



Evaluation of the beneficial effects of ETAS® on normal aging or mild cognitive impairment subjects: A pilot randomized controlled trial

[Evaluación de los efectos beneficiosos de ETAS® en sujetos con envejecimiento normal o deterioro cognitivo leve: Un ensayo piloto controlado aleatorizado]

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Abstract

Context: Recently, new intervention tools at the mild cognitive impairment (MCI) stage are needed to prevent dementia, including Alzheimer's disease (AD).

Aims: To evaluate whether a standardized *Asparagus officinalis* stem (ETAS) extract, as a functional ingredient, effectively maintains cognitive functions in subjects with normal aging and MCI.

Methods: A pilot randomized controlled trial was conducted on thirty subjects that were randomly allocated to the experimental group (n = 15), which received ETAS supplementation (1,000 mg) daily, and a control group, which received placebo (dextrin) in the same form (n = 15) for a period of 12 months. All subjects were evaluated using neuropsychological questionnaires comprised of Mini-Mental State Examination (MMSE), Frontal Assessment Battery (FAB), Clock Drawing Test (CDT), and Hospital Anxiety and Depression Scale (HADS) at all visit times. Brain tests (MRI, EEG and EPB) and blood tests (hematology, hemochemistry, immunological status and HSP70) were also performed. Primary endpoints were the change in the neuropsychological questionnaire scores. Secondary endpoints such as immunological and molecular biomarkers of dementia, neurological imaging, and electrophysiological outcomes were measured.

Results: The ETAS group showed a significant improvement in scores on MMSE, FAB, and HADS, tended to improve in scores on CDT, and could show a slight increase in blood HSP70. The ETAS group also maintained normal levels of CD4/CD8 immune complex. No adverse events were detected during the study period.

Conclusions: ETAS supplementation could prevent the progression of cognitive decline and anxiety/depression expression associated with the prevention of AD.

Keywords: Alzheimer's disease; dietary supplements; functional food; heat-shock proteins; mild cognitive impairment.

Resumen

Contexto: Recientemente, se necesitan nuevas herramientas de intervención en la fase de deterioro cognitivo leve (DCL) para prevenir la demencia, incluida la enfermedad de Alzheimer (EA).

Objetivos: Evaluar si un extracto estandarizado de tallo de *Asparagus officinalis* (ETAS), como ingrediente funcional, mantiene eficazmente las funciones cognitivas en sujetos con envejecimiento normal y DCL.

Métodos: Se realizó un ensayo piloto controlado aleatorizado en treinta sujetos que fueron asignados al azar al grupo experimental (n = 15), que recibió suplementación de ETAS (1.000 mg) diariamente, y a un grupo de control, que recibió placebo (dextrina) en la misma forma (n = 15) durante un período de 12 meses. Todos los sujetos fueron evaluados mediante cuestionarios neuropsicológicos compuestos por el Mini-Mental State Examination (MMSE), la Frontal Assessment Battery (FAB), el Clock Drawing Test (CDT) y la Hospital Anxiety and Depression Scale (HADS) en todas las visitas. También se realizaron pruebas cerebrales (IRM, EEG y EPB) y análisis de sangre (hematología, hemoquímica, estado inmunológico y HSP70). Los criterios de valoración primarios fueron los cambios en las puntuaciones del cuestionario neuropsicológico. Se midieron criterios de valoración secundarios como los biomarcadores inmunológicos y moleculares de la demencia, las imágenes neurológicas y los resultados electrofisiológicos.

Resultados: El grupo ETAS mostró una mejora significativa en las puntuaciones en MMSE, FAB y HADS, tendió a mejorar en las puntuaciones en CDT, y pudo mostrar un ligero aumento de HSP70 en sangre. El grupo ETAS también mantuvo niveles normales del complejo inmunitario CD4/CD8. No se detectaron acontecimientos adversos durante el período de estudio.

Conclusiones: La suplementación con ETAS podría prevenir la progresión del deterioro cognitivo y la expresión de ansiedad/depresión asociada a la prevención de la EA.

Palabras Clave: alimentos funcionales; deterioro cognitivo leve; Enfermedad de Alzheimer; proteínas de choque térmico; suplementos dietéticos.

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INTRODUCTION

Alzheimer's disease (AD) accounts for 50-80% of cognitive decline diseases and is characterized by changes of tauopathy and amyloid beta ($A\beta$) amyloidosis (Abbott, 2011). AD's typical symptoms are memory and learning disorders through steadily progressive episodic memory decline. The disease progression significantly impairs independence and quality of life (QOL) due to causes of social cognitive dysfunction such as apraxia, agnosia, aphasia, apathy, a reversal of day and night, delirium incontinence and personality changes (Igarashi and Ikeda, 2022). It has been reported that mild cognitive impairment (MCI) exists as a precursor to the onset of AD and that approximately 16.5% of MCI patients transition to dementia, including AD (Petersen et al., 2010). Since $A\beta$ is known to be one of the causes of MCI, any clinical treatment of $A\beta$ at the MCI stage is important to prevent AD.

The development of the amyloid vaccine focused on immunotherapy was expected to be an AD's fundamental remedy. Aducanumab, one of the newest antibody drugs, has been reported to reduce $A\beta$ in the brain in clinical trials and conditionally approved by the FDA. Unfortunately, unlike $A\beta$ reduction, improvement in dementia with Aducanumab should be studied continuously (Sevigny et al., 2016). Recently, a new antibody drug, Lecanemab, was developed and showed a moderate reduction in measures of cognition and function with adverse events in the clinical trial (Dyck et al., 2023).

On the other hand, treatment at the early stage of dementia is critical for QOL maintenance. It is regarded that the efficacy of investigational drugs against the progressed AD stage is insufficient due to the brain has already suffered considerable damage by the accumulation of the $A\beta$ tangle, which had occurred 20 to 30 years before the appearance of cognitive decline. Thus, it is important to provide care that includes not only the milder stages of pathological change, such as MCI and the prodromal stage of AD, but also the normal aging stage with declining cognitive function (Selkoe and Hardy, 2016).

