

## PAPER

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# *Perilla frutescens* seed oil combined with *Anredera cordifolia* leaf powder attenuates age-related cognitive decline by reducing serum triglyceride and glucose levels in healthy elderly Japanese individuals: a possible supplement for brain health†

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We have shown that *Anredera cordifolia* extract improves learning and memory in a senescence-accelerated mouse model, and that  $\alpha$ -linolenic acid (ALA)-rich *Perilla frutescens* seed oil (PO) improves brain function in healthy Japanese adults and elderly individuals. Herein, we present a 12-month, randomised, double-blind, parallel-armed intervention trial examining the effects of PO supplementation alone or in combination with *A. cordifolia* leaf powder on brain function in healthy elderly Japanese individuals. Participants were randomly divided into two groups: the PO group received 1.47 mL PO (0.88 g ALA) daily via soft gelatine capsules, and the POAC group received 1.47 mL PO and 1.12 g *A. cordifolia* leaf powder (1.46 mg vitexin and 1.12 mg adenosine) daily. After 12 months of intervention, the POAC group showed generally higher cognitive index scores than the PO group. The beneficial effects of combined supplementation on cognitive function were associated with increased ALA and eicosapentaenoic acid levels in red blood cell plasma membranes, increased serum biological antioxidant potential, and decreased serum triglyceride, glucose, and *N*-(epsilon)-carboxymethyl-lysine (CML), an advanced glycation end-product and biochemical marker of oxidative stress levels. The effects of combined supplementation on cognitive function also showed a significant negative correlation with serum CML levels after 12 months of intervention. Our findings suggest that combined long-term supplementation with PO and *A. cordifolia* more effectively ameliorates age-related cognitive decline than PO alone. These findings may serve as a basis for the development of new supplements for brain health. Clinical Trial Registry, UMIN000040863.

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## 1. Introduction

Aging is characterised by a progressive decline in brain function, evidenced by impaired learning and memory, along with changes in mental health. Concurrently, neuronal plasticity and synaptic function begin to decline with age, affecting cognition.<sup>1,2</sup> These neuronal dysfunctions are associated with a decrease in plasma antioxidant power and an increase in neuronal oxidative stress.<sup>3</sup> Observational studies have suggested a wide variety of potentially modifiable risk factors for cognitive impairment and dementia<sup>4,5</sup> as targets for preventive care. For example, cardiovascular risk factors and lifestyle choices, including diet, exercise, tobacco use, and coffee consumption have been shown to directly affect cognitive decline. Interestingly, several nutrients have been shown to



influence neural cell function,<sup>6</sup> and several randomised clinical trials have proposed nutritional interventions as preventive or therapeutic trials to slow the progression of cognitive impairment or reduce the risk of Alzheimer's disease in the elderly. Although many efforts have been made to elucidate the pathogenesis of neuronal dysfunction, few effective therapies and interventions exist to target aging.

Several naturally derived supplements have been suggested to influence mental health and cognitive function.<sup>7–10</sup> The basellaceous perennial *Anredera cordifolia* (AC) has been used as a medicinal plant in East Asia for centuries. In preclinical studies, AC extracts have been shown to exert neuroprotective effects associated with antioxidant and anti-inflammatory functions and to improve *N*-methyl-D-aspartate receptor antagonist MK-801-induced memory impairment in mice.<sup>11–15</sup> We recently found that the administration of AC extract enhances learning and memory in senescence-accelerated mouse-prone 8 (SAMP8) mice.<sup>16</sup> The treatment resulted in increased levels of neuronal plasticity-related proteins, and caused no noticeable side effects.<sup>16</sup> These findings suggest that long-term supplementation of AC ameliorates the age-related cognitive and mental health decline in healthy elderly individuals. However, no intervention studies have verified the effects of AC on cognitive function in this group.

Another plant-based supplement for cognitive function, *Perilla frutescens* seed oil (PO), is characterised by high levels of  $\alpha$ -linolenic acid (ALA, C18:3; 54–64% by weight), an essential  $\omega$ -3 polyunsaturated fatty acid (PUFA).<sup>17</sup> ALA exhibits anti-inflammatory<sup>18</sup> and neuroprotective<sup>19</sup> properties, and long-term administration has been shown to improve spatial learning, memory, and synaptic plasticity in aging rats.<sup>20</sup> ALA is converted to other  $\omega$ -3 PUFAs, such as eicosapentaenoic acid (EPA, C20:5) and docosahexaenoic acid (DHA, C22:6) by the rate-limiting enzymes  $\Delta$ 5- and  $\Delta$ 6-desaturases in animals.<sup>21</sup> DHA is well-known for its beneficial effects on brain function.<sup>21,22</sup> Our previous randomised, double-blind, placebo-controlled studies revealed that the administration of DHA-rich foods prevents cognitive decline in elderly Japanese individuals.<sup>23,24</sup> However, in humans, the rate of metabolic conversion from ALA to EPA and DHA is very low.<sup>25</sup> Therefore, it is important to investigate whether dietary ALA can supply EPA and DHA to various tissues. Interestingly, flavonoids have been reported to modulate the expression of  $\Delta$ 5- and  $\Delta$ 6-desaturases and improve the conversion of ALA to EPA and DHA.<sup>26–28</sup> However, a controversy exists regarding the effects of flavonoids on such conversion.

We previously reported that the long-term supplementation of dietary PO has beneficial effects on psychological conditions such as apathy, and on age-related cognitive decline in healthy Japanese elderly individuals by enhancing the antioxidant potential.<sup>29</sup> In addition, we found that the administration of PO combined with nobiletin-rich ponkan powder improved cognitive function in healthy elderly Japanese individuals.<sup>30</sup> Because of the high flavonoid levels in AC, the combined supplementation with PO and AC leaf powder may have a greater beneficial effect on cognitive function than PO supplementa-

tion alone. However, to the best of our knowledge, there have been no interventional studies on the combined effects of PO and AC leaf powder on brain health in the elderly. It is also unclear whether AC promotes the conversion of ALA to EPA or DHA in humans. Therefore, we aimed to investigate whether (1) PO + AC have a synergistic effect on brain health and (2) AC promotes the conversion of ALA to EPA and DHA, resulting in improved cognitive function in the elderly. In this study, two types of soft gelatine capsules (SGCs) containing either PO alone or combined with AC leaf powder (POAC) were prepared as easy-to-consume dietary supplements to prevent dementia and ameliorate age-related cognitive decline. We conducted a 12-month, randomised, double-blind, interventional trial in healthy elderly Japanese individuals to compare the effects of the two supplements on cognitive function and health. Our findings present POAC treatment as a beneficial and easy-to-consume dietary supplement to prevent and maintain cognitive function in the elderly population.

## 2. Materials and methods

### 2.1. Participants and study design

This study was approved by the Shimane University Ethics Committee (Study No. 3194, 3497) and was performed in accordance with the principles of the Declaration of Helsinki and Good Clinical Practice. All volunteers provided written informed consent before participating in the study. The intervention trial was conducted between 2018 and 2020.

Details of the study design, intervention, recruitment of subjects, enrolment, and randomisation have been recently described.<sup>30</sup> A total of 32 healthy elderly volunteers (17 women, mean age  $67.7 \pm 1.1$ ; 15 men, mean age  $68.5 \pm 1.6$ ) living in Shimane Prefecture, Japan, were recruited for this 12-month, randomised, double-blind, parallel-armed study. The volunteers were subjected to physical (*e.g.*, body weight and height, blood pressure, and waist circumference), clinical (hepatic and renal function, serum lipids, glucose, and haematological parameters), cognitive, and mental assessments. Additionally, they were asked to respond to a self-reported life-style questionnaire regarding their medical/drug history.

