

# Decreased N-Acetyl Aspartate/Myo-Inositol Ratio in the Posterior Cingulate Cortex Shown by Magnetic Resonance Spectroscopy May Be One of the Risk Markers of Preclinical Alzheimer's Disease: A 7-Year Follow-Up Study

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**Abstract.** Although molecular positron emission tomography imaging of amyloid and tau proteins can facilitate the detection of preclinical Alzheimer's disease (AD) pathology, it is not useful in clinical practice. More practical surrogate markers for preclinical AD would provide valuable tools. Thus, we sought to validate the utility of conventional magnetic resonance spectroscopy (MRS) as a screening method for preclinical AD. A total of 289 older participants who were cognitively normal at baseline were clinically followed up for analysis of MRS metabolites, including N-acetyl aspartate (NAA) and myo-inositol (MI) in the posterior cingulate cortex (PCC) for 7 years. The 289 participants were retrospectively divided into five groups 7 years after baseline: 200 (69%) remained cognitively normal; 53 (18%) developed mild cognitive impairment (MCI); 21 (7%) developed AD; eight (2%) developed Parkinson's disease with normal cognition, and seven (2%) developed dementia with Lewy bodies (DLB). The NAA/MI ratios of the PCC in the AD, MCI, and DLB groups were significantly decreased compared with participants who maintained normal cognition from baseline to 7 years after baseline. MMSE scores 7 years after baseline were significantly correlated with MI/Cr and NAA/MI ratios in the PCC. These results suggest that cognitively normal elderly subjects with low NAA/MI ratios in the PCC might be at risk of progression to clinical AD. Thus, the NAA/MI ratio in the PCC measured with conventional <sup>1</sup>H MRS should be reconsidered as a possible adjunctive screening marker of preclinical AD in clinical practice.

**Keywords:** Magnetic resonance spectroscopy, myo-inositol, N-acetyl aspartate, posterior cingulate cortex, preclinical Alzheimer's disease, screening marker

## INTRODUCTION

Accumulating data from large-scale studies, such as the Alzheimer's Disease Neuroimaging Initiative

(ADNI) and the Dominantly Inherited Alzheimer Network (DIAN), and the results of clinical trials of disease modifying drugs, suggest that the pathology of Alzheimer's disease (AD) often begins approximately 20 years before the clinical onset of mild cognitive impairment (MCI) and AD [1, 2]. Thus, treatment with disease-modifying drugs after the onset of AD/MCI may already be too late. Therefore,

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developing methods for screening and preventive intervention in the preclinical stages of AD is a critical issue. Specifically, establishing a system for identifying subjects at risk of prodromal AD could play a valuable role in enabling preventive therapy at the preclinical stages of AD. Cerebrospinal fluid (CSF) biomarkers, including  $A\beta_{40}$ ,  $A\beta_{42}$ , total tau (t-tau), and phosphorylated tau (p-tau) obtained by lumbar puncture have been established as reliable biomarkers reflecting the underlying pathogenesis of AD; however, obtaining these biomarkers is invasive for patients [3–8]. Recently developed neuroimaging techniques, such as molecular positron emission tomography (PET) for visualizing abnormal amyloid and tau deposition in the brain, are powerful methods not only for detecting molecular pathology at asymptomatic stages in AD but also for differential diagnosis of other tauopathies, such as progressive supranuclear palsy, corticobasal syndrome, and frontotemporal dementia [9–15].

However, although amyloid and tau imaging techniques enable direct observation of AD pathogenesis, they are not appropriate methods for easily screening dementia in most hospitals, and have not been widely applied in clinical practice. Ideal screening methods for capturing subjects at risk of early MCI/AD or preclinical AD must be accessible, easy to perform, non-invasive and low-cost. Novel blood-based biomarkers for AD/MCI have been proposed as an alternative method; however, there are currently no verified blood-based biomarkers that are specific to AD/MCI [16–19].