Heat Shock Proteins (HSPs) are intracellular proteins and have the role as molecular chaperones induced by stresses such as environmental conditions, pathogens, toxins, and diseases. Induced HSPs restore misfolding protein caused by this stress. Proteins lose their functions by collapsing their higher-order structure via misfolding and/or physicochemical stresses. HSPs bind to the denaturing and/or dysfunctional proteins and show the roles of repairing and protecting the higher-order protein structures. If it is impos-

sible to repair the protein structure, it will be degraded by the proteasome after ubiquitination. Especially, HSP70 is essential to the recovery and survival of the cells and to maintain normal cellular functions (Kampinga and Craig, 2010). It is hypothesized that correction of misfolded proteins by HSP70 may be an effective means of preventing amyloid- β accumulation in suppressing cognitive decline (Selkoe, 2004).

A standardized extract of *Asparagus officinalis* stem (trademarked as ETAS[®], Amino Up Co., Ltd., Sapporo, Japan) used in the present study is standardized as containing over 50 $\mu\text{g/g}$ of asparagus-derived proline- containing 3-alkyldiketopiperazines in its specification: cyclo (L-Phe-L-Pro), cyclo (L-Tyl-L-Pro), and cyclo (L-Leu-L-Pro) (Inoue et al., 2020). Until now, many studies have been conducted on neuroprotective and antioxidant effects of ETAS. Recent investigations have demonstrated that ETAS ameliorates cognitive impairment by inhibiting $A\beta$ deposition via BACE-1 in senescence-accelerated mice, and it indicated normalizing circadian rhythm signaling via MT1 and MT2. ETAS attenuates $A\beta$ -Induced cellular disorder in PC12 cells. Moreover, several lines of evidence indicated that ETAS has neuroprotective effects *in vitro* and *in vivo* studies (Chan et al., 2019; Koda et al., 2017; Sakurai et al., 2014).

This study aims to investigate the effects of ETAS on normal aging or MCI subjects through a pilot randomized, double-blind, placebo-controlled study that includes the evaluation of HSP70 protein concentrations in blood.

MATERIAL AND METHODS

Subjects

The subjects for this study were recruited by the principal investigator at NEVRON International Medical Center. The recruited subjects were socially intact and healthy, who did not require any support in their daily lives, but whose cognitive function levels were recognized to be declining by themselves or informants who knew the subjects well, such as family members or caregivers. The subjects needed to have objective cognitive performance scores and clinical histories that were consistent with normal aging and MCI (i.e., mild neurocognitive disorder) and inconsistent with dementia (i.e., major neurocognitive disorder) (American Psychiatric Association, 2013; Petersen, 2004). The patients with moderate and severe AD judged by the principal investigator at the time of recruitment by means of brain MRI, electroencephalography, evoked potentials of the brain, brain blood flow, blood test, and neuropsychological question-

naires were excluded. In addition, the exclusion criteria included subjects requiring the use of contraindicated medicines during the study period, subjects with a history of food allergies, subjects with severe hepatic, renal, cardiac, or hypertensive diseases, subjects with acute infectious diseases, cancer patients, and pregnant or potentially pregnant and/or breast-feeding women. Before the beginning of this study, valid informed written consent was obtained from each subject. The total sample size was thirty ($n = 30$: two men and twenty-eight women). Subjects were randomly divided into two groups, the ETAS group ($n = 15$) and the placebo group ($n = 15$), using a random number generator (Randomus.ru) for a simple method. The age range was from 51 to 79 years old (average age: 66 years). The study results were analyzed by dividing the subjects into two groups: MCI (Mini-Mental State Examination (MMSE) score of 23 or higher, Frontal Assessment Battery (FAB) score of 14 or higher, and Clock Drawing Test (CDT) score of six or higher) and advanced MCI (MMSE, FAB, or CDT score are less than MCI criteria). MCI group included the socially intact subjects with normal aging. The study was conducted under the principles of ethical standards set out in the Declaration of Helsinki of the World Medical Association, and Ethical Committee approved the adequacy of Nonprofit Organization TACTICS. The institution's IRB approval number was 2018-124.

Test sample

The test sample was produced by Amino Up Co., Ltd. (Sapporo, Japan), according to Good Manufacturing Practice (GMP) standards for dietary supplements and ISO9001:2015 and ISO22000:2018 criteria. The safety of ETAS was proven by the Ames test, the bone marrow micronucleus test in mice, and the acute and subacute oral toxicity tests in rats (Ito et al., 2014b).

The test sample was prepared in a capsule containing ETAS powder as ETAS50: comprising 50% solid content of asparagus extract (elemental ETAS) and 50% dextrin.

Study design

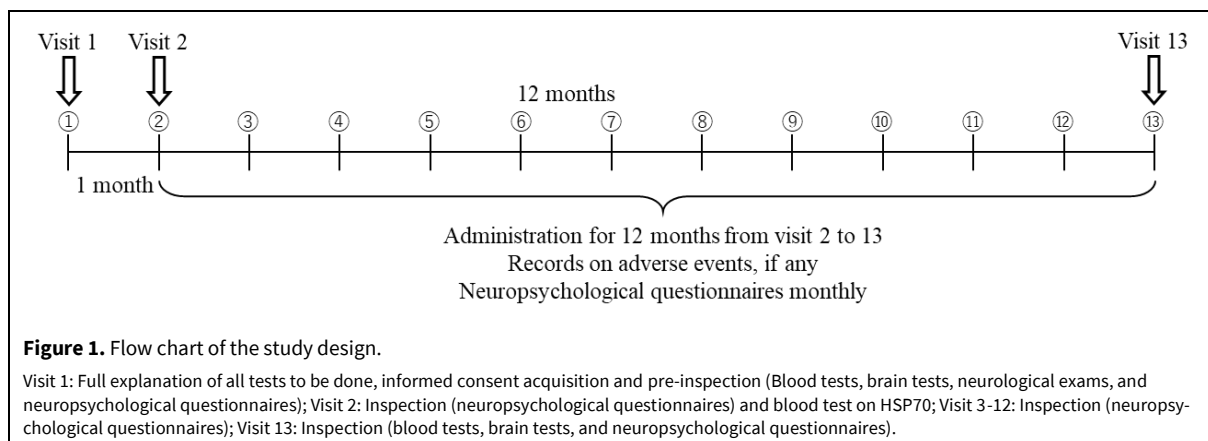
The study was a randomized, double-blind, placebo-controlled clinical study with normal aging and MCI. Subjects ingested either placebo (dextrin) or ETAS (containing 1,000 mg of ETAS50 per 3 capsules a day) after the evening meal for 12 months, and the evaluations were performed as described in Fig. 1. The test samples, both placebo and ETAS were encapsulated, and color and taste did not differ from each other. Potential adverse events and intake of the test samples were checked every month to monitor safety and compliance. A treatment period of 12 months to determine the long-term cognitive effects of ETAS. The study period was from October 1, 2018, to September 30, 2019. The inspection items were as follows.

