acid requirements for adults. *Ann. Nutr. Metab.* **55**, 56-75 (2009).
  - 16) Daiello, L. A.; Gongvatana, A.; Dunsiger, S.; Cohen, R. A.; Ott, B. R.; Alzheimer's Disease Neuroimaging Initiative. Association of fish oil supplement use with preservation of brain volume and cognitive function. *Alzheimers Dement.*, in press.
  - 17) Titova, O. E.; Sjogren, P.; Brooks, S. J.; Kullberg, J.; Ax, E.; Kilander, L.; Riserus, U.; Cederholm, T.; Larsson, E. M.; Johansson, L.; Ahlstrom, H.; Lind, L.; Schioth, H. B.; Benedict, C. Dietary intake of eicosapentaenoic and docosahexaenoic acids is linked to gray matter volume and cognitive function in elderly. *Age*. **35**, 1495-1505 (2013).
  - 18) Gao, Q.; Niti, M.; Feng, L.; Yap, K. B.; Ng, T. P. Omega-3 polyunsaturated fatty acid supplements and cognitive decline: Singapore Longitudinal Aging Studies. *J. Nutr. Health Aging* **15**, 32-35 (2011).
  - 19) van Gelder, B. M.; Tijhuis, M.; Kalmijn, S.; Kromhout, D. Fish consumption, n-3 fatty acids, and subsequent 5-y cognitive decline in elderly men: the Zutphen Elderly Study. *Am. J. Clin. Nutr.* **85**, 1142-1147 (2007).
  - 20) Beydoun, M. A.; Kaufman, J. S.; Sloane, P. D.; Heiss, G.; Ibrahim, J. n-3 Fatty acids, hypertension and risk of cognitive decline among older adults in the Atherosclerosis Risk in Communities (ARIC) study. *Public Health Nutr.* **11**, 17-29 (2008).
  - 21) Whalley, L. J.; Fox, H. C.; Wahle, K. W.; Starr, J. M.; Deary, I. J. Cognitive aging, childhood intelligence, and the use of food supplements: possible involvement of n-3 fatty acids. *Am. J. Clin. Nutr.* **80**, 1650-1657 (2004).
  - 22) Griskova, I.; Dapsys, K.; Andruskevicius, S.; Ruksenas, O. Does electroconvulsive therapy (ECT) affect cognitive components of auditory evoked P300? *Acta Neurobiol. Exp (Wars)*. **65**, 73-77 (2005).
  - 23) Polich, J.; Ladish, C.; Burns, T. Normal variation of P300 in children: age, memory span, and head size. *Int. J. Psychophysiol.* **9**, 237-248 (1990).
  - 24) Hirayasu, Y.; Samura, M.; Ohta, H.; Ogura, C. Sex effects on rate of change of P300 latency with age. *Clin. Neurophysiol.* **111**, 187-194 (2000).
  - 25) Konagai, C.; Yanagimoto, K.; Hayamizu, K.; Han, L.; Tsuji, T.; Koga, Y. Effects of krill oil containing n-3 polyunsaturated fatty acids in phospholipid form on human brain function: a randomized controlled trial in healthy elderly volunteers. *Clin. Interv. Aging.* **8**, 1247-1257 (2013).
  - 26) Tokuda, H.; Kontani, M.; Kawashima, H.; Akimoto, K.; Kusumoto, A.; Kiso, Y.; Koga, Y.; Shibata, H. Arachidonic acid-enriched triacylglycerol improves cognitive function in elderly with low serum levels of arachidonic acid. *J. Oleo Sci.* **63**, 219-227 (2014).
  - 27) Otsuka, R.; Kato, Y.; Imai, T.; Ando, F.; Shimokata, H. Higher serum EPA or DHA, and lower ARA compositions with age independent fatty acid intake in Japanese aged 40 to 79. *Lipids* **48**, 719-727 (2013).
  - 28) Sakuradani, E.; Kobayashi, M.; Shimizu, S. Delta 9-fatty acid desaturase from arachidonic acid-producing fungus. Unique gene sequence and its heterologous expression in a fungus, *Aspergillus*. *Eur. J. Biochem.* **260**, 208-216 (1999).
  - 29) Folch, J.; Lees, M.; Sloane Stanley, G. H. A simple method for the isolation and purification of total lipids from animal tissues. *J. Biol. Chem.* **226**, 497-509 (1957).
  - 30) Kakutani, S.; Ishikura, Y.; Tateishi, N.; Horikawa, C.; Tokuda, H.; Kontani, M.; Kawashima, H.; Sakakibara, Y.; Kiso, Y.; Shibata, H.; Morita, I. Supplementation of arachidonic acid-enriched oil increases arachidonic acid contents in plasma phospholipids, but does not increase their metabolites and clinical parameters in Japanese healthy elderly individuals: a randomized controlled study. *Lipids Health Dis.* **10**, 241 (2011).
  - 31) Murakami, K.; Mizoue, T.; Sasaki, S.; Ohta, M.; Sato, M.; Matsushita, Y.; Mishima, N. Dietary intake of folate, other B vitamins, and omega-3 polyunsaturated fatty acids in relation to depressive symptoms in Japanese adults. *Nutrition* **24**, 140-147 (2008).
  - 32) Hirota, S.; Adachi, N.; Gomyo, T.; Kawashima, H.; Kiso, Y.; Kawabata, T. Low-dose arachidonic acid intake increases erythrocytes and plasma arachidonic acid in young women. *Prostaglandins Leukot. Essent. Fatty Acids* **83**, 83-88 (2010).
  - 33) Zhang, X.; Ni, X.; Chen, P. Study about the effects of different fitness sports on cognitive function and emotion of the aged. *Cell Biochem. Biophys.* **70**, 1591-1596 (2014).
  - 34) Semlitsch, H. V.; Anderer, P.; Saletu, B.; Binder, G. A.; Decker, K. A. Cognitive psychophysiology in nootropic drug research: effects of Ginkgo biloba on event-related potentials (P300) in age-associated memory impairment. *Pharmacopsychiat* **28**, 134-142 (1995).
  - 35) Stough, C.; Downey, L.; Silber, B.; Lloyd, J.; Kure, C.; Wesnes, K.; Camfield, D. The effects of 90-day supplementation with the omega-3 essential fatty acid docosahexaenoic acid (DHA) on cognitive function and visual acuity in a healthy aging population. *Neurobiol. Aging* **33**, 824 e1-3 (2012).

- 36) Mahmoudi, M. J.; Hedayat, M.; Sharifi, F.; Mirarefin, M.; Nazari, N.; Mehrdad, N.; Ghaderpanahi, M.; Tajalizadekhoob, Y.; Badamchizade, Z.; Larijani, B.; Alatab, S.; Alizadeh, M.; Arzaghi, SM.; Najafi, B.; Fakhrzadeh, H. Effect of low dose  $\omega$ -3 poly unsaturated fatty acids on cognitive status among older people: a double-blind randomized placebo-controlled study. *J. Diabetes Metab. Disord.* **13**, 34(2014).
- 37) van de Rest, O.; Geleijnse, J. M.; Kok, F. J.; van Sta-veren, W. A.; Dullemeijer, C.; Olderikkert, M. G.; Beekman, A. T.; de Groot, C. P. Effect of fish oil on cognitive performance in older subjects: a randomized, controlled trial. *Neurology* **71**, 430-438(2008).
- 38) Dangour, A. D.; Allen, E.; Elbourne, D.; Fasey, N.; Fletcher, A. E.; Hardy, P.; Holder, G. E.; Knight, R.; Letley, L.; Richards, M.; Uauy, R.; Effect of 2-y n-3 long-chain polyunsaturated fatty acid supplementation on cognitive function in older people: a randomized, double-blind, controlled trial. *Am. J. Clin. Nutr.* **91**, 1725-1732(2010).
- 39) Polich, J. Normal variation of P300 from auditory stimuli. *Electroencephalogr. Clin. Neurophysiol.* **65**, 236-240(1986).
- 40) Ito, J. Auditory event-related potentials (ERPs) in healthy subjects and patients with dementia. *Rinsho Byori* **39**, 859-864(1991).
- 41) Kobayashi, M.; Sasaki, S.; Kawabata, T.; Hasegawa, K.; Akabane, M.; Tsugane, S. Single measurement of serum phospholipid fatty acid as a biomarker of specific fatty acid intake in middle-aged Japanese men. *Eur. J. Clin. Nutr.* **55**, 643-650(2001).
- 42) Yamori, Y.; Nara, Y.; Iritani, N.; Workman, R. J.; Inagami, T. Comparison of serum phospholipid fatty acids among fishing and farming Japanese populations and American inlanders. *J. Nutr. Sci. Vitaminol.* **31**, 417-422(1985).
- 43) Ogura, T.; Takada, H.; Okuno, M.; Kitade, H.; Matsuura, T.; Kwon, M.; Arita, S.; Hamazaki, K.; Itomura, M.; Hamazaki, T. Fatty acid composition of plasma, erythrocytes and adipose: their correlations and effects of age and sex. *Lipids* **45**, 137-144(2010).
- 44) Takahashi, R.; Ito, H.; Horrobin D. F. Fatty acid composition of serum phospholipids in an elderly institutionalized Japanese population. *J. Nutr. Sci. Vitaminol.* **37**, 401-409(1991).
- 45) Ninomiya, T.; Nagata, M.; Hata, J.; Hirakawa, Y.; Ozawa, M.; Yoshida, D.; Ohara, T.; Kishimoto, H.; Mukai, N.; Fukuhara, M.; Kitazono, T.; Kiyohara, Y. Association between ratio of serum eicosapentaenoic acid to arachidonic acid and risk of cardiovascular disease: the Hisayama Study. *Atherosclerosis* **231**, 261-267(2013).
- 46) Nishizaki, Y.; Shimada, K.; Tani, S.; Ogawa, T.; Ando, J.; Takahashi, M.; Yamamoto, M.; Shinozaki, T.; Miyachi, K.; Nagao, K.; Hirayama, A.; Yoshimura, M.; Komuro, I.; Nagai, R.; Daida, H. Significance of imbalance in the ratio of serum n-3 to n-6 polyunsaturated fatty acids in patients with acute coronary syndrome. *Am. J. Cardiol.* **113**, 441-445(2014).
- 47) Folstein, M. F.; Folstein, S. E.; McHugh, P. R. "Mini-Mental State" A practical method for grading the cognitive state of patients for the clinician. *J. Psychiatr. Res.* **12**, 189-198(1975).
- 48) Beck, A. T.; Steer, R. A.; Brown, G. K. (translated into Japanese by Kojima, M.; Furukawa, T.) Manual for Beck Depression Inventory-Second Edition (Japanese edition). Nihon Bunka Kagakusha(2003).
- 49) Sklare, D. A.; Lynn, G. E. Latency of the P3 event-related potential: normative aspects and within-subject variability. *Electroencephalogr. Clin. Neurophysiol.* **59**, 420-424(1984).
- 50) Polich, J.; McIsaac, H. K. Comparison of auditory P300 habituation from active and passive conditions. *Int. J. Psychophysiol.* **17**, 25-34(1994).
- 51) Takahashi, M. Somatosensory P300s in Stroke Patients. *Jpn. J. Rehabil. Med.* **33**, 710-718(1996).