Volunteers with a total Mini-Mental State Examination (MMSE; see section 2.4) score of 23 or less; medical disorders, including respiratory, hepatic, renal, and/or cardiac disease; diabetes mellitus; endocrine, metabolic, or haematological diseases; allergies or hypersensitivity; or a history of any psychotropic drug or supplement use that might significantly affect the results of the study were excluded.

Two types of SGCs were produced by Sankyo Holdings Co. Ltd (Fuji, Japan). Considering the ease of swallowing one capsule, the maximum amount of PO per capsule was set at 0.098 mL (58.8 mg ALA). Therefore, one SGC contained 0.098 mL PO alone, and the other contained both 0.098 mL PO and 0.075 g AC leaf powder (0.098 mg of vitexin). The nutrient composition of the AC leaf powder and PO is shown in Tables 1 and 2, respectively.



**Table 1** Nutrient composition of dried *Anredera cordifolia* leaf powder

| Proximate analysis |      | Mineral and materials |      |
|--------------------|------|-----------------------|------|
| Energy (kcal)      | 287  | Sodium (mg)           | 464  |
| Protein (g)        | 26.3 |                       |      |
| Fat (g)            | 6.1  | Vitexin (mg)          | 130  |
| Carbohydrate (g)   | 18.0 | Adenosine (mg)        | 100  |
| Fiber (g)          | 27.5 | Polyphenol (mg)       | 1560 |
| Moisture (g)       | 3.4  |                       |      |
| Ash (g)            | 18.7 |                       |      |

Nutritional values per 100 g of dry powder. Data on proximate analysis were obtained from Japan Food Research Laboratories (JFRL, Tokyo, Japan).

**Table 2** Nutrient composition of perilla seed oil

| Proximate analysis | N = 5    | Fatty acids (g per 100 g)        | N = 5        |
|--------------------|----------|----------------------------------|--------------|
| Energy (kcal)      | 931 ± 22 | Palmitic acid (C16:0) (g)        | 5.8 ± 0.1    |
| Protein (g)        | 0        | Stearic acid (C18:0) (g)         | 2.1 ± 0.1    |
| Fat (g)            | 101 ± 2  | Oleic acid (C18:1 ω-9) (g)       | 13.4 ± 0.6   |
| Carbohydrate (g)   | 0        | Linoleic acid (C18:2 ω-6) (g)    | 13.2 ± 0.4   |
| Fiber (g)          | 0        | α-Linolenic acid (C18:3 ω-3) (g) | 62.9 ± 1.5   |
| Moisture (g)       | 0        |                                  |              |
| Ash (g)            | 0        | Vitamin E (mg)                   | 67.8 (N = 1) |

Values are means ± SE. Nutritional values per 100 g of *perilla* seed oil (O-san Farm Co., Kawamoto, Shimane, Japan). Data on proximate analysis were obtained from the Shimane Institute for Industrial Technology (Matsue, Japan), and data on fatty acids and vitamin E were obtained from Japan Food Research Laboratories (JFRL, Tokyo, Japan). N, number of samples analysed.

Thirty-two participants were randomly divided into two groups, and each received 15 SGCs in three even doses each day for 12 months, between or immediately after meals. The PO group ( $n = 15$ ) received 1.47 mL of PO daily, and the POAC group ( $n = 17$ ) received both 1.47 mL of PO and 1.12 g of AC leaf powder daily. Group allocation was performed by stratified random assignment according to the total MMSE score, sex, and age, as described previously.<sup>31–33</sup> Randomised code lists were generated by the medical statistics advisor, and the investigators, participants, and sponsors were blinded to these codes. Neither the participants nor the researchers knew which capsules were consumed. Prior to the intervention trial, a blind sensory test was performed to confirm that no differences existed in the appearance or taste of the SGCs (data not shown).

## 2.2. Anthropometry, body composition, and intake analysis

The height, body weight, and waist circumference of all participants were measured. Body composition was determined using a bioelectrical impedance analyser, WB-150 (Tanita Co., Tokyo, Japan).

To determine the interactions of PO or POAC supplementation with health status and lifestyle, all participants were

asked to self-report their daily SCG intake and health/mental status during the entire 12-month intervention trial. Dietary intake before and after the trial was estimated using a brief-type self-administered diet history questionnaire (BDHQ) designed and validated for the Japanese population.<sup>34</sup>

## 2.3. Blood sampling

Before and after the trial, blood samples were collected either in the morning or afternoon, after confirming that the participants had not eaten breakfast or lunch. Blood samples were separated into serum and erythrocyte (red blood cell, RBC) aliquots by centrifugation. RBC samples were collected to monitor the fatty acid profiles of plasma membranes (RBC-PMs). Fresh serum samples were used to measure blood biochemistry, as well as *N*-(epsilon)-carboxymethyl-lysine (CML), the biological antioxidant potential (BAP), and brain-derived neurotrophic factor (BDNF) levels. The RBC and serum samples were stored at  $-80^{\circ}\text{C}$  within 8 h of collection.

## 2.4. Cognitive function and mental health

Cognitive function was evaluated using three previously described methods.<sup>30</sup> The first involved Hasegawa's Dementia Scale-Revised (HDS-R),<sup>35</sup> which comprises nine simple questions and has been widely accepted for epidemiological screening of cognitive dysfunction in Asian populations. The second, the MMSE test,<sup>36</sup> is commonly used to assess cognitive impairments associated with dementia status, especially in patients with Alzheimer's disease or mild cognitive impairment (MCI). The third method used the Japanese version of the Montreal Cognitive Assessment (MoCA-J),<sup>37</sup> a reliable and valid cognitive screening test designed to assist health professionals in detecting MCI.

Apathy and depression were assessed using the Japanese version of the apathy scale<sup>38</sup> and the Zung Self-Rating Depression Scale (SDS),<sup>39</sup> respectively. The apathy scale was developed as a tool for measuring apathy resulting from brain-related pathology, and the SDS is an established norm-referenced screening measure used worldwide to identify the presence of depressive symptoms in adults.<sup>38,39</sup>

All tests were performed before treatment (baseline) and again after the 12-month intervention.

## 2.5. Blood biochemical analysis, fatty acid profile, and apolipoprotein E (APOE) genotyping

Serum biochemical analyses included the evaluation of gamma-glutamyl transpeptidase, alanine aminotransferase, aspartate aminotransferase, albumin, total cholesterol, blood urea nitrogen, triglyceride (TG), creatinine, blood sugar, and high- and low-density lipoprotein cholesterol levels using a BiOLis 24i automatic analyser (Tokyo Boeki Medisys, Tokyo, Japan). Haemoglobin A1c (HbA1c) levels in blood were determined using a commercially available kit (MetaboLead HbA1c, TFB Inc., Tokyo, Japan).

Haematological analyses included RBC, white blood cell, and platelet counts, as well as the evaluation of haemoglobin, mean corpuscular volume (MCV), mean corpuscular haemo-



globin (MCH), and mean corpuscular haemoglobin concentration (MCHC) using an automated haematology analyser, XS-1000i (Sysmex Corporation, Kobe, Japan).