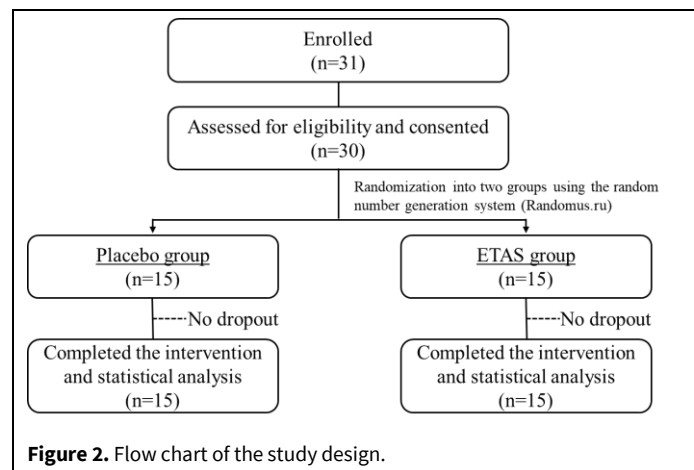
1. Neuropsychological questionnaires: MMSE, CDT, FAB and Hospital Anxiety and Depression Scale (HADS).
2. Brain tests: MRI brain scan, brain EEG, and EPB.
3. Blood tests: complete blood count, biochemistry, lipid profile blood test, immunological status and HSP70.

The primary endpoints were neuropsychological exams: MMSE, FAB, HADS, and CDT. The secondary endpoints were blood and brain examinations.

Neuropsychological questionnaires

Neuropsychological questionnaires comprised of MMSE, FAB, CDT, and HADS were carried out with all visit timing described in Fig. 2.





Blood and brain examinations

To assess the safety of ETAS supplementation, hematological and biochemical parameters were measured. The blood samples drawn from the subjects at visit 1 and visit 13 were used for the measurements as follows: Complete blood count (red blood cells, white blood cells, hemoglobin, hematocrit, and plasma), Biochemistry (total protein, albumin, total bilirubin, AST, ALT, LDH, ALP, gamma-GTP, CK, Cr, BUN, Na, K, Cl, uric acid, glucose, and insulin), Lipid profile blood test (total cholesterol, triglycerides, LDL, HDL, and VLDL). Secondary efficacy variables of ETAS included immunologic status (CD4/CD8), humoral immunity testing (IgA, IgM, IgG, CD3 and CD4, circulating immune complex), and blood HSP70. Human Heat Shock Protein 70 (Chundubio, Wuhan Purity Biotechnology Co., Ltd, China) was used to measure HSP70 in blood cells of peripheral blood. In the brain examinations, MRI scans, EEG, and EPB were performed at the same timings of blood tests. The EEG evaluated according to as follows changes; the negative changes are represented by an increase in slow-wave activity and its amplitude, sharp waves in frontal lobes, and a decrease of α -rhythm frequency and power, and the positive changes are represented by an increase of α -rhythm frequency and organization and decrease irritation of subcortical structures.

Statistical analysis

According to the minimum sample size of the pilot test reported in the article (Steven, 2005), the total sample size of subjects is determined as $n = 30$. All subjects will be analyzed on a full analysis set basis. Statistical analysis was performed using EZR with variance analysis by f-test followed by t-test. EZR is a modified version of R commander designed to add statistical functions frequently used in biostatistics (Kanda, 2013). Differences were statistically significant or tended at * $p < 0.05$, ** $p < 0.01$, † $p < 0.1$ vs. base-

line, and # $p < 0.05$, ## $p < 0.01$, ‡ $p < 0.1$ vs. placebo. Results are expressed as the mean standard error of the mean (SEM).

RESULTS

In this study, 30 out of 31 subjects enrolled in the study completed the study (Fig. 2). As a result of the intervention, three subjects in the ETAS group presented allergic symptoms. Although these subjects had a history of allergies, a causal relationship with ETAS was ruled out, as there was no association between the allergic symptoms and the ingredients contained in the test samples. Also, four subjects in the ETAS group showed transient nausea, satiety, and abdominal pain without stool changes. A causal relationship was ruled out because they had a clinical history of gastrointestinal disorders, and the changes were not correlated with the administration of ETAS.

The results of the comparison of age, neuropsychological questionnaire scores, CD4/CD8 ratios, and HSP70 concentrations in WBCs at baseline of subjects enrolled in the placebo and ETAS groups were listed in Table 1.

There was no significant difference in the data of the two groups at baseline. The stratified analysis was performed as follows: Placebo group consisted of MCI ($n = 9$), Advanced MCI ($n = 6$), and Total (MCI and Advanced MCI, $n = 15$), and ETAS group consisted of MCI ($n = 8$), Advanced MCI ($n = 7$), and Total (MCI and advanced MCI, $n = 15$). All the data are shown in Table 2, and the boxplots of Total, MCI, and advanced MCI in Figs. 3-5.

The delta changes between 2 groups, placebo and ETAS, are shown in Fig. 6. Regarding the pre/post comparison in Total, the ETAS group showed significant improvement in MMSE from 24.3 ± 0.5 to 26.3 ± 0.3 , $p = 0.001$, and in FAB from 15.2 ± 0.5 to 17.2 ± 0.3 , $p = 0.001$ (Fig. 3A-B). The results of HADS Depression and Anxiety in the placebo group showed a signifi-

cant worsening, while those in the ETAS group showed significant improvement in HADS Depression from 8.5 ± 0.8 to 7.4 ± 0.5 , $p = 0.03$, and in HADS Anxiety from 9.7 ± 0.9 to 7.6 ± 0.4 , $p = 0.014$ (Fig. 3D-E). The CD4/CD8 ratio in the placebo group showed a significant increase from 1.97 ± 0.22 to 2.58 ± 0.31 , $p = 0.001$, whereas no significant change in the ETAS group (Fig. 3F). Also, HSP70 in WBC showed a significant increase in both groups, from 249.71 ± 10.92 pg/mL to 332.65 ± 15.34 pg/mL, $p = 0.0001$ in placebo, and from 222.12 ± 13.07 pg/mL to 337.91 ± 12.85 pg/mL, $p = 0.00001$ in ETAS (Fig. 3G).

The MMSE, FAB, HADS Depression and Anxiety, CD4/CD8 ratio, and HSP70 in WBC in MCI showed similar changes to comparisons in Total (Fig. 4A-B, D-F). CDT score improved significantly from 7.1 ± 0.4 to 8.1 ± 0.2 , $p = 0.03$, in the ETAS group (Fig. 4C).

In advanced MCI, the ETAS group showed a significant improvement or increase in MMSE from 23.1 ± 1.0 to 25.6 ± 0.6 , $p = 0.03$, in FAB from 14.1 ± 0.8 to 16.6 ± 0.6 , $p = 0.047$, and in HSP70 in WBC from 230.75 ± 27.45 pg/mL to 334.33 ± 13.59 pg/mL, $p = 0.014$ (Fig. 5A-B and G). The HADS Anxiety of ETAS also showed a trend of improvement (Fig. 5E). HSP70 in WBC showed a significant increase in placebo as well, from 225.45 ± 17.36 pg/mL to 338.79 ± 17.77 pg/mL, $p = 0.00002$, and the CD4/CD8 ratio was also increased significantly in the placebo group, from 2.05 ± 0.33 to 2.75 ± 0.46 , $p = 0.042$ (Fig. 5F-G).

Before and after the intervention, the delta change showed that the ETAS group improved significantly in MMSE, HADS Depression, and Anxiety compared to the placebo group, and prevented significantly the increasing CD4/CD8 ratio (Fig. 6A, D-F). Besides, ETAS group showed a trend of improvement and increase in FAB, CDT, and HSP70 in WBC (Fig. 6B-C, G).

There were no remarkable changes in the safety blood tests and brain examinations such as MRI and EPB during the test period (data not shown).

Regarding EEG of the brain examination in the ETAS group, 87.5% of MCI and 42.9% of advanced MCI subjects showed a positive change. In the placebo group, only 11% of the MCI subjects showed a positive change, while 44% in the MCI stage and 66% in the advanced MCI subjects showed a negative change (data not shown).

DISCUSSION

In this study, neuropsychological questionnaires consisting of MMSE, FAB, CDT, and HADS, blood and brain tests on normal aging, and MCI subjects were performed to evaluate the beneficial effects of ETAS supplementation for cognitive dysfunction.

For the analysis, the subjects were stratified into MCI (MMSE score of 23 or higher, FAB score of 14 or higher, and CDT score of 6 or higher) and advanced MCI (MMSE, FAB, or CDT scores below MCI stage criteria). At MMSE evaluation, the number of subjects with a score of 23 points or higher considered to have a normal cognitive function was 11 out of 15 subjects at the baseline and 12 out of 15 subjects at 12 months in the placebo groups. The subjects considered to have normal cognitive function in the ETAS group were 11 out of 15 subjects at the baseline and all subjects at 12 months. ETAS significantly improved MMSE scores in MCI, advanced MCI, and Total. Moreover, ETAS showed a significant difference compared to the placebo group in MCI. MMSE is a widely used assessment for cognitive functions such as orientation, attention, memory, language, and visual-spatial skills. Bracco et al. (1998) reported that the MMSE score decreases by 1.8-4.5 points per year.

Table 1. Characteristics of the subjects.

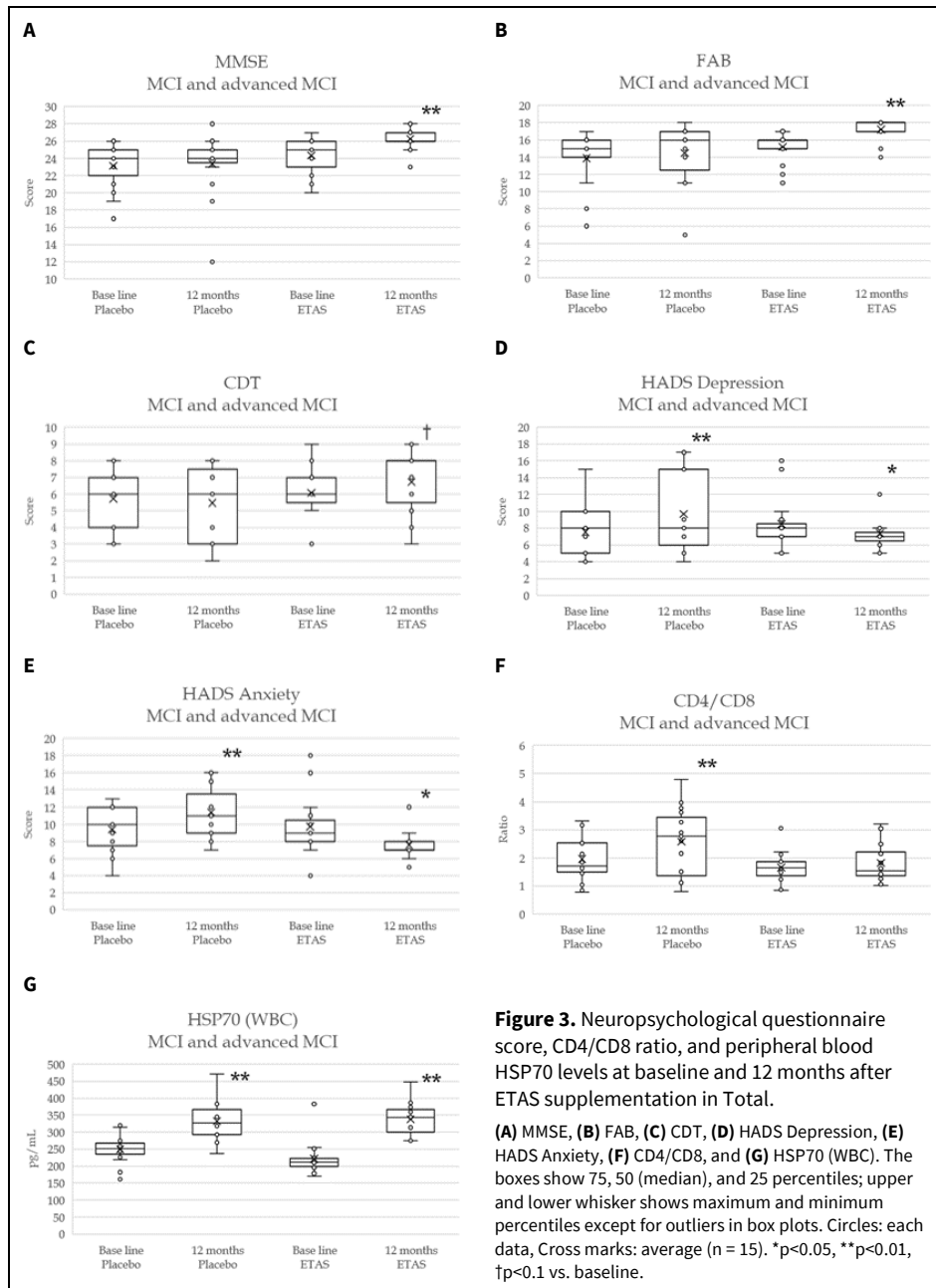
Measurement Items	Baseline		P values vs. placebo
	Placebo	ETAS	
Age	66.1 ± 1.9	64.0 ± 1.8	0.42
MMSE	23.1 ± 0.7	24.3 ± 0.5	0.19
FAB	13.9 ± 0.8	15.2 ± 0.5	0.17
CDT	5.7 ± 0.5	6.1 ± 0.5	0.63
HADS Depression	7.5 ± 0.8	8.5 ± 0.8	0.42
HADS Anxiety	9.4 ± 0.7	9.7 ± 0.9	0.77
CD4/CD8	1.94 ± 0.22	1.66 ± 0.15	0.243
HSP70 (WBC)	249.71 ± 10.92	222.12 ± 13.07	0.116