However, standard 1.5-Tesla (T) MRI is popular in clinical settings in Japan, and could be applied for voxel-based morphometry (VBM)-MRI for detection of AD-specific structural brain changes, especially medial temporal atrophy. The utility of VBM-MRI has been confirmed for a range of applications, such as voxel-based specific regional analysis systems for Alzheimer's disease (VSRAD), which enables the detection of hippocampal atrophy [20–25], and proton magnetic resonance spectroscopy ( $^1\text{H}$  MRS) for capturing early brain biochemical abnormalities useful for early diagnosis and tracking subjects with dementia caused by AD [25]. Furthermore, a number of previous studies have demonstrated that detecting metabolite abnormalities with brain  $^1\text{H}$  MRS brain is useful not only for the early differential diagnosis of dementia-related diseases, including MCI and AD, but also for predicting conversion from MCI into AD [25–47].

A previous cross-sectional study in our laboratory demonstrated the utility of VSRAD, combined with  $^1\text{H}$  MRS of posterior cingulate cortex (PCC) relevant CSF biomarkers ( $A\beta_{42}$ , p-tau, and  $A\beta_{42}/\text{p-tau}$ ) as adjunct early screening methods for dementia caused by AD in clinical practice [25]. The results revealed that the peak values of each metabolite of  $^1\text{H}$  MRS, particularly N-acetylaspartate (NAA) and myo-inositol (MI) of the PCC, were altered in MCI/AD subjects compared with normal controls before cognitive decline, and hippocampal atrophy was revealed with VSRAD from the very early stages [25]. In that study, VSRAD incidentally revealed that 118 elderly subjects showed very mild cognitive decline without brain atrophy. Of 118 subjects, 79 (67%) were cognitively normal, 18 (15%) had MCI, and six (5%) had early AD. The NAA/Cr ratio and NAA/MI ratio in the PCC on  $^1\text{H}$  MRS in the 18 MCI and six AD patients was significantly decreased, compared with 79 normal age-matched controls. Thus, we proposed that not only VSRAD but also analysis of  $^1\text{H}$  MRS metabolites in the PCC might be useful for the diagnosis of AD/MCI in clinical practice. Moreover, we speculated that alterations of metabolites of  $^1\text{H}$  MRS in the PCC, which may reflect biochemical alterations caused by AD, could precede the clinical onset and structural alterations in AD/MCI and may be helpful for prediction of preclinical AD. The aim of this 7-year follow-up study was to validate whether  $^1\text{H}$  MRS analysis in the PCC is useful for the prediction of AD progression in cognitively normal, elderly individuals.

## MATERIALS AND METHODS

A sample of 320 healthy and cognitively normal individuals with no organic brain disorders (aged 69–89 years) who voluntarily attended a medical checkup including a brain examination at our hospital was recruited for this study. All study subjects provided informed consent. Individuals with a history of stroke, white matter lesions including periventricular hyperintensity on T2 weighted MRI, cardiac dysfunction, and other systemic diseases including diabetes mellitus and psychiatric diseases were excluded from the study. Thirty-one of the 320 participants withdrew from the study within 5 years from baseline because of complications due to other significant sicknesses or personal circumstances. As a result, 289 subjects were consistently followed up for 7 years, from April 2010

to April 2017. All 289 subjects fulfilled the following inclusion criteria: 1) Absence of memory-related complaints, as verified by a study partner; 2) Mini-Mental State Examination (MMSE) scores between 24 and 30; 3) Clinical Dementia Rating (CDR)=0; 4) Normal cognitive function, based on an absence of significant impairment in cognitive functions or activities of daily living. The exclusion criteria were as follows: any significant neurological disease, such as Parkinson’s disease (PD), multi-infarct dementia, Huntington’s disease, normal pressure hydrocephalus, brain tumor, progressive supranuclear palsy, seizure disorder, subdural hematoma, multiple sclerosis, or history of significant head trauma followed by persistent neurological deficits or known structural brain abnormalities.

At baseline, 289 cognitively normal elderly subjects (mean age: 74.8 ± 5.2 years, age range: 69–82 years, female: 164, male: 125, MMSE: 28.2 ± 1.8, CDR: 0) were selected.

A general medical examination by interview, in addition to counseling, routine blood testing, and electrocardiography, was performed annually.

MMSE, regular MRI, VSRAD, and <sup>1</sup>H MRS of the PCC were performed at baseline, and at 5 and 7 years after baseline as the final observation points of the study. CSF was obtained by lumbar puncture at the final point, 7 years after baseline. The APOE genotype was tested in all subjects using standard procedures, which included obtaining informed consent from all research subjects and donors [48]. None of the subjects was taking acetylcholinesterase inhibitors or memantine at baseline. At 7 years after baseline, the 289 participants were divided into five groups based on neurological status (Fig. 1): *Remained Normal*: Subjects remaining cognitively normal (n = 200, 69.2%); *Progressor MCI*: Subjects who developed MCI (n = 53, 18.3%); *Progressor AD*: Subjects who developed AD (n = 21, 7.3%); *Progressor PD*: Subjects who developed PD without dementia (n = 8, 2.8%); and *Progressor DLB*: Subjects who developed DLB (n = 7, 2.4%). The diagnoses were based on clinical criteria [49–53]. The diagnosis of DLB was confirmed based on the results of <sup>123</sup>I-MIBG myocardial scintigraphy showing that myocardial <sup>123</sup>I-MIBG uptake was impaired

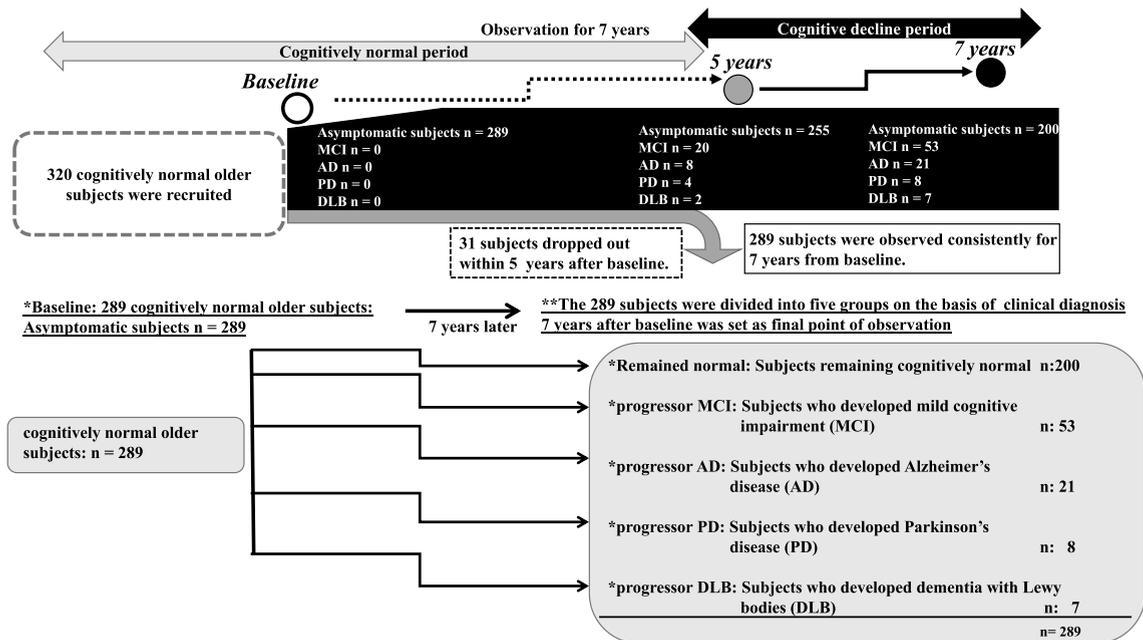


Fig. 1. Outline of the follow up study. An outline of this follow up study is shown in a flowchart. We recruited 320 participants who previously voluntarily visited the memory clinic and were deemed cognitively normal without organic brain disorder, ranging from 69 to 89 years old. Informed consent was obtained from all participants. Of these participants, 31 dropped out within 5 years after baseline. Thus, a total of 289 cognitively normal older participants were consistently followed up for 7 years from April 2010 to April 2017. At 7 years after baseline, the 289 participants were divided into the following five groups on the basis of neurological status: \*Remained normal: Subjects remaining cognitively normal (n = 200); \*Progressor MCI: Subjects who developed MCI 7 years later (n = 53); \*Progressor AD: Subjects who developed AD 7 years later (n = 21); \*Progressor PD: Subjects who developed PD 7 years later (n = 8); and \*Progressor DLB: Subjects who developed DLB 7 years later (n = 7). At 5 years later, there were 20 MCI, eight AD, four PD, and two DLB patients.

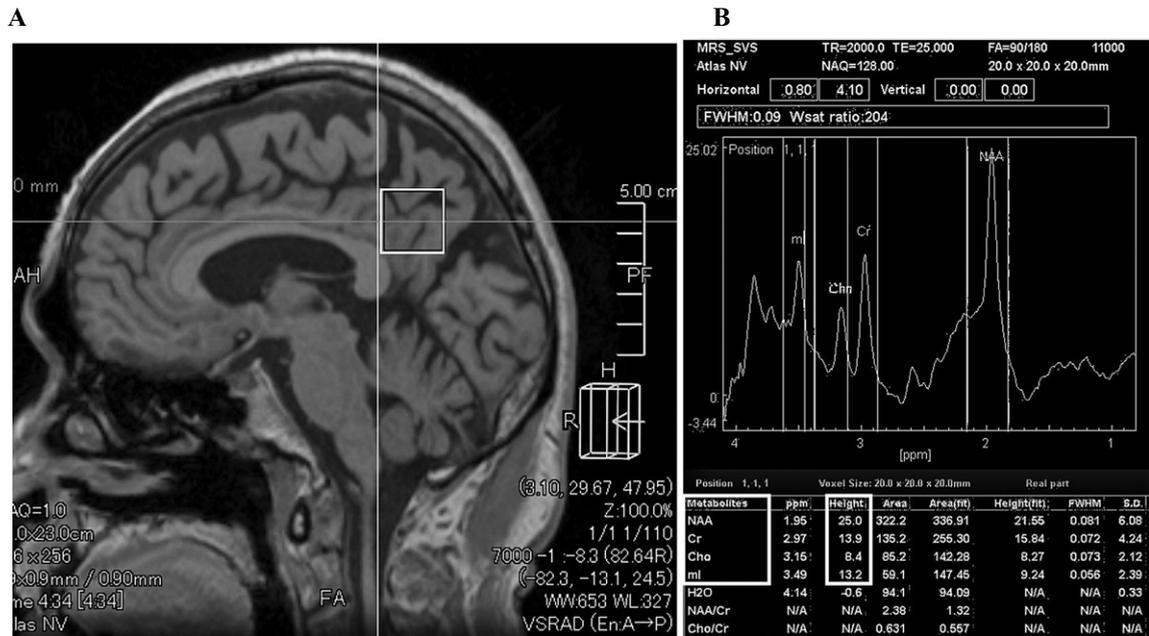


Fig. 2.  $^1\text{H}$  MRS examination. Sagittal and axial MRI images showing the PCC, defined as a volume of interest (VOI) in  $^1\text{H}$  MRS examination (A). Upper picture of (B) shows the analyzed peak of each metabolite. The lower table shows the measured peak height of each metabolite: myo-inositol (MI), choline (cho), creatine (Cr), and N-acetylaspartate (NAA) using  $^1\text{H}$  MRS in the posterior cingulate cortex (PCC). We calculated the NAA/Cr, Cho/Cr, MI/Cr, and NAA/MI ratios, as described in the Materials and Methods.

in early-stage DLB [54]. Moreover, all subjects with AD/MCI, PD and DLB at 7 years after baseline, undertook examinations of CSF  $\text{A}\beta_{1-42}$  and CSF p-tau proteins for accurate diagnosis [3–8].

Informed consent was provided by each participant and a relative. The protocol was approved by the Ethics Committees of the Higashi Matsudo Municipal Hospital. The study was conducted over 7 years, from April 2010 to April 2017.

#### MR and $^1\text{H}$ MRS examination

MRI (T1, T2, and fluid attenuated inversion recovery [FLAIR]) and  $^1\text{H}$  MRS were performed with a 1.5 T MR system (Vantage Titan; Toshiba Medical Systems, Tochigi, Japan) with a 14-channel phased array head coil (Toshiba Medical Systems).

$^1\text{H}$  MRS was performed at baseline and at 5 years and 7 years after baseline, using the vendor-supplied (Toshiba Medical Systems) automated MRS package single-voxel method with a spin echo sequence. A point resolved spectroscopy sequence with TR = 2,000 ms, TE = 25 ms, 2,048 data points, and 128 excitations was used for the examinations. An 8-cm<sup>3</sup> (2 × 2 × 2 cm)  $^1\text{H}$  MRS voxels that mainly included the posterior cingulate and precuneus region

were prescribed on a midsagittal 3D-GRE image, including the right and left PCC (Fig. 2A,B) [26]. The quality control procedure for the  $^1\text{H}$  MRS was as follows: 1) The spectra with full width at half maximum were  $\leq 0.2$  ppm; 2) The flip angle of water suppression pre-pulse for minimizing the water signal in the volume of interest (VOI) was optimized, i.e., the water suppression ratio of the water signal to the residual water signal was set to approximately 80–300.

We selected the PCC as the VOI in  $^1\text{H}$  MRS in this study because previous studies have shown that the PCC is a well-defined midline structure from which reproducible, high-quality, single-voxel spectra can be acquired using regular 1.5-T MRI. In this respect, it differs from the hippocampus, which is anatomically small and sometimes difficult to analyze because of CSF contamination from the inferior horn of the lateral ventricle. The PCC also has significant metabolite measurement reliability and higher sensitivity to early AD pathology compared with other regions of the brain, including the hippocampus [26, 55–58]. The peak height ratios of NAA, choline (Cho), and MI normalized to Cr levels (described as NAA/Cr, Cho/Cr, MI/Cr, and NAA/MI) were evaluated as described previously (Fig. 2B) [26]. Voxel

placement for spectroscopy and all data analysis were carried out by one neurologist and two trained radiologists who were blinded to each subject's diagnosis.

### CSF analysis

CSF analysis was performed in subjects with MCI/AD, PD, and DLB at the endpoint: 7 years from baseline. All CSF samples were obtained by lumbar puncture between 2 P.M. and 3 P.M., and were collected into polypropylene tubes. The CSF samples were centrifuged ( $1,000 \times g$  for 10 min at  $4^\circ\text{C}$ ) to remove any debris, then stored in small aliquots at  $-80^\circ\text{C}$ . CSF  $\text{A}\beta_{1-40}$  and  $\text{A}\beta_{1-42}$  were measured by enzyme-linked immunosorbent assay (ELISA) using a Human Beta Amyloid (1–40) ELISA Kit (Wako, 292-62301; Wako Chemical Co.) and a Human Beta Amyloid (1–42) ELISA Kit (Wako, 298-62401; Wako Chemical Co.), respectively. CSF p-tau protein levels were measured using INNOTEST<sup>®</sup> PHOSPHO-TAU (181P; Innogenetics, Belgium).

### Statistics

Statistical calculations were performed using GraphPad Prism<sup>®</sup> software (GraphPad Software). Values are presented as the mean  $\pm$  standard deviation (SD) and the median  $\pm$  interquartile deviation. Groups were compared using  $\chi^2$  tests (two groups) or Kruskal–Wallis tests (three and four groups). Correlations between each factor were examined using Spearman's rank correlation analyses. A linear model was applied to the data to obtain the correlation coefficients (rs) and *p*-values.

## RESULTS

### *NAA/Cr, MI/Cr, and NAA/MI in the PCC at baseline, and 5 and 7 years from baseline (i.e., the end of follow-up)*

The NAA/Cr ratio at baseline was already lower in subjects with progressor MCI who developed MCI 7 years later ( $p=0.01$ ) and in subjects with progressor AD ( $p<0.0001$ ) who developed AD 7 years later compared with subjects who remained cognitively normal (Fig. 3A). The NAA/Cr ratio was lower in subjects with MCI and AD compared with subjects who remained cognitively normal throughout the observation period. Seven years after baseline,

the NAA/Cr ratio was significantly lower in AD and DLB subjects compared with subjects who remained cognitively normal, MCI subjects, and PD subjects (Table 1A and Fig. 3A). The MI/Cr ratio in subjects with progressor AD and DLB was already significantly higher compared with subjects who remained cognitively normal ( $p=0.046$ ) at baseline. In subjects with progressor AD and DLB, the MI/Cr ratios at 5- and 7-year follow-up were higher than at baseline (Table 1B and Fig. 3B). The NAA/MI ratio was significantly lower in subjects with MCI ( $p<0.0001$ ), AD ( $p<0.0001$ ), and DLB ( $p<0.0001$ ) compared with subjects who remained cognitively normal (Table 1C and Fig. 3C). Importantly, the NAA/MI ratio of progressor AD and DLB patients was already significantly decreased at baseline compared with the progressor MCI ( $p=0.022$ ) and progressor PD ( $p=0.012$ ) groups, and subjects who remained cognitively normal ( $p<0.0001$ ) (Fig. 3C).

Mean values  $\pm$  SD for each parameter are presented in Table 1.

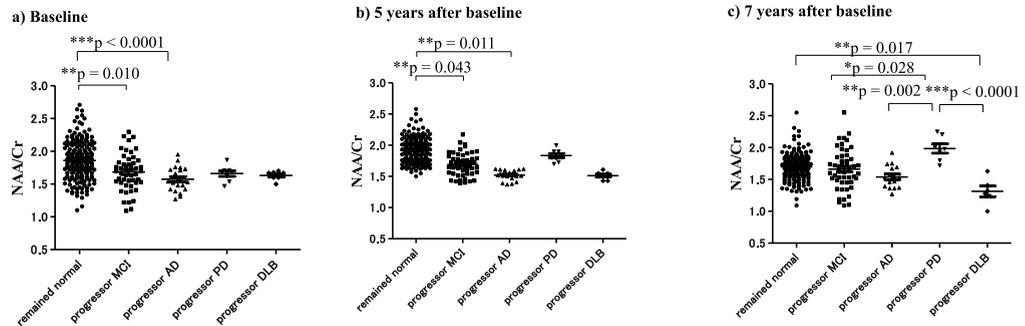
### *ROC analysis of the NAA/MI ratio distinguishing subjects with progressor AD from those who remained cognitively normal*

ROC analysis revealed that the NAA/MI ratio in the PCC at baseline could differentiate subjects who developed AD 7 years later from subjects who remained cognitively normal, with an area under the curve (AUC) of 0.95, sensitivity 90.5%, and specificity of 90.3% at a cut-off value of  $<1.67$  (Fig. 4A). In contrast, the NAA/MI ratio that distinguished subjects with progressor MCI from those who remained cognitively normal had an AUC of 0.78 with a sensitivity of 67.9% and a specificity of 67.9% at a cut-off value of  $<1.87$  (Fig. 4B). The results of the ROC analysis revealed that cognitively normal subjects whose NAA/MI ratio in the PCC was  $<1.67$  may be likely to develop AD in the future. Importantly, our follow up results suggest that a low NAA/MI ratio ( $<1.67$ ) in the PCC may be a risk factor for the future onset of AD.

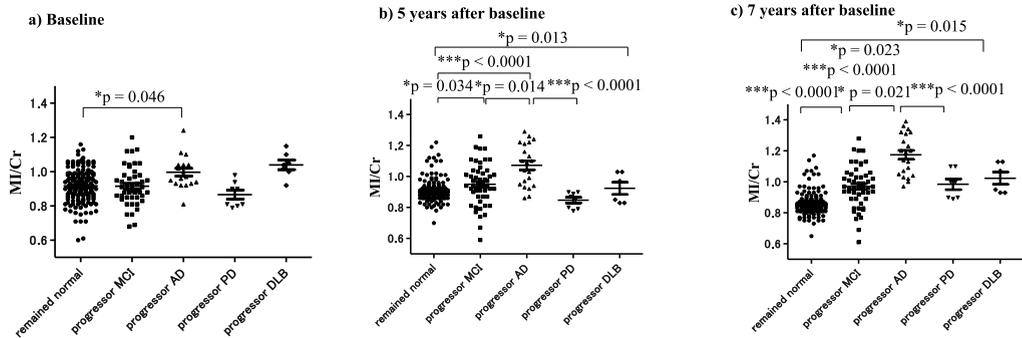
### *MMSE score*

Subjects showed no apparent changes in cognition based on MMSE scores until 5 years after baseline. Declining MMSE scores were found in subjects with progressor MCI:  $27.8 \pm 1.3$  (28.0) ( $***p<0.0001$ ), AD:  $27.6 \pm 0.9$  (28.0) ( $***p<0.0001$ ), and DLB:  $25.1 \pm 0.7$  (25.0) ( $***p<0.0001$ ) compared

## A) NAA/Cr



## B) MI/Cr



## C) NAA/MI

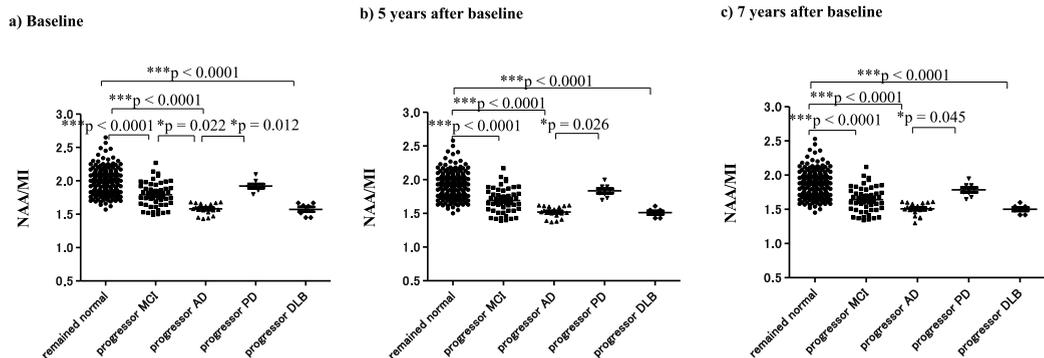


Fig. 3. Metabolites of  $^1\text{H}$  MRS at baseline, and 5 and 7 years later in each group. The NAA/Cr ratio was significantly decreased in the progressor MCI and the progressor AD groups compared with subjects who remained normal and those who exhibited progression of PD from baseline. The NAA/Cr ratio became significantly decreased in the DLB group compared with subjