The role of neuroinflammation in Alzheimer's disease and evaluation of the effect of the Standardized Extract of Asparagus Officinalis Stem in mild cognitive impairment

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Abstract. Recently, new intervention tools at the mild cognitive impairment (MCI) stage are needed to prevent dementia, including Alzheimer's disease (AD). This study aimed to evaluate the role of neuroinflammation in AD and whether the Standardized Extract of Asparagus Officinalis Stem (ETAS), as a functional ingredient, effectively maintains cognitive functions in subjects with subjective cognitive decline (SCD) and MCI. This study was conducted as a pilot randomized controlled trial lasting 12 months. 30 subjects were randomly divided into two groups, with 15 subjects in the control group and 15 subjects in the ETAS group. All subjects received Blood tests (including immunological status and HSP70) and Brain tests in the beginning and in the end of study, and also received neuropsychological questionnaires at all visit timing. The intervention of ETAS slight increase in the expression of HSP70 in blood. The ETAS group also maintained normal levels of CD4/CD8 immune complex. It showed a significant improvement in scores on MMSE, FAB, HADS and tend to improve in scores on CDT. No adverse events of any kind were detected during the study period. ETAS supplementation could prevent the progression of cognitive decline and anxiety/depression expression associated with the prevention of Alzheimer's disease.

1. Introduction

Cognitive impairment is one of the most common disorders in the elderly, with severe cognitive impairment (dementia) occurring in 5% of the elderly population. According to forecasts, in 2050 the number of patients with dementia may exceed 150 million [1]. There are a large number of diseases that can lead to the development of dementia, the majority (70-80%) of dementia cases are Alzheimer's disease (AD), cerebrovascular diseases and their combinations [2]. The development of dementia over several years is usually preceded by subjective cognitive decline (SCD) and mild cognitive impairment (MCI) [2]. MCI are cognitive impairments identified during neuropsychological examination, which do not lead to loss of functional activity, but may cause difficulties in learning new forms of activity [3, 4]. The prevalence of MCI increases with age, amounting to 6.7% at the age of 60-64 years and 25.2% at the age of 80-84 years [3].

Diagnostics of the presence and degree of cognitive impairments is based on a thorough assessment of the patient's medical history and complaints, as well as those of others (relatives), the results of neuropsychological research methods, and an assessment of the impact of cognitive impairment on daily (professional, social, and household) activity [2]. In the case of dementia, there are pronounced difficulties in at least one of the areas of daily life while the patient with MCI may experience minor difficulties compared to past experience, which do not limit his independence.

When cognitive impairment is detected, in order to determine their potential causes and exclude concomitant conditions/diseases that worsen cognitive impairment, it is necessary to conduct a laboratory examination, including complete blood count, a biochemical blood test, a lipid profile and immunologic tests. Neuroimaging examination: computer tomography (CT) or magnetic resonance imaging (MRI) of the brain - allows you to identify the cause of cognitive impairments in the case of certain diseases (cerebrovascular disease, tumor, subdural hematoma, normotensive hydrocephalus, etc.). AD is characterized by atrophic changes, which are most pronounced in the medial parts of the temporal lobes and in the parietal parts of the brain. However, it should be borne in mind that atrophic changes are often observed in healthy elderly and senile people. In recent years, biological markers have been used to diagnose AD, and if available, it is proposed to identify several stages of AD [5].

Table 1. Stages of AD when biological markers of the disease are present

Stage 1	asymptomatic
Stage 2	subjective cognitive decline (SCD)
Stage 3	mild cognitive impairment (MCI)
Stage 4	mild dementia
Stage 5	moderate dementia
Stage 6	severe dementia

Epidemiological studies show that the presence of SCD is associated with the risk of progression of cognitive impairment, the development of MCI, and later dementia [6]. The progression of SCD to MCI during the year averages 6.7%, the progression of SCD to MCI or dementia occurs over an average of 15 years [7]. In patients with SCD, the risk of developing MCI is increased 4.5–fold, and the risk of developing AD is 6.5-fold, compared with people who do not notice cognitive decline [8]. In cases where biological markers of AD are detected in patients with SCD, the risk of progression of SCD to MCI and dementia increases significantly [9]. A study of biological markers of AD shows that changes characteristic of AD are found in 7-40% of patients with SCD [10]. Observation of patients with SCD and the presence of biological markers of AD demonstrates that approximately half of them develop MCI or dementia within 3 years [11]. The identification of biological markers of AD in patients with SCD, according to some authors, allows to regard this condition as an early clinical stage of AD, preceded by an asymptomatic stage of AD [12, 13, 10].

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 impossible 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 [14]. The misfolded protein accumulation leads to a folding disease, and AD is a kind of such disease [15].

A standardized extract of Asparagus officinalis stem (trademarked as Enzyme –treated asparagus extract (ETAS®), Amino Up Co., Ltd., Sapporo, Japan), used in the present study is standardized as containing over 50 μg/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) [16]. One ETAS 320 mg capsule contains: 129 mg of the main substance of asparagus extract and auxiliary components (dexitrin, calcium stearate and crystalline cellulose).Until now many studies have been conducted on neuroprotective and antioxidant effects of ETAS [17-19].

We present our own clinical study that aims to investigate the role of neuroinflammation in Alzheimer's disease and evaluation of the effects of ETAS in the early stages of the AD by a randomized, double-blind, placebo-controlled pilot study that includes the evaluation of HSP70 protein concentrations in blood.

2. Material and methods

2.1 Subjects

The subjects for this study were recruited 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 SCD and MCI (i.e., mild neurocognitive disorder) and inconsistent with dementia (i.e., major neurocognitive disorder) [20, 21]. The patients with moderate and severe AD at the time of recruitment by means of brain MRI, electroencephalography, evoked potentials of the brain, brain blood flow, blood test, and neuropsychological questionnaires 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 breastfeeding 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 is 23 or higher, Frontal Assessment Battery (FAB) score is 14 or higher, and Clock Drawing Test (CDT) score is six or higher) and advanced MCI (MMSE, FAB, or CDT score are less than MCI criteria). 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 is 2018-124.

2.2 Study design

The study was a randomized, double-blind, placebo-controlled clinical study with SCD 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 foods were checked every month to monitor safety and compliance. A treatment period was 12 month - this is a scientifically proven minimum time (the gold standard) to obtain convincing evidence of a long-term safety profile and to determine the long-term cognitive

effects of ETAS, based on deep physiological processes. The study period was from Oct 1, 2018, to Sep 30, 2019. The inspection items were as follows.

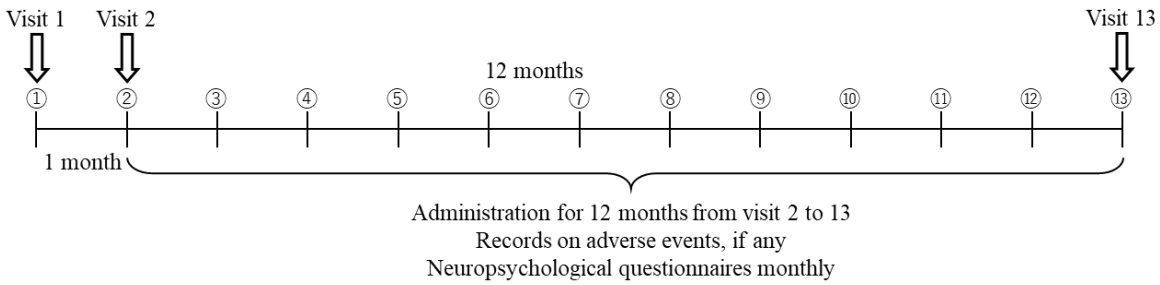

Figure 1. Flow chart of the study design.

Visit 1: Full explanation of all tests to be done, informed consent acquisition and pre-inspection (Blood tests, brain tests, neurological exams, and neuropsychological questionnaires); Visit 2: Inspection (neuropsychological questionnaires) and blood test on HSP70; Visit 3-12: Inspection (neuropsychological questionnaires); Visit 13: Inspection (blood tests, brain tests, and neuropsychological questionnaires).

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

2.3 Neuropsychological questionnaires

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

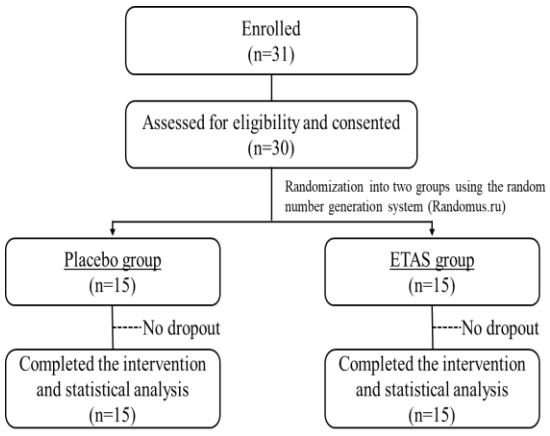

Figure 2. Flow chart of the study design.

Blood and brain examinations

The blood samples of 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), Immunological status (CD4/CD8), Humoral immunity test (IgA, IgM, IgG, CD3, and CD4, circulating immune complex) and HSP70 in blood. 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 increase 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.

3. Statistical analysis

According to the minimum sample size of the pilot test reported in the article [22], 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 [23]. Differences were considered to be statistically significant or tended at*: $p < 0.05$, **: $p < 0.01$, †: $p < 0.1$ vs. Baseline, and #: $p < 0.05$, ##: $p < 0.01$, ‡: $p < 0.1$ vs. Placebo. Results are expressed as the mean ± Standard Error.

4. 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 foods. 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 enrolled in the placebo and ETAS groups were listed in Table 2

Measurement Items	**Baseline**		
	Placebo	**ETAS**	***P* values vs. Placebo**
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
Comparison of age, neuropsychological questionnaires score, CD4/CD8 ratio, and peripheral blood HSP70 levels at Baseline. ± SE.			

Table 2. Characteristics of the subjects. of subjects *: $p < 0.05$, **: $p < 0.01$, †: $p < 0.1$ vs. Baseline. #: $p < 0.05$, ##: $p < 0.01$, ‡: $p < 0.1$ vs. Placebo. ± SE.

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 were shown in Table 3, and the boxplots of Total, MCI, and advanced MCI were shown in Figures 3, 4 and 5.

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). The delta changes between 2 groups: placebo and ETAS, were shown in Figure 5. 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. 3, A and B). The results of HADS Depression and Anxiety in the placebo group showed a significant 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 (Figure 3, D and 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. 3, F). 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. 3, G).

The MMSE, FAB, HADS Depression and Anxiety, CD4/CD8 ratio, and HSP70 in WBC in MCI showed similar changes to comparisons in Total (Fig. 4, A, B, D-F). CDT score improved significantly from 7.1 ± 0.4 to 8.1 ± 0.2, p = 0.03, in the ETAS group (Figure 4, C). 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. 5, A, B and G). The HADS Anxiety of ETAS also showed a trend of improvement (Figure 4, E). 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. 5, F and 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. 6, A, D-F). Besides, ETAS group showed a trend of improvement and increase in FAB, CDT, and HSP70 in WBC (Fig. 6, B, C, G).There were no remarkable changes in the blood and brain examination such as MRI and EPB during the test period (data not shown).Regarding EEG of the brain examination in the ETAS group, 87.5% in 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).

5. Discussion

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 SCD was 11 out of 15 subjects at the baseline and 12 out of 15 subjects at 12 months in the placebo groups. Whereas the subjects considered to have a SCD in the ETAS group were 11 out of 15 subjects at the baseline and all subjects at 12 months. ETAS showed significant improvements in 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. reported that the MMSE score decreases by 1.8 - 4.5 points per year [24]. 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 [25]. The significant improvements in the MMSE score observed in ETAS group suggest that ETAS may improve cognitive functions in SCD 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. Since the FAB assesses frontal lobe function [26, 27], 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 maintain or improve 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 [28, 29]. 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 [30, 31]. The significant improvement of CDT scores in the MCI stage subjects alone 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 impairment 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 [32]. Therefore, significant improvement in HADS score in ETAS group resulted from the beneficial effect on the subject's autonomic nerve.

Recently, several reports have been published on the relationship between immune-response molecules and AD. Larbi A et al. 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 naïve cells, elevated memory cells, and increased proportions of CD4+ but not CD8+ T cells lacking the essential costimulatory receptor CD28 [33]. Moreover, Pellicanò M et al. 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 of CD4+ T cells may result from chronic stimulation by Aβ present in the blood [34]. 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 is increased with the AD symptoms progression 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 of ETAS did not show remarkable elevation of the CD4/CD8 values, and the 8 out of 15 subjects (2 out of 15 in the placebo group) maintained an average level such as 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 the placebo group in the MCI stage subjects. Magrané et al. reported that HSP70 overexpression rescued toxic effects of intracellular Aβ accumulation in the neurons [35]. Also, Hoshino T. et al. reported that HSP70 overexpression in the neurons of Aβ overexpressed AD model mice suppresses cognitive decline effects in behavioral experiments with reducing Aβ expression, Aβ tissue deposition, neuronal degeneration, and synapse reduction [36]. 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 Aβ-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 of peripheral blood of the MCI subjects. The results of this study suggest that ETAS supplementation is well tolerated for elderly subjects and beneficial for neuroprotection and suppression of SCD or MCI subjects through HSP70 induction and maintaining the CD4/CD8 immune complex within a normal level.

6. Conclusion

Our results show a significant role of neuroinflammation and, as a result, increased activation of the immune system that occurs in AD,

which were previously confirmed by various researchers. The most promising biomarker seems to be HSP70 in blood, Immunological status (CD4/CD8), with the highest number of correlations of their plasma levels compared to other biomarkers in the blood. This study shows that taking ETAS prevents cognitive decline, and ETAS is expected to become an effective functional food to alleviate the decline in brain function leading to Alzheimer's disease.

Conflict of interests

Author declared no competing interests.

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Table 3. The change of neuropsychological questionnaires values by ETAS

Items	subgroups	Placebo		*p* values vs. Baseline	ETAS		*p* values vs. Baseline	*p* values 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

Figure 3. Neuropsychological questionnaires score, CD4/CD8 ratio, and peripheral blood HSP70 levels at baseline and 12 months after ETAS supplementation in Total. 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, *: $p < 0.05$, **: $p < 0.01$, †: $p < 0.1$ vs. Baseline.

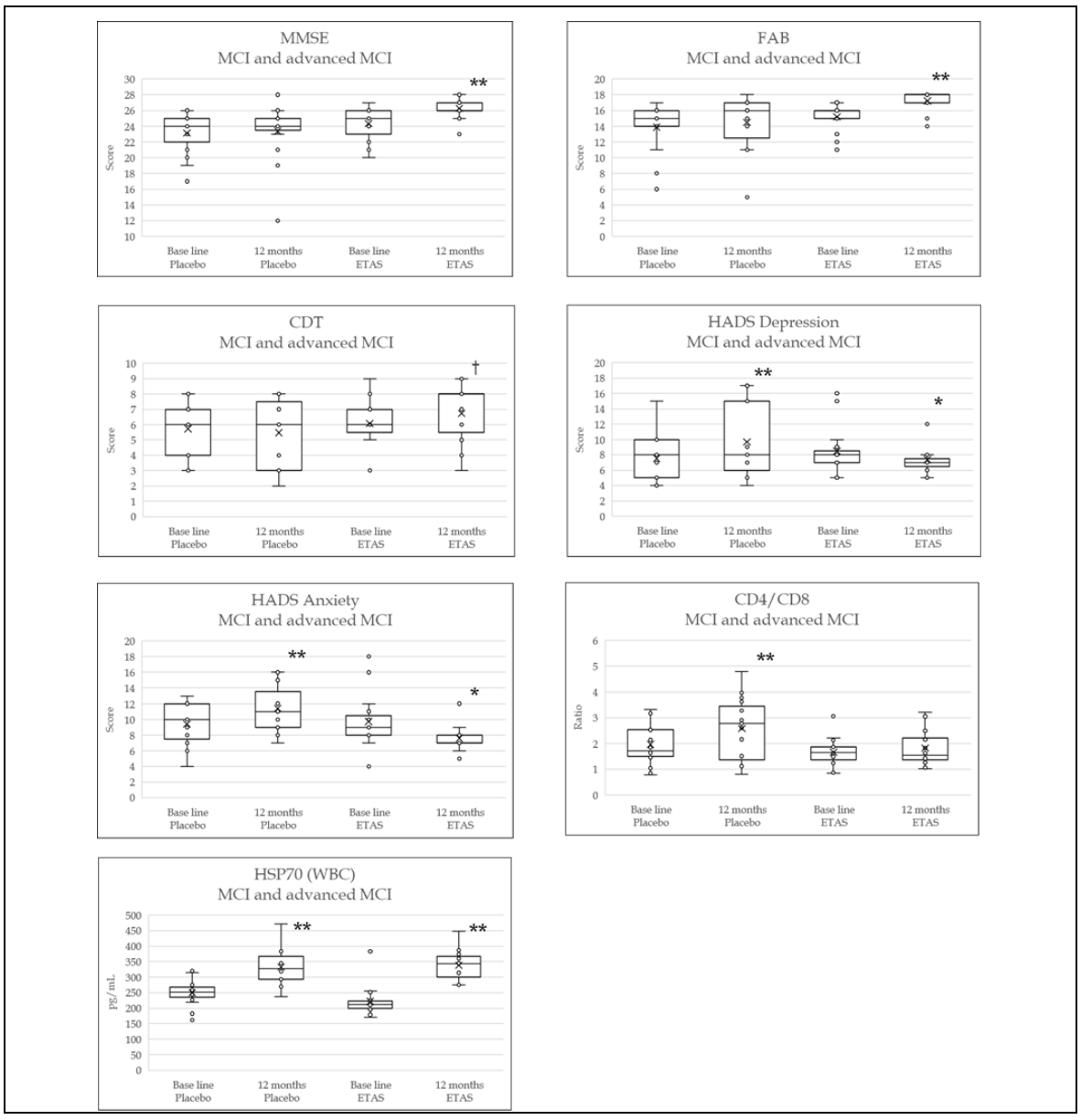

Figure 4. Neuropsychological questionnaires score, CD4/CD8 ratio, and peripheral blood HSP70 levels at baseline and 12 months after ETAS supplementation in MCI. A: MMSE, B: FAB, C: CDT, D: HADS Depression, E: HADS Anxiety, F: CD4/CD8, and G: HSP70 (WBC). The boxes show 75, 50 (median), and 25 percentiles; upper and lower whisker shows maximum and minimum percentiles except for outliers in box plots. Circles: each data, Cross marks: average, *: $p < 0.05$, **: $p < 0.01$ vs. Baseline.

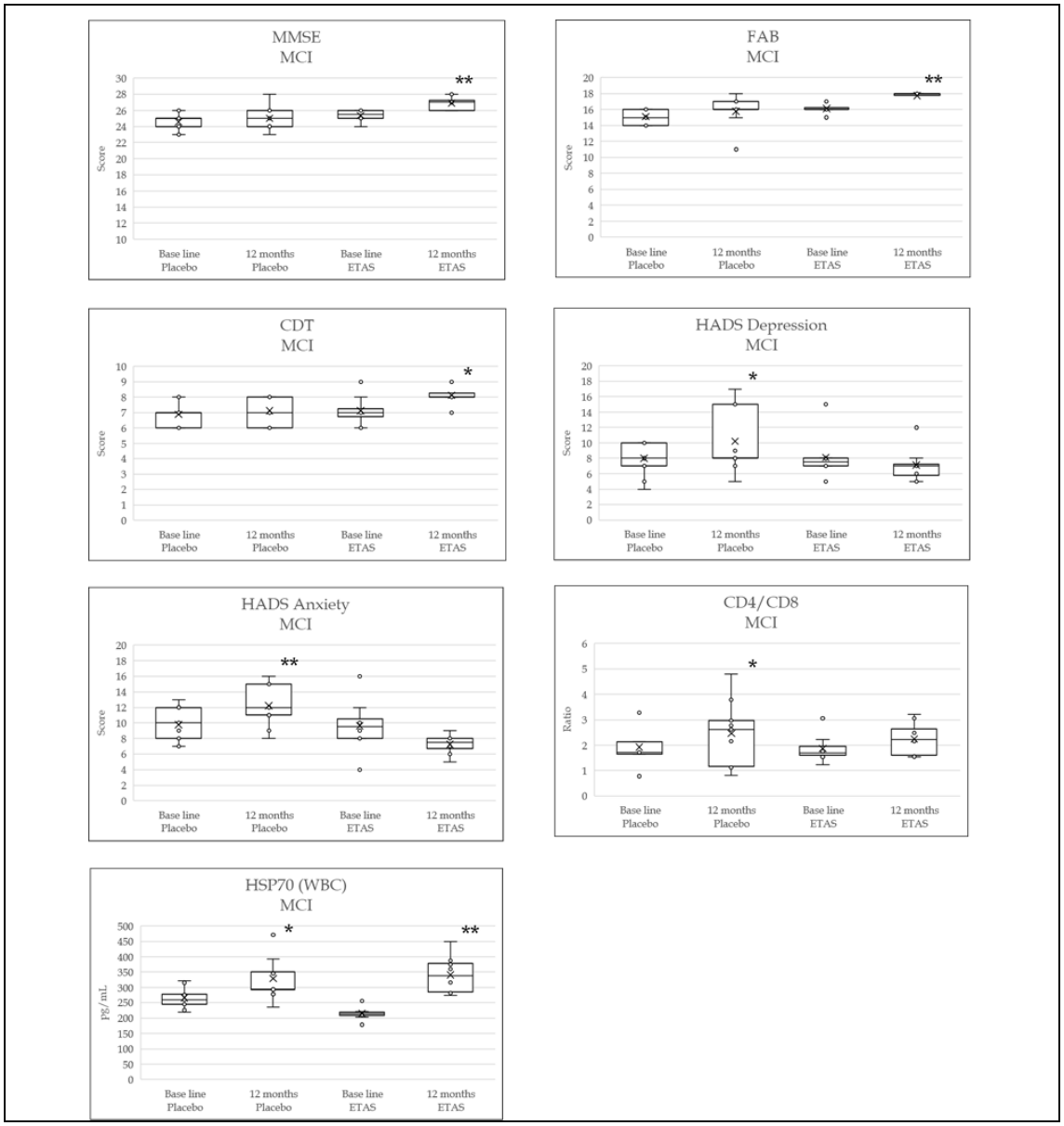

Figure 5. Neuropsychological questionnaires score, CD4/CD8 ratio, and peripheral blood HSP70 levels at baseline and 12 months after ETAS supplementation in advanced MCI. A: MMSE, B: FAB, C: CDT, D: HADS Depression, E: HADS Anxiety, F: CD4/CD8, and G: HSP70 (WBC). The boxes show 75, 50 (median), and 25 percentiles; upper and lower whisker shows maximum and minimum percentiles except for outliers in box plots. Circles: each data, Cross marks: average, *: $p < 0.05$, **: $p < 0.01$, †: $p < 0.1$ vs. Baseline.

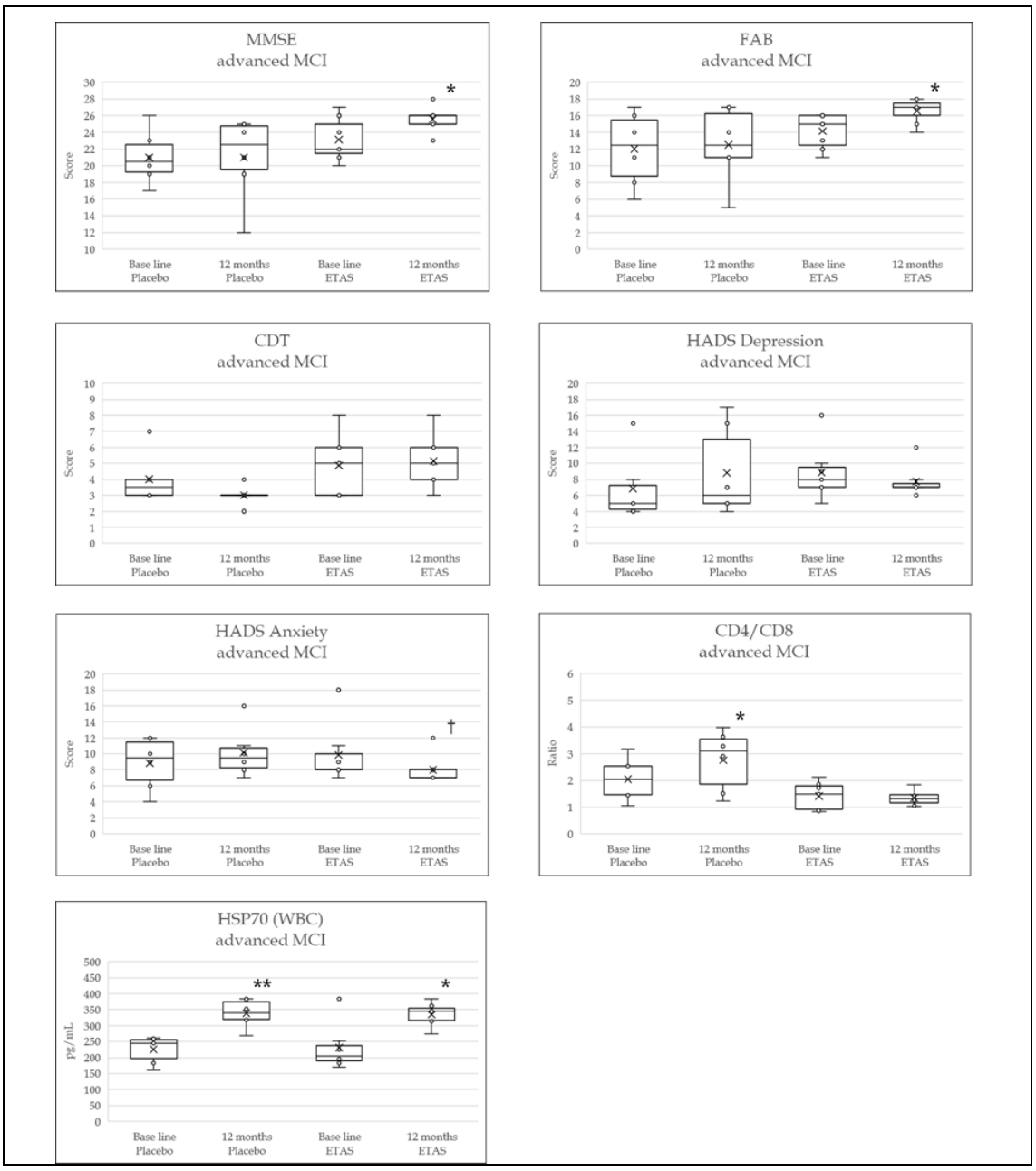

Figure 6. The delta (Δ) change of Neuropsychological questionnaires score, CD4/CD8 ratio, and peripheral blood HSP70 levels at 12 months after ETAS supplementation compared to baseline in each class. A: MMSE, B: FAB, C: CDT, D: HADS Depression, E: HADS Anxiety, F: CD4/CD8, and G: HSP70 (WBC). #: $p < 0.05$, ##: $p < 0.01$, ‡: $p < 0.1$ vs. Placebo for the corresponding class. Error bar: ± SE.