Data represent the mean \pm SEM ($n = 15$). Comparison of age, neuropsychological questionnaire score, CD4/CD8 ratio, and peripheral blood HSP70 levels at baseline.

Table 2. The change of neuropsychological questionnaire values by ETAS intervention for the normal aging or MCI subjects.

Items	Subgroup	Placebo		p value vs. baseline	ETAS		p value vs. baseline	p value vs. placebo
		Baseline	12 months		Baseline	12 months		
MMSE	MCI and advanced MCI	23.1 ± 0.7	23.4 ± 1.0	0.75	24.3 ± 0.5	26.3 ± 0.3	0.001**	0.08‡
	MCI	24.6 ± 0.4	25.0 ± 0.5	0.22	25.4 ± 0.3	26.9 ± 0.3	0.003**	0.04#
	Advanced MCI	21.0 ± 1.3	21.0 ± 2.0	1.00	23.1 ± 1.0	25.6 ± 0.6	0.03*	0.28
FAB	MCI and advanced MCI	13.9 ± 0.8	14.5 ± 0.9	0.26	15.2 ± 0.5	17.2 ± 0.3	0.001**	0.06‡
	MCI	15.1 ± 0.3	15.8 ± 0.7	0.32	16.1 ± 0.2	17.8 ± 0.2	0.002**	0.21
	Advanced MCI	12.0 ± 1.8	12.5 ± 1.9	0.62	14.1 ± 0.8	16.6 ± 0.6	0.047*	0.19
CDT	MCI and advanced MCI	5.7 ± 0.5	5.5 ± 0.6	0.52	6.1 ± 0.5	6.7 ± 0.5	0.07†	0.09‡
	MCI	6.9 ± 0.3	7.1 ± 0.3	0.51	7.1 ± 0.4	8.1 ± 0.2	0.03*	0.14
	Advanced MCI	4.0 ± 0.6	3.0 ± 0.3	0.30	4.9 ± 0.7	5.1 ± 0.6	0.63	0.22
HADS depression	MCI and advanced MCI	7.5 ± 0.8	9.7 ± 1.2	0.007**	8.5 ± 0.8	7.4 ± 0.5	0.03*	0.0004##
	MCI	8.0 ± 0.8	10.2 ± 1.4	0.04*	8.1 ± 1.0	7.1 ± 0.8	0.14	0.012#
	Advanced MCI	6.8 ± 1.7	8.8 ± 2.3	0.12	8.9 ± 1.3	7.7 ± 0.7	0.14	0.03#
HADS anxiety	MCI and advanced MCI	9.4 ± 0.7	11.4 ± 0.8	0.004**	9.7 ± 0.9	7.6 ± 0.4	0.014*	0.0002##
	MCI	9.8 ± 0.7	12.2 ± 0.9	0.006**	9.6 ± 1.2	7.3 ± 0.5	0.11	0.004##
	Advanced MCI	8.8 ± 1.3	10.2 ± 1.3	0.26	9.9 ± 1.4	8.0 ± 0.7	0.06†	0.03#
CD4/CD8	MCI and advanced MCI	1.97 ± 0.22	2.58 ± 0.31	0.001**	1.66 ± 0.15	1.82 ± 0.17	0.240	0.039#
	MCI	1.92 ± 0.30	2.46 ± 0.44	0.023*	1.87 ± 0.20	2.24 ± 0.23	0.078	0.517
	Advanced MCI	2.05 ± 0.33	2.75 ± 0.46	0.042*	1.41 ± 0.20	1.35 ± 0.11	0.743	0.031#
HSP70 (WBC)	MCI and advanced MCI	249.71 ± 10.92	332.65 ± 15.34	0.0001**	222.12 ± 13.07	337.91 ± 12.85	0.00001**	0.161
	MCI	265.88 ± 11.85	328.55 ± 23.45	0.025*	214.56 ± 7.61	341.05 ± 21.85	0.0003**	0.053‡
	Advanced MCI	225.45 ± 17.36	338.79 ± 17.77	0.00002**	230.75 ± 27.45	334.33 ± 13.59	0.014*	0.763

This study's subjects were divided into MCI (MMSE score of 23 or higher, FAB score of 14 or higher, and CDT score of 6 or higher) and advanced MCI (MMSE, FAB, or CDT score are less than MCI stage criteria). Data represent the mean ± SEM (n = 15). *p<0.05, **p<0.01, †p<0.1 vs. baseline. #p<0.05, ##p<0.01, ‡p<0.1 vs. placebo.