Serum CML levels were measured using a CircuLex CML/Ne-(carboxymethyl) lysine ELISA kit (MBL Ltd, Tokyo, Japan). Serum was diluted 1 : 4 in the included dilution buffer and the enzymatic reaction was stopped after 5 min. Serum CML concentrations were calculated using SoftMax Pro software (Molecular Devices).<sup>40</sup> Serum BAP levels,<sup>29,30,41</sup> RBC-PM fatty acid profiles,<sup>23,42</sup> and (APOE) gene statuses<sup>24</sup> were measured as previously described.<sup>30</sup>

## 2.6. Statistical analysis

Per-protocol analysis was performed as previously described.<sup>30</sup> Data distribution was assessed by the Shapiro–Wilk test. Comparisons between the baseline and 12-month values for each group were assessed by paired *t*-tests or the Wilcoxon signed-rank tests. Comparisons between the two groups were performed by the independent *t*-test or Mann–Whitney *U*-test. Allelic distribution of the APOE gene was analysed by the Pearson chi-square test. Analysis of covariance was used to compare the differences between groups regarding cognitive outcomes and serum BDNF, CML, and BAP levels. Pearson partial correlation coefficients were used to assess associations between cognitive outcome (MoCA-J “total” and subscale “language”) scores and serum CML levels, and Spearman partial correlation coefficients were used to assess associations between changes ( $\Delta$ ) in MoCA-J total scores, and in  $\Delta$ BAP and  $\Delta$ RBC-PM ALA levels. Analyses were adjusted for age, sex, BMI, and educational level of the participants. All analyses were performed using PASW Statistics software (version 23.0, SPSS Inc., Chicago, IL, USA). All statistical tests were two-tailed, and significance was set at  $p < 0.05$ .

## 3. Results

### 3.1. Demographic and clinical characteristics and assessment of dietary intake

Thirty-one participants completed the full 12-month intervention (Fig. 1). The one participant who dropped out of the study did so voluntarily, and not due to adverse effects, but due to moving to another area where he could not continue the intervention trial. Based on self-administration records, the protocol adherence of the final 31 participants to the study during 12 months was  $93.4 \pm 2.8\%$  for the PO group and  $94.5 \pm 2.4\%$  for the POAC group.

No remarkable differences were observed between the PO and POAC groups regarding the general questionnaire on medical/medication history and lifestyle habits before or after the trial. The participants did not report adverse effects such as stomach irritation, palpitations, or allergic reactions that influenced their daily lives. No significant differences were observed between the groups regarding the baseline anthropometry, blood pressure, blood biochemical parameters, or haematological parameters (Table 3).

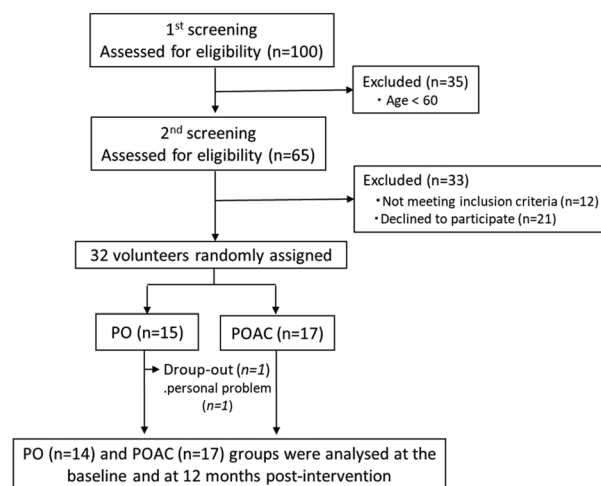


Fig. 1 Flow diagram of participant selection. PO, *perilla* seed oil group; POAC, PO and *Anredera cordifolia* leaf powder group.

The frequency of APOE2/2, APOE2/3, or APOE3/3 genotypes was 78.6% (11/14) in the PO group and 64.7% (11/17) in the POAC group, and that of APOE2/4 or APOE3/4 genotypes was 21.4% (3/14) in the PO group and 35.3% (6/17) in the POAC group. The APOE4/4 genotype was not detected in either group. These results indicated that significant differences in the distribution of APOE alleles were not observed between the two groups ( $p > 0.05$ ).

The average dietary nutritional intake based on BDHQ reports was not significantly different between the baseline and 12-month responses for either the PO or POAC group ( $p > 0.05$ , Supplement 1). No overall change was detected for either group (Supplement 1), indicating that the intake of these supplements did not influence nutritional intake during the interventional trial.

When compared to the baseline levels, the serum albumin ( $p = 0.003$ ), blood urea nitrogen ( $p = 0.005$ ), and creatinine ( $p = 0.049$ ) levels were significantly increased, and the blood sugar levels were significantly ( $p = 0.044$ ) decreased in the PO group after 12 months of treatment, and serum TG levels ( $p = 0.064$ ) tended to decrease over time (Table 3). Similarly, serum TG ( $p < 0.001$ ), blood sugar ( $p = 0.035$ ), and MCV ( $p < 0.001$ ) levels were significantly lower, and MCHC levels were significantly higher ( $p = 0.001$ ) in the POAC group at 12 months than at the baseline. The remaining parameters were unchanged in both the PO and POAC groups (Table 3). The mean change in the MCV levels was lower in the POAC group than in the PO group, and mean changes in the remaining parameters showed no significant differences between the two groups after 12 months of treatment.

Although serum TG, blood sugar, MCV, and MCHC levels at 12 months showed significant changes compared to the baseline levels in both groups, values remained within clinical standard ranges. Taken together, these results show that no adverse events were reported, and that neither PO alone nor POAC treatment altered lipid metabolism, hepatic or renal





**Table 3** Participant characteristics at the baseline and after 12 months of intervention

|   | PO ( <i>n</i> = 14) |              | POAC ( <i>n</i> = 17) |               | Change (12 months-baseline) |                           |
|---|---------------------|--------------|-----------------------|---------------|-----------------------------|---------------------------|
|   | Baseline            | 12 months    | Baseline              | 12 months     | PO                          | POAC                      |
| <b>Anthropometry</b>                          |                     |              |                       |               |                             |                           |
| Sex (male/female)                             | 14 (7/7)            | —            | 17 (8/9)              | —             |                             |                           |
| Age (years)                                   | 68.9 ± 1.4          | 70.2 ± 1.4   | 67.0 ± 1.2            | 68.1 ± 1.2    |                             |                           |
| Height (cm)                                   | 157.2 ± 1.9         | —            | 158.4 ± 2.6           | —             |                             |                           |
| Body Weight (kg)                              | 54.3 ± 2.6          | 54.1 ± 2.6   | 58.2 ± 3.3            | 58.2 ± 3.3    | −0.3 ± 0.3                  | 0.0 ± 0.3                 |
| Body Mass Index (kg m <sup>−2</sup> )         | 21.9 ± 0.8          | 21.8 ± 0.8   | 22.9 ± 0.7            | 22.9 ± 0.7    | −0.1 ± 0.1                  | 0.0 ± 0.2                 |
| Waist Circumference (cm)                      | 81.5 ± 2.2          | 80.7 ± 2.1   | 84.6 ± 2.2            | 83.5 ± 1.9    | −0.8 ± 0.4                  | −1.0 ± 0.5                |
| Body fat (%)                                  | 26.1 ± 1.6          | 25.8 ± 1.6   | 28.1 ± 1.4            | 28.2 ± 1.4    | −0.4 ± 0.5                  | 0.1 ± 0.4                 |
| <b>Blood pressure (BP)</b>                    |                     |              |                       |               |                             |                           |
| Systolic BP (mmHg)                            | 146 ± 7             | 141 ± 6      | 141 ± 6               | 143 ± 5       | −4 ± 4                      | 2 ± 6                     |
| Diastolic BP (mmHg)                           | 90 ± 5              | 86 ± 4       | 82 ± 3                | 84 ± 4        | −4 ± 3                      | 1 ± 3                     |
| <b>Education</b>                              |                     |              |                       |               |                             |                           |
| ≤12 years (%)                                 | 9 (64.3)            |              | 10 (58.8)             |               |                             |                           |
| >12 years (%)                                 | 5 (35.7)            |              | 7 (41.2)              |               |                             |                           |
| <b>Blood biochemistry</b>                     |                     |              |                       |               |                             |                           |
| GOT (U L <sup>−1</sup> )                      | 24.9 ± 1.4          | 23.4 ± 0.8   | 23.5 ± 1.4            | 23.8 ± 1.6    | −1.5 ± 1.0                  | 0.4 ± 0.7                 |
| GPT (U L <sup>−1</sup> )                      | 20.5 ± 1.5          | 18.4 ± 1.6   | 20.8 ± 2.2            | 20.8 ± 2.6    | −2.1 ± 1.4                  | 0.0 ± 0.7                 |
| γ-GTP (IU L <sup>−1</sup> )                   | 23.7 ± 3.3          | 23.0 ± 3.1   | 38.4 ± 9.8            | 31.6 ± 5.0    | −0.7 ± 0.8                  | −6.8 ± 5.8                |
| Albumin (g dL <sup>−1</sup> )                 | 4.2 ± 0.07          | 4.5 ± 0.07** | 4.3 ± 0.09            | 4.3 ± 0.11    | 0.21 ± 0.06                 | −0.01 ± 0.10              |
| Total cholesterol (mg dL <sup>−1</sup> )      | 218.7 ± 8.6         | 223.8 ± 10.0 | 205.6 ± 7.5           | 204.6 ± 7.1   | 5.1 ± 6.5                   | −1.0 ± 7.0                |
| Triglyceride (mg dL <sup>−1</sup> )           | 117.1 ± 11.5        | 90.9 ± 9.8*  | 108.6 ± 6.9           | 79.9 ± 4.7**  | −26.2 ± 13.0                | −28.8 ± 5.5               |
| Blood urea nitrogen (mg dL <sup>−1</sup> )    | 16.2 ± 0.8          | 18.6 ± 1.0** | 16.0 ± 0.8            | 17.1 ± 1.0    | 2.4 ± 0.7                   | 1.1 ± 0.7                 |
| Creatinine (mg dL <sup>−1</sup> )             | 0.7 ± 0.03          | 0.8 ± 0.03** | 0.8 ± 0.05            | 0.8 ± 0.06    | 0.03 ± 0.01                 | −0.01 ± 0.02              |
| Blood sugar (mg dL <sup>−1</sup> )            | 106.5 ± 7.8         | 90.6 ± 2.0** | 110.4 ± 4.1           | 102.2 ± 2.1** | −15.9 ± 7.1                 | −8.1 ± 3.5                |
| HDL-C (mg dL <sup>−1</sup> )                  | 70.0 ± 4.5          | 69.4 ± 4.3   | 74.8 ± 4.3            | 73.6 ± 3.9    | −0.6 ± 2.5                  | −1.2 ± 2.6                |
| LDL-C (mg dL <sup>−1</sup> )                  | 129.1 ± 6.7         | 135.7 ± 8.1  | 112.1 ± 6.1           | 114.4 ± 5.7   | 6.6 ± 5.5                   | 2.2 ± 5.7                 |
| HbA1c (NGSP) (%)                              | 5.6 ± 0.08          | 5.6 ± 0.09   | 5.6 ± 0.06            | 5.6 ± 0.08    | −0.04 ± 0.04                | −0.05 ± 0.05              |
| <b>Haematological parameters</b>              |                     |              |                       |               |                             |                           |
| WBC (×10 <sup>3</sup> μL <sup>−1</sup> )      | 5.9 ± 0.6           | 5.8 ± 0.5    | 5.6 ± 0.3             | 5.5 ± 0.3     | −0.09 ± 0.3                 | −0.12 ± 0.2               |
| RBC (×10 <sup>4</sup> μL <sup>−1</sup> )      | 442.9 ± 11.7        | 448.2 ± 12.0 | 439.3 ± 7.6           | 442.2 ± 10.2  | 5.2 ± 4.8                   | 2.9 ± 4.8                 |
| Haemoglobin (g dL <sup>−1</sup> )             | 13.7 ± 0.3          | 13.9 ± 0.2   | 13.8 ± 0.3            | 13.9 ± 0.3    | 0.1 ± 0.1                   | 0.1 ± 0.1                 |
| Haematocrit (%)                               | 41.3 ± 0.8          | 41.5 ± 0.7   | 41.3 ± 0.7            | 41.0 ± 0.9    | 0.2 ± 0.5                   | −0.3 ± 0.4                |
| Platelet (×10 <sup>4</sup> μL <sup>−1</sup> ) | 21.3 ± 1.7          | 21.0 ± 1.7   | 21.0 ± 0.7            | 22.5 ± 1.5    | −0.3 ± 0.8                  | 1.6 ± 1.5                 |
| MCV (fL)                                      | 93.5 ± 1.1          | 93.0 ± 1.2*  | 94.1 ± 0.9            | 92.7 ± 0.9**  | −0.5 ± 0.3                  | −1.4 ± 0.2 <sup>###</sup> |
| MCH (pg)                                      | 31.1 ± 0.4          | 31.1 ± 0.4   | 31.4 ± 0.5            | 31.4 ± 0.5    | −0.0 ± 0.2                  | −0.0 ± 0.1                |
| MCHC (g dL <sup>−1</sup> )                    | 33.2 ± 0.3          | 33.4 ± 0.2   | 33.3 ± 0.2            | 33.8 ± 0.2**  | 0.2 ± 0.2                   | 0.5 ± 0.1                 |

Values are means ± SE. Significant differences from the baseline values, \*\**p* < 0.05, 0.05 < \**p* < 0.1. Significant differences from the PO group, <sup>###</sup>*p* < 0.05. BUN, Blood urea nitrogen; GOT, glutamate oxaloacetate transaminase; GPT, glutamic pyruvic transaminase; γ-GTP, γ-glutamyl transpeptidase; HbA1c, haemoglobin A1c; HDL-C, high-density lipoprotein cholesterol; LDL-C, low-density lipoprotein cholesterol; MCH, mean corpuscular haemoglobin; MCHC, mean corpuscular haemoglobin concentration; MCV, mean corpuscular volume; NGSP, national glycohemoglobin standardization program; PO, *perilla* seed oil group; POAC, PO and *Anredera cordifolia* leaf powder group; RBC, red blood cell; WBC, white blood cell.

function, or haematopoiesis, suggesting the safety of long-term administration.

### 3.2. Cognitive function and mental health assessment

The mean HDS-R, MMSE, and MoCA-J total scores of the final 31 participants at the baseline of the interventional trial were (28.5 ± 0.2)/30, (28.8 ± 0.2)/30, and (25.8 ± 0.5)/30, respectively. After 12 months of treatment, the MoCA-J total scores were significantly increased in the POAC group (*p* = 0.049), but remained unchanged in the PO group (Table 4). Neither HDS-R nor MMSE total scores showed a significant change after 12 months of PO or POAC treatment.

When analysing the scores for the HDS-R and MMSE sub-items and the MoCA-J subscales, the MoCA-J subscale “language” scores in the POAC group showed a significant increase over the 12 months (*p* = 0.007). The HDS-R sub-item “SMRT” score in the PO group and the “recalling 5 objects” score in the POAC group tended to increase over the 12 months (*p* = 0.100 and 0.096, respectively) (Table 4). The mean change in the MoCA-J subscale “language” score tended to be higher in the POAC group than in the PO group (*p* = 0.100) (Table 4). The remaining sub-item and subscale scores were not significantly different before and after intervention in either group.



**Table 4** Cognitive and mental scores at the baseline and after 12 months of intervention

|                               | PO ( <i>n</i> = 14) |              | POAC ( <i>n</i> = 17) |               | Change (12 months-baseline) |                          |
|-------------------------------|---------------------|--------------|-----------------------|---------------|-----------------------------|--------------------------|
|                               | Baseline            | 12 months    | Baseline              | 12 months     | PO                          | POAC                     |
| <i>Cognitive index</i>        |                     |              |                       |               |                             |                          |
| MMSE                          |                     |              |                       |               |                             |                          |
| Total                         | 28.8 ± 0.3          | 29.2 ± 0.4   | 28.8 ± 0.3            | 29.2 ± 0.3    | 0.4 ± 0.5                   | 0.5 ± 0.4                |
| HDS-R                         |                     |              |                       |               |                             |                          |
| Total                         | 28.6 ± 0.3          | 28.7 ± 0.4   | 28.5 ± 0.3            | 29.0 ± 0.3    | 0.1 ± 0.5                   | 0.5 ± 0.4                |
| Subitem "recalling 5 objects" | 4.86 ± 0.10         | 4.93 ± 0.07  | 4.59 ± 0.15           | 4.88 ± 0.12*  | 0.07 ± 0.13                 | 0.29 ± 0.17              |
| MoCA-J                        |                     |              |                       |               |                             |                          |
| Total                         | 25.9 ± 0.7          | 26.8 ± 0.9   | 25.8 ± 0.8            | 27.5 ± 0.7**  | 0.9 ± 0.7                   | 1.8 ± 0.8                |
| Subscale "SMRT"               | 2.50 ± 0.53         | 3.43 ± 0.45* | 2.59 ± 0.42           | 3.29 ± 0.45   | 0.93 ± 0.54                 | 0.71 ± 0.49              |
| Subscale "language"           | 4.00 ± 0.15         | 4.07 ± 0.22  | 4.06 ± 0.20           | 4.71 ± 0.11** | 0.07 ± 0.20                 | 0.65 ± 0.21 <sup>#</sup> |
| <i>Emotional index</i>        |                     |              |                       |               |                             |                          |
| SDS                           | 35.0 ± 2.2          | 33.0 ± 1.6   | 35.5 ± 2.0            | 33.9 ± 1.7    | −2.0 ± 1.7                  | −1.6 ± 1.6               |
| Apathy                        | 9.6 ± 1.5           | 9.6 ± 1.3    | 11.9 ± 1.2            | 12.5 ± 1.2    | 0.0 ± 0.9                   | 0.6 ± 0.9                |

Values are means ± SE. Significant differences from the baseline values, \*\* $p < 0.05$ ,  $0.05 < *p < 0.1$ . Tendency of significant differences from the PO,  $0.05 < \#p < 0.1$ . HDS-R, Hasegawa's dementia scale-revised; MMSE, mini-mental state examination; MoCA-J, Japanese version of Montreal cognitive assessment; PO, *perilla* seed oil group; POAC, PO and *Anredera cordifolia* leaf powder group. SDS, self-rating depression scale; SMRT, short-term memory recall task.

**Table 5** Fatty acid profiles (mol%) of the red-blood cell plasma membrane at the baseline and after 12 months of intervention

|                 | PO ( <i>n</i> = 14) |               | POAC ( <i>n</i> = 17) |               | Change (12 months – baseline) |             |
|-----------------|---------------------|---------------|-----------------------|---------------|-------------------------------|-------------|
|                 | Baseline            | 12 months     | Baseline              | 12 months     | PO                            | POAC        |
| PLA (C16:0)     | 22.5 ± 0.42         | 22.4 ± 0.34   | 21.9 ± 0.26           | 21.8 ± 0.29   | −0.1 ± 0.45                   | −0.1 ± 0.27 |
| STA (C18:0)     | 17.1 ± 0.25         | 16.7 ± 0.36   | 17.3 ± 0.42           | 17.4 ± 0.53   | −0.4 ± 0.45                   | 0.1 ± 0.65  |
| OLA (C18:1 ω-9) | 13.0 ± 1.43         | 15.3 ± 0.44   | 15.2 ± 0.29           | 15.2 ± 0.27   | 2.3 ± 1.39                    | −0.0 ± 0.29 |
| LLA (C18:2 ω-6) | 12.9 ± 0.35         | 12.5 ± 0.39   | 12.2 ± 0.35           | 11.9 ± 0.36   | −0.4 ± 0.44                   | −0.3 ± 0.25 |
| ALA (C18:3 ω-3) | 0.23 ± 0.01         | 0.32 ± 0.01** | 0.22 ± 0.02           | 0.29 ± 0.01** | 0.08 ± 0.01                   | 0.07 ± 0.02 |
| AA (C20:4 ω-6)  | 13.4 ± 0.45         | 13.2 ± 0.40   | 13.2 ± 0.29           | 13.8 ± 0.26*  | −0.2 ± 0.48                   | 0.6 ± 0.29  |
| EPA (C20:5 ω-3) | 1.9 ± 0.14          | 2.1 ± 0.20    | 1.7 ± 0.11            | 2.0 ± 0.13**  | 0.1 ± 0.17                    | 0.3 ± 0.08  |
| DPA (C22:5 ω-3) | 1.8 ± 0.04          | 1.8 ± 0.06    | 1.6 ± 0.06            | 1.7 ± 0.05**  | 0.0 ± 0.05                    | 0.1 ± 0.04  |
| C24:0           | 4.8 ± 0.13          | 4.2 ± 0.12**  | 4.5 ± 0.13            | 4.2 ± 0.11**  | −0.5 ± 0.12                   | −0.3 ± 0.11 |
| DHA (C22:6 ω-3) | 8.2 ± 0.27          | 7.8 ± 0.25    | 8.0 ± 0.23            | 8.0 ± 0.21    | −0.4 ± 0.25                   | 0.1 ± 0.20  |
| C24:1           | 3.8 ± 0.09          | 3.3 ± 0.09**  | 3.7 ± 0.13            | 3.3 ± 0.10**  | −0.5 ± 0.09                   | −0.3 ± 0.11 |
| ω-6/ω-3         | 2.2 ± 0.08          | 2.2 ± 0.11    | 2.3 ± 0.10            | 2.2 ± 0.08    | 0.0 ± 0.10                    | −0.1 ± 0.06 |

Values are means ± SE. Significant differences from the baseline values, \*\* $p < 0.05$ ,  $0.05 < *p < 0.1$ . AA: arachidonic acid; ALA: α-linolenic acid; C24:0: lignoceric acid; C24:1: nervonic acid; DHA: docosahexaenoic acid; DPA: docosapentaenoic acid; EPA: eicosapentaenoic acid; LLA: linoleic acid; OLA: oleic acid; PLA: palmitic acid; PO, *perilla* seed oil group; POAC, PO and *Anredera cordifolia* leaf powder group; STA: stearic acid.

No significant differences were observed between the baseline and 12-month apathy or SDS scores in either group (Table 4).

### 3.3. Fatty acid profiles of RBC-PMs

In the PO group, the RBC-PM ALA levels significantly increased from the baseline to 12 months ( $p < 0.001$ ), whereas the levels of both lignoceric ( $p = 0.001$ ) and nervonic ( $p < 0.001$ ) acids significantly decreased; DHA and remaining fatty acid levels remained unchanged (Table 5). Similarly, in the POAC group, ALA ( $p = 0.001$ ), EPA ( $p = 0.004$ ), and docosapentaenoic acid ( $p = 0.01$ ) levels were significantly higher at 12 months than at the baseline. Arachidonic acid levels were generally higher after treatment ( $p = 0.074$ ), whereas lignoceric ( $p = 0.01$ ) and nervonic ( $p = 0.01$ ) acid levels were significantly lower. DHA and the remaining fatty acid levels did not change over time.

The mean changes in the RBC-PM fatty acid profiles were not significantly different between the two groups (Table 5).

### 3.4. Serum BAP, BDNF, and CML levels

No noticeable differences were observed between groups in the baseline serum BAP, BDNF, or CML levels (Table 6). The BAP levels significantly increased over 12 months in both the PO ( $p = 0.004$ ) and POAC ( $p = 0.008$ ) groups, whereas serum BDNF levels remained unchanged in both groups. The CML levels significantly increased over time in the PO group ( $p = 0.009$ ) and tended to decrease in the POAC group ( $p = 0.076$ ).

The mean change in serum CML levels over 12 months was significantly lower ( $p = 0.004$ ) in the POAC group than in the PO group, whereas the mean changes in BAP and BDNF were not significantly different between the two groups (Table 6).



**Table 6** Serum BAP, CML and BDNF levels at the baseline and after 12 months of intervention

|                                | PO ( <i>n</i> = 14) |                  | POAC ( <i>n</i> = 17) |                 | Change (12 months-baseline) |                  |
|--------------------------------|---------------------|------------------|-----------------------|-----------------|-----------------------------|------------------|
|                                | Baseline            | 12 months        | Baseline              | 12 months       | PO                          | POAC             |
| BAP ( $\mu\text{mol L}^{-1}$ ) | 2759 $\pm$ 41       | 2964 $\pm$ 46**  | 2820 $\pm$ 59         | 3007 $\pm$ 41** | 205 $\pm$ 58                | 187 $\pm$ 61     |
| CML ( $\mu\text{g mL}^{-1}$ )  | 9.5 $\pm$ 0.6       | 10.3 $\pm$ 0.4** | 10.1 $\pm$ 0.5        | 9.5 $\pm$ 0.4*  | 0.8 $\pm$ 0.3               | -0.6 $\pm$ 0.3## |
| BDNF ( $\text{pg mL}^{-1}$ )   | 4104 $\pm$ 513      | 4398 $\pm$ 621   | 3616 $\pm$ 346        | 4083 $\pm$ 405  | 294 $\pm$ 399               | 466 $\pm$ 293    |

Values are means  $\pm$  SE. Significant differences from the baseline values, \*\* $p < 0.05$ ,  $0.05 < *p < 0.1$ . Significant differences from the PO, ## $p < 0.05$ . BAP, biological antioxidant potential; CML, *N*-(epsilon)-carboxymethyl-lysine; BDNF, brain-derived neurotrophic factor; PO, *perilla* seed oil group; POAC, PO and *Anredera cordifolia* leaf powder group.

### 3.5. Correlation between cognitive function scores, serum characteristics, and serum BAP and CML levels

After 12 months of intervention, serum blood sugar levels tended to be negatively correlated (Fig. 2A) with RBC-PM EPA levels ( $r = -0.346$ ,  $p = 0.066$ ). At 12 months, serum BAP levels were negatively correlated ( $r = -0.415$ ,  $p = 0.02$ ) with serum TG levels (Fig. 2B). When adjusted for age, sex, and BMI, the MoCA-J total scores ( $r = -0.384$ ,  $p = 0.043$ ) and the MoCA-J subscale “language” scores ( $r = -0.397$ ,  $p = 0.037$ ) showed significantly negative correlations with serum CML levels (Table 7). Additionally,  $\Delta\text{MoCA-J}$  total scores were positively associated with  $\Delta\text{RBC-PM ALA}$  levels ( $r = 0.416$ ,  $p = 0.038$ ), whereas no significant correlations were observed between  $\Delta\text{MoCA-J}$  subscale “language” scores and  $\Delta\text{RBC-PM ALA}$  levels.

## 4. Discussion

In this study, we showed that a 12-month intervention with combined PO and AC leaf powder supplementation improved certain parameters of age-related cognitive decline in healthy elderly Japanese individuals more effectively than PO alone. This improvement was associated with an increase in RBC-PM

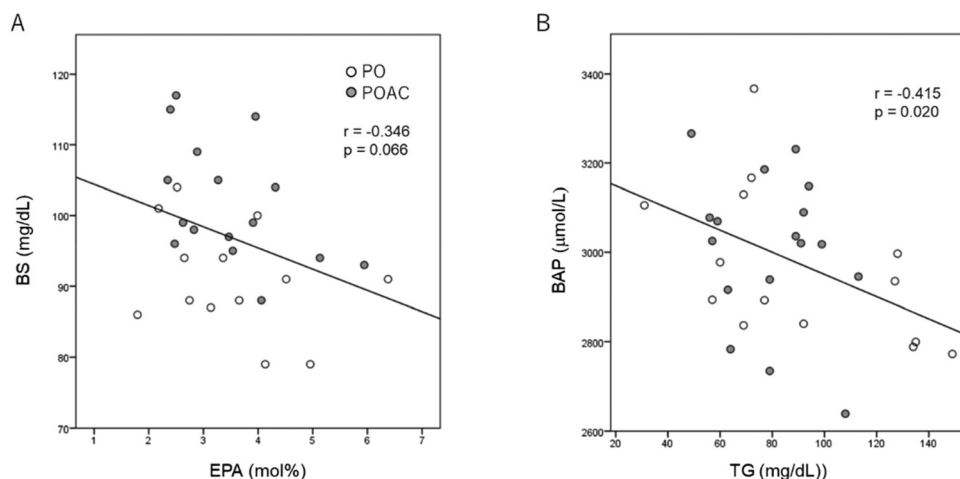
**Table 7** Pearson's partial correlation of cognitive outcomes with serum CML or RBC-PM ALA levels

| Associated variables |                         | Coefficient <i>r</i> | <i>p</i> |
|----------------------|-------------------------|----------------------|----------|
| Moca-J total         | CML <sup>a</sup>        | -0.384               | 0.043    |
|                      | RBC-PM ALA <sup>b</sup> | 0.416                | 0.038    |
| Moca-J language      | CML <sup>a</sup>        | -0.397               | 0.037    |
|                      | RBC-PM ALA <sup>b</sup> | 0.099                | 0.630    |

Adjusted by age, sex, BMI and education levels. <sup>a</sup> Values at the end of the study. <sup>b</sup> Differences between the extent of change (12-month-baseline values). CML, *N*-(epsilon)-carboxymethyl-lysine; ALA,  $\alpha$ -linolenic acid; RBC-PM, red-blood cell plasma membrane.

ALA and EPA levels and serum BAP levels, as well as a decrease in serum TG, glucose, and CML levels. To the best of our knowledge, this is the first long-term, double-blind, randomised, parallel-armed study to demonstrate the combined effects of ALA-rich PO and AC leaf powder on cognitive ability and blood biochemical parameters in healthy elderly Japanese individuals.

Participant compliance in consuming 15 SGCs daily for 12 months was excellent. This high compliance was evidenced by increased RBC-PM ALA levels in both the PO and POAC



**Fig. 2** Scatter plot of the relationship (A) between BS and EPA levels in erythrocyte plasma membranes and (B) between serum BAP and TG levels at 12 months after the intervention. PO (open circle), *perilla* seed oil (PO) group; POAC (gray circle), PO and *Anredera cordifolia* leaf powder groups. BAP, biological antioxidant potential; BS, blood sugar; EPA, eicosapentaenoic acid; TG, triglyceride.



groups (Table 5), indicating the effective incorporation of  $\omega$ -3 PUFAs into RBC-PMs by PO ingestion. This result is consistent with those of our recent intervention,<sup>29</sup> in which the dietary intake of 7 mL of PO daily for 12 months increased RBC-PM ALA levels in association with ameliorated cognitive decline in healthy Japanese elderly participants. Similarly, a prospective study in Japan reported that serum ALA levels, but not EPA or DHA levels, showed a negative correlation with the risk of dementia.<sup>43</sup> ALA is thought to enhance neuroprotection and brain plasticity.<sup>44</sup> Interestingly, however, the increased RBC-PM ALA levels seen in the present study did not appear to affect cognitive performance in participants (Table 4). Similarly, it has been reported that PO does not influence cognitive performance in participants with mild to moderate dementia.<sup>45</sup> The exact reason why the PO group in this study did not show the same changes in cognitive performance seen in our previous study<sup>29</sup> remains unclear. However, the discrepancy may be related to the differences in PO dosage between the two interventions. The amount of PO administered in this study was 1.47 mL daily, compared to 7 mL daily in the previous study. The dose was reduced due to logistical reasons, as detailed in a recent article.<sup>30</sup>

AC contains bioactive compounds, such as flavonoids, saponins, tannins, and terpenoids.<sup>46</sup> Flavonoids and saponins contain many bioactive substances, though it is not known which contribute to reducing cognitive decline. It has also been reported that adenosine and its derivative, cordysinin B (2'-O-methyl adenosine), abundant in AC, may be involved in cognitive improvement by activating the signalling pathway associated with memory formation (*i.e.*, cAMP/PKA/CREB-pathway).<sup>47</sup> Previous studies have suggested a link between flavonoid intake and cognitive function in humans and animals.<sup>48</sup> Similarly, saponins have been reported to prevent synaptic loss and reverse learning-memory deficits in APP/PS1 transgenic mice, suggesting an improvement of cognitive function.<sup>49</sup> It has been reported that AC extract administration improved MK-801-induced memory impairment in mice.<sup>15</sup> Moreover, we recently reported that AC extract administration enhanced the levels of neuronal plasticity-related proteins such as hippocampal brain-derived neurotrophic factor (BDNF), PSD95, and NR2A in SAMP8 mice, suggesting benefits for learning and memory.<sup>16</sup> After 12 months of treatment, the MoCA-J total scores and subscale "language" scores had significantly increased compared to the baseline levels in the POAC group, but not in the PO group (Table 4). Taken together, these findings suggest that POAC supplementation is more effective for reducing age-related cognitive decline than PO supplementation alone. Further studies are necessary to clarify the bioactive compounds of interest in AC leaves.

The brain is sensitive to changes in oxidative balance, and age-related declines in memory and cognition are associated with reduced plasma antioxidative power and increased oxidative stress.<sup>3</sup> Cognitive impairment is remarkably correlated with oxidative damage, suggesting that enhancing antioxidant abilities can defend cognitive function in the elderly.<sup>3</sup> In this study, serum BAP levels, which reflect the total antioxidant

power,<sup>50</sup> significantly increased compared to the baseline values after 12 months of both PO and POAC treatment (Table 6). We recently reported that dietary PO supplementation for 12 months enhances cognitive ability in healthy elderly Japanese individuals in association with raised serum BAP levels. This supports the idea that dietary antioxidants prevent cognitive decline in the elderly.<sup>29</sup> PO contains vitamin E, a well-known antioxidant (Table 2), and AC leaves exhibit antioxidant activity *via* flavonoids and saponins.<sup>51,52</sup> These compounds likely contributed to the increased serum BAP seen in both the PO and POAC groups. Furthermore, the AC leaf powder used in this study contained large amounts vitexin (Table 1). Vitexin has a variety of beneficial effects, including antioxidant, anti-inflammatory, and neuroprotective properties, and functions as an antioxidant in oxidative stress-related diseases, including memory impairment, cerebral ischaemia, and neurotoxicity.<sup>53</sup> The difference in cognition-related outcomes between the PO and POAC groups observed in this study may have occurred due to the flavonoids, saponins, and polyphenols in AC leaf powder.

The formation of advanced glycation end-products, the irreversible end-products of non-enzymatic glycation, has been linked to neuronal diseases such as vascular dementia and Alzheimer's disease.<sup>54</sup> CML is a well-studied advanced glycation end-product<sup>55</sup> and biochemical marker of oxidative stress to represent early pathological changes in the brain during Alzheimer's disease.<sup>56</sup> Ahmed *et al.*<sup>57</sup> reported that cerebrospinal fluid CML levels were significantly higher in patients with Alzheimer's disease than in age-matched control individuals, with a clear link to cognitive impairment. Zhang *et al.*<sup>58</sup> reported a negative correlation between plasma CML and cognitive function scores in older patients with type 2 diabetes and MCI, suggesting an important regulatory role of CML in the manifestation of diabetic cognitive impairment. In this study, serum CML levels after the 12-month interventional trial were significantly decreased in the POAC group, but increased in the PO group compared to the respective baseline values (Table 6). POAC supplementation also significantly increased the MoCA-J total scores and subscale "language" scores after 12 months of intervention, whereas the administration of PO alone did not affect these parameters (Table 4). Significant negative correlations were observed between serum CML levels and both MoCA-J total and "language" scores (Table 7). In addition, the mean changes in the MoCA-J total scores were positively correlated with the mean changes in RBC-PM ALA levels (Table 7). These results suggest that POAC supplementation may have improved the cognitive function in the elderly by reducing the CML levels. However, the reason why serum CML levels increased after PO intake alone remains to be explained, as this was not observed in previous studies.

ALA is a precursor molecule for EPA and DHA, which are thought to prevent cognitive decline with aging.<sup>59</sup> However, their conversion ability in humans is low, with only 0.1–21% of ALA converted to EPA and 0.1–0.9% to DHA.<sup>25</sup> In this study, the administration of ALA-rich PO for 12 months significantly increased the RBC-PM ALA, but not EPA or DHA, levels in par-





participants (Table 5). This result was consistent with that of Hamazaki *et al.*,<sup>60</sup> who reported that dietary PO administration did not influence EPA or DHA levels in the human serum phospholipid fraction. Similarly, we previously reported<sup>29</sup> that dietary PO intake (7 mL daily) for 12 months did not change the RBC-PM EPA or DHA levels in healthy elderly Japanese participants. In contrast, our recent report<sup>30</sup> showed that the administration of 1.47 mL of PO daily for 12 months significantly increased RBC-PM ALA and EPA levels in healthy elderly Japanese participants. This may be related to the difference in the number of participants (14 vs. 21 subjects), since this and the previous<sup>30</sup> intervention trial were conducted at about the same time, and the participants were healthy elderly volunteers living in the same local area of Shimane Prefecture in Japan. If the number of participants increased, the RBC-PM EPA levels at 12 months may have increased in the PO group. In the present study, the administration of POAC, as opposed to PO alone, significantly increased RBC-PM ALA and EPA, but not DHA, levels in participants (Table 5). Generally, the RBC-PM EPA levels will increase if the levels of both the desaturation and elongation enzymes of the hepatic tissues increase upon AC administration, and/or if intestinal EPA absorption increases. Along with aging, sex, body weight, consumption of alcohol, smoking status, and genetics,<sup>61</sup> research suggests that dietary polyphenols may influence the desaturase/elongase enzymes controlling the EPA or DHA concentration. Some studies with rodents and humans have suggested that polyphenols increase the levels of EPA or DHA, probably by stimulating the activities of hepatic  $\Delta 5$  and/or  $\Delta 6$  desaturases.<sup>28,62–65</sup> Vauzour *et al.*<sup>66</sup> reported that anthocyanins, polyphenols abundant in plants, do not influence the levels of EPA, mRNA of fatty acid synthesis genes (*Fads1/2*), or fatty acid synthesis proteins, such as  $\Delta 5$ - and  $\Delta 6$ -desaturases. Moreover, resveratrol, a stilbenoid polyphenol found in the skin and seeds of grapes,<sup>67</sup> was found to decrease EPA levels with concurrent reduction or null effects on the mRNA levels of  $\Delta 5$  and  $\Delta 6$  desaturases. These reports thus suggest that *in vitro* effects of polyphenols do not reflect the *in vivo* effects mentioned above. However, the differences in the bioavailability of polyphenols in the body and the concentration of polyphenols used in these *in vitro* studies may be attributed to these discrepancies regarding the effects of polyphenols. The expression of  $\Delta 5$ - or  $\Delta 6$ -desaturase and elongase enzymes is regulated by transcription factors, such as peroxisome proliferator-activated receptor  $\alpha$  (PPAR- $\alpha$ ), sterol regulatory element binding protein 1c (SREBP-1c), retinoid X receptor (RXR), and carbohydrate response element binding protein (ChREBP),<sup>68,69</sup> the effects of polyphenols on these regulatory proteins need to be clearly delineated. Whatever the exact mechanism, consistent with the *in vivo* reports is as mentioned above,<sup>62–65</sup> POAC supplementation likely increased the RBC-PM EPA levels more than PO supplementation alone due to the flavonoids contained in AC.

Epidemiological studies have reported an association between serum BDNF levels and cognitive function,<sup>70,71</sup> though the results have been inconsistent. The administration

of EPA and DHA improved cognitive impairment and increased serum BDNF levels in a rat model of Alzheimer's disease.<sup>72</sup> Furthermore, we recently found that the administration of AC extract enhanced learning and memory in association with increased hippocampal BDNF levels in SAMP8 mice.<sup>16</sup> Håkansson *et al.*<sup>73</sup> reported that a physical exercise-induced increase in serum BDNF levels was correlated with improved cognitive performance, concluding that the production of peripheral BDNF reflects the availability of BDNF in the brain. These results suggest that changes to serum BDNF levels in elderly individuals are associated with a decline in cognition during aging. In this study, however, neither PO nor POAC supplementation had an effect on serum BDNF levels in healthy elderly participants after 12 months. Further studies are needed to elucidate the relationship between serum BDNF levels and cognitive ability under PO and/or POAC treatment.

Several observational studies have suggested that cardiovascular risk factors such as hypertriglyceridaemia and hyperglycaemia are potential risk factors for the onset and/or progression of MCI and dementia.<sup>4</sup> In the present study, serum TG and glucose levels significantly decreased in both the PO and POAC groups after 12 months of treatment (Table 3). This reduction in serum TG levels was associated with an increase in the RBC-PM EPA levels in the POAC group, but not in the PO group (Table 3). Many clinical studies have shown that  $\omega$ -3 PUFAs, particularly EPA, lower TG levels. The administration of icosapent ethyl, a high-purity form of EPA, for 12 weeks significantly increased the RBC EPA levels and was associated with TG reduction in patients with hypertriglyceridaemia.<sup>74,75</sup> Similarly, Egert *et al.*<sup>76</sup> reported that serum TG levels significantly decreased after ALA intervention in normolipidemic individuals. Furthermore, Yamanaka *et al.*<sup>77</sup> reported in a randomised, double-blind, controlled crossover study that a single oral ingestion of ALA-rich diacylglycerol oil suppressed postprandial serum TG levels in humans. These results suggest that the decrease in serum TG levels observed in both the PO and POAC groups after 12 months of treatment was caused by the increased ALA intake. However, no significant relationship was observed between serum TG levels and RBC-PM ALA levels in this study. Further studies are needed to elucidate the connection between ALA and serum TG levels as they relate to dementia and MCI.

AC leaves are traditionally used in Indonesia to reduce blood glucose.<sup>78,79</sup> The AC leaf extract has been shown to reduce blood glucose levels in rats with high-fat diet-induced diabetes mellitus by regulating fatty acid metabolism.<sup>78,79</sup> To our knowledge, no data have been published concerning the effects of AC intake on serum glucose levels in healthy elderly individuals. In this study, we observed significantly reduced serum glucose levels in association with increased RBC-PM ALA levels in both the PO and POAC groups after 12 months of treatment. After 12 months of POAC treatment, serum glucose levels tended to negatively correlate with RBC-PM EPA levels ( $r = -0.346$ ,  $p = 0.066$ ) (data not shown), but not with ALA levels. These findings suggest that chronic administration of PO or



POAC supplements lowers serum glucose levels by increasing the RBC-PM EPA levels.

The APOE-ε4 allele is the strongest and most prevalent genetic risk factor for sporadic late-onset Alzheimer's disease due to its influence on amyloid-beta deposition, neuro-inflammation, neurogenesis, synaptic function, and lipid metabolism.<sup>80,81</sup> The potential of APOE-ε4 allele frequency to affect results of the present study made it meaningful to measure. The distribution of different APOE-ε4 alleles showed no significant differences between the PO and POAC groups, indicating that the outcome of this intervention trial was not influenced by allele frequency in the two groups.

This study has several limitations. First, because the volunteers were healthy elderly individuals without neuronal dysfunction, the observed cognitive and mental health decline was mostly related to age, rather than pathology. This is reflected in the total test scores for cognitive performance and mental health, which did not differ significantly in the PO or POAC groups. A wider range of baseline cognitive function scores could lead to more visible effects of intervention, and we plan to investigate the effects of PO and POAC on individuals with more diverse cognitive abilities. Second, to elucidate the exact mechanisms of ALA action, it is necessary to determine whether it is ALA alone, or its conversion to EPA and/or DHA, that yields effects. However, this distinction cannot be clarified by human intervention studies alone, nor by rodent model studies because the conversion efficiency of ALA to EPA and DHA in humans is much lower than that in rodents. Investigating human gene polymorphisms as they relate to conversion enzymes may provide insights into the mechanisms of ALA action. Third, the sample size of this study was relatively small, with participants from a single population, which limits the generalisability of the current results. Finally, this intervention study lacked two controls: a placebo group and a group taking only AC leaf powder. The small participant population limited our ability to divide patients into more than two groups, and study volunteers were unwilling to take 15 potentially ineffective (*i.e.*, placebo) SGCs daily for a year. The lack of control groups may have decreased the power of this intervention trial, and larger trials are needed in the future.

## 5. Conclusions

Twelve months of POAC supplementation showed no adverse clinical effects and improved cognitive function in healthy Japanese elderly individuals, presumably by lowering the CML, TG, and glucose levels. Nutritional interventions that prevent cardiovascular risk factors, such as hypertriglyceridaemia and hyperglycaemia, are potential preventive agents against the onset and/or progression of MCI and dementia. Although further research is needed to elucidate the mechanisms underlying the beneficial effects of PO and POAC on cognitive performance, these effective and easy-to-consume supplements may be viable and accessible options for maintaining cognitive

and cardiovascular function in the elderly. Future research will clarify the impact of PO alone or with AC on patients with clinical cognitive impairments and mental illness, including neurodegenerative disorders.

## Author contributions

Michio Hashimoto: conceptualization, data curation, formal analysis, funding acquisition, investigation, methodology, project administration, resources, supervision, visualization, writing – original draft, writing – review and editing. Kentaro Matsuzaki: data curation, investigation, methodology, software, formal analysis, visualization, writing-original draft, writing – review and editing. Koji Maruyama: conceptualization, project administration, funding acquisition, validation. Eri Sumiyoshi: validation, visualization, writing – review and editing. Shahdat Hossain: validation, writing – original draft, visualization, writing – review and editing. Harumi Wakatsuki: data curation, formal analysis, investigation, methodology. Setsushi Kato: methodology, resources, supervision, validation. Miho Ohno: investigation, methodology, resources. Yoko Tanabe: investigation. Yoko Kuroda: investigation, methodology. Shuhei Yamaguchi: supervision. Koji Kajima: conceptualization, funding acquisition, supervision. Yasushi Ohizumi: conceptualization, project administration, funding acquisition, writing – review and editing, supervision. Osamu Shido: supervision, validation.

## Conflicts of interest

Koji Maruyama and Koji Kajima, employees of Sankyo Holdings Co., Ltd, contributed to the experiments. Other authors have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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