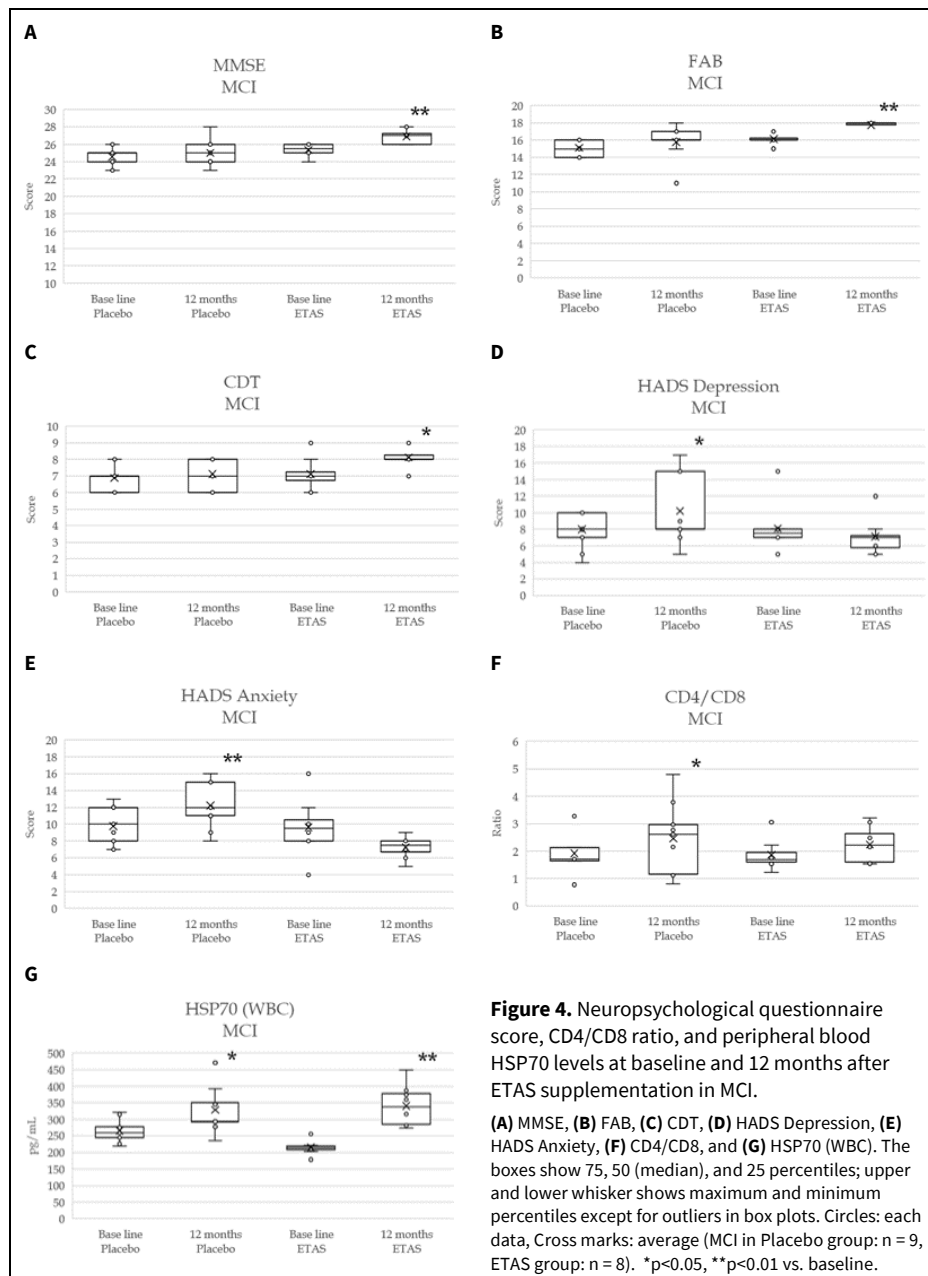


It is known that the degree of MMSE score reduction and symptom progression are correlated with each other. Furthermore, Liu et al. reported that the hippocampal volume is associated with MMSE scores (Liu et al., 2015). The significant improvements in the MMSE score observed in the ETAS group suggest that ETAS may improve cognitive functions in normal aging or MCI subjects.

At FAB evaluation, ETAS showed significant improvements in MCI, advanced MCI, and Total, whereas there were no significant changes with the placebo group. Psychotic symptoms such as hallucinations, delusions, anxiety, and depression are associated with frontal lobe function (Nakano et al., 2006; Sultzer et al., 2003) and are detected in patients with

dementia, including AD. Since the FAB assesses frontal lobe function, the improvement in group-specific FAB scores for the ETAS suggests that ETAS may maintain and improve the psychotic symptoms due to reduced frontal lobe function, resulting in maintained or improved QOL of AD patients and their caregivers.

CDT has been used to evaluate the visuospatial function, but it is recently used to screen for cognitive function (Shulman, 2000; Tuokko et al., 1992). In CDT evaluation, ETAS also showed significant improvements in the MCI subjects. Improvement of CDT scores in MCI is also important to prevent progression to dementia, including AD, because it correlates with conceptual deficits and cognitive decline in AD



patients (Parsey and Schmitter-Edgecombe, 2011; Rouleau et al., 1996). The significant improvement of CDT scores in the MCI stage subjects along with ETAS intervention suggests the importance of the treatment during MCI.

The HADS Depression and Anxiety in the placebo group worsened significantly in the Total and MCI subjects. On the other hand, ETAS showed significant improvements not only in HADS depression but also in HADS anxiety in Total. Furthermore, HADS Depression and Anxiety scores were significantly improved in the ETAS group compared to the placebo group in all stratified analyses. When MCI progresses to AD, cognitive dysfunction with various behavioral and psychological symptoms and memory impair-

ment associated with dementia were observed. And especially, in the psychological aspect, symptoms of anxiety and depression have been observed. Supplementation with ETAS was reported to improve autonomic nerve condition by taking a balance between sympathetic and parasympathetic activity (Ito et al., 2014a). Therefore, significant improvement in HADS score in ETAS group resulted from the beneficial effect on the subject's autonomic nervous system.

Recently, several reports have been published on the relationship between immune-response molecules and AD. Larbi et al. (2009) reported a drastic change in the naive and memory subsets of CD4⁺ T cells in a study of mild AD patients, with a significant decrease of naive cells, elevated memory cells, and increased

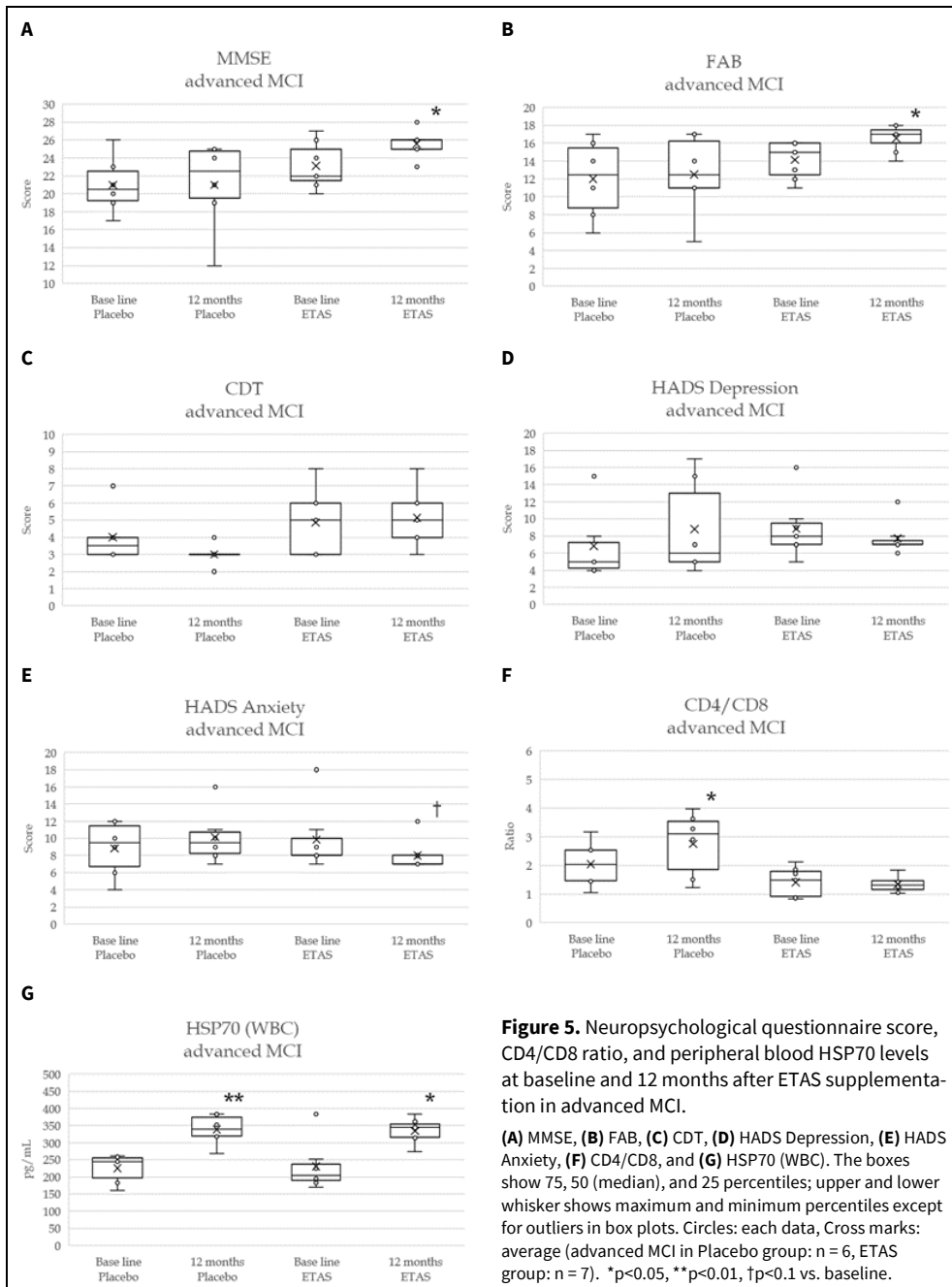


Figure 5. Neuropsychological questionnaire score, CD4/CD8 ratio, and peripheral blood HSP70 levels at baseline and 12 months after ETAS supplementation in advanced MCI.

(A) MMSE, (B) FAB, (C) CDT, (D) HADS Depression, (E) HADS Anxiety, (F) CD4/CD8, and (G) HSP70 (WBC). The boxes show 75, 50 (median), and 25 percentiles; upper and lower whisker shows maximum and minimum percentiles except for outliers in box plots. Circles: each data, Cross marks: average (advanced MCI in Placebo group: n = 6, ETAS group: n = 7). *p<0.05, **p<0.01, †p<0.1 vs. baseline.

proportions of CD4⁺ but not CD8⁺ T cells lacking the essential costimulatory receptor CD28. Moreover, Pellicanò et al. (2012) reported that the differences between AD patients and age-matched normal subjects were in the CD4⁺ rather than the CD8⁺ T cell compartment. They proposed that the changes in CD4⁺ T cells may result from chronic stimulation by Aβ in the blood. Regarding the change of the CD4/CD8 through the intervention period, it was elevated in the placebo group alone, and this result is not inconsistent with the levels of CD4⁺ T cell increase with the progression of AD symptoms, as several studies have reported. An increase in CD4⁺ T cells indicates activation of immune systems, and excessive

activation of the immune systems related to AD progression might be leading to the abnormal destruction of own tissues such as the brain, likewise an autoimmune disease. The subjects supplemented with ETAS did not show remarkable elevation of the CD4/CD8 values, and 8 out of 15 subjects (2 out of 15 in the placebo group) maintained an average level of 1.5-2.5. Hence, it is suggested that ETAS has a protective effect on MCI subject's brain tissue by suppressing an excessive immune response.

HSP70 levels in the WBC increased significantly in both groups, and the change in the ETAS group was greater than that in the placebo group. Furthermore, ETAS tended to increase WBC HSP70 compared to

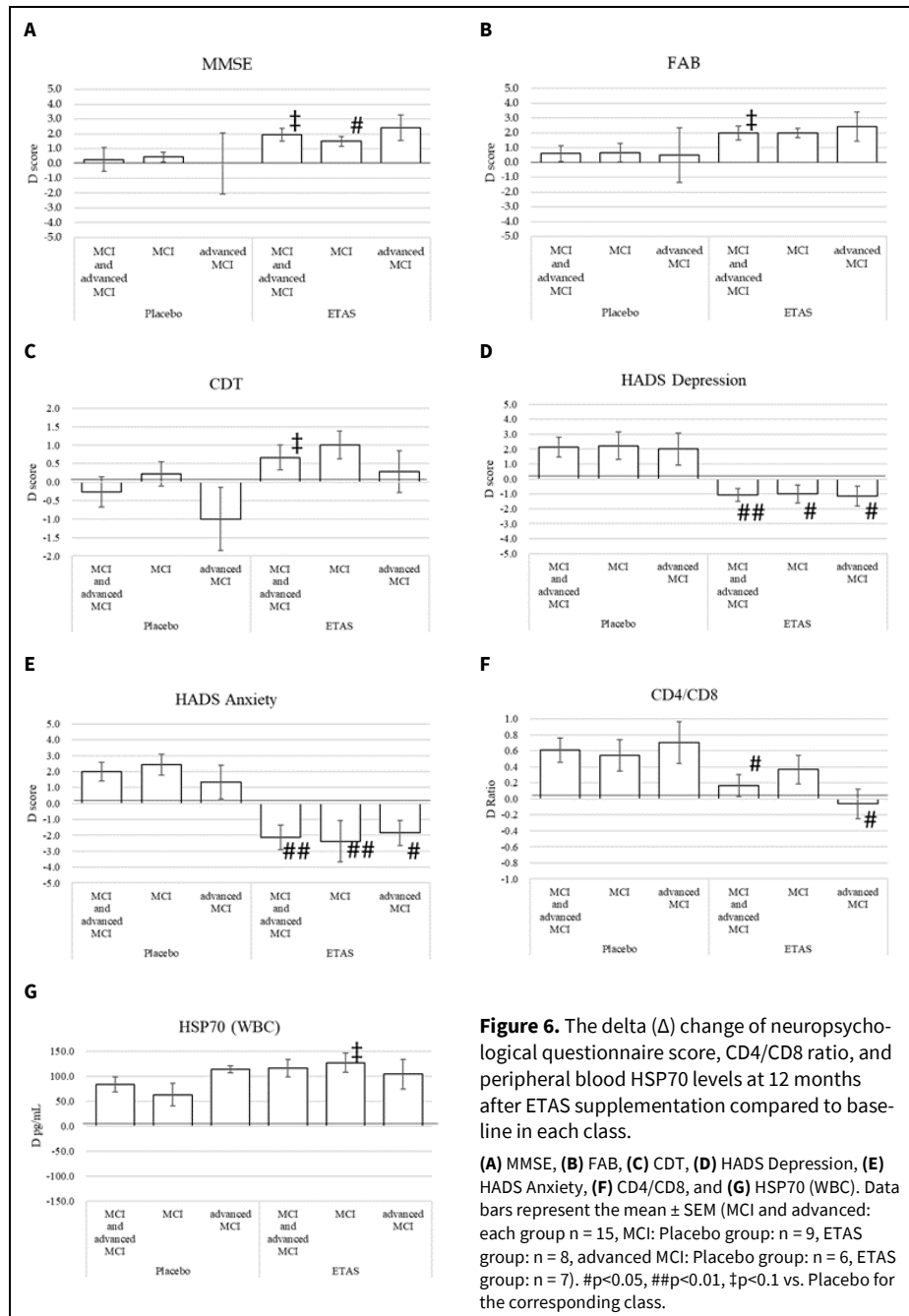


Figure 6. The delta (Δ) change of neuropsychological questionnaire score, CD4/CD8 ratio, and peripheral blood HSP70 levels at 12 months after ETAS supplementation compared to baseline in each class.

(A) MMSE, (B) FAB, (C) CDT, (D) HADS Depression, (E) HADS Anxiety, (F) CD4/CD8, and (G) HSP70 (WBC). Data bars represent the mean \pm SEM (MCI and advanced: each group n = 15, MCI: Placebo group: n = 9, ETAS group: n = 8, advanced MCI: Placebo group: n = 6, ETAS group: n = 7). #p<0.05, ##p<0.01, †p<0.1 vs. Placebo for the corresponding class.

the placebo group in the MCI stage subjects. Magrané et al. (2004) reported that HSP70 overexpression rescued toxic effects of intracellular A β accumulation in the neurons. Also, Hoshino et al. (2011) reported that HSP70 overexpression in the neurons of A β overexpressed AD model mice suppresses cognitive decline effects in behavioral experiments with reducing A β expression, A β tissue deposition, neuronal degeneration, and synapse reduction. Induction of HSP70 expression by ETAS intake may have a protective and beneficial effect on brain neurons in MCI as the gateway to AD, because it has been suggested that A β -induced damage may occur more than 20 years before the onset of AD.

Recent investigations have demonstrated that ETAS has been able to induce HSP70, and this study also showed that ETAS supplementation increased WBC HSP70 in the peripheral blood of the MCI subjects. The results of this study suggest that ETAS supplementation is well tolerated by elderly subjects and beneficial for neuroprotection and suppression of a cognitive decline in normal aging or MCI subjects through HSP70 induction and maintaining the CD4/CD8 immune complex within a normal level.

Although on a pilot scale, this study provided data on the efficacy and safety of ETAS for cognitive functions. Therefore, it is necessary to verify the effective-

ness of ETAS by conducting further studies on a larger scale in the future.

CONCLUSION

This study shows that ingestion of ETAS prevents cognitive decline, and ETAS is expected to be an effective functional ingredient to alleviate the decline in brain function leading to Alzheimer's disease.

CONFLICT OF INTEREST

TT, JT, and KG are employees of Amino Up Co., Ltd. All the other authors declared no competing interests.

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AUTHOR CONTRIBUTION:

Contribution	Mikhailichenko N	Lai TJ	Wei JC	Takahashi T	Takanari J	Goto K
Concepts or ideas	x					
Design	x	x	x		x	
Definition of intellectual content	x					
Literature search	x			x		x
Clinical trial	x	x	x			
Experimental studies	x					
Data acquisition			x			
Data analysis		x		x		x
Statistical analysis		x		x		
Manuscript preparation	x					x
Manuscript editing		x	x	x		x
Manuscript review	x	x	x	x	x	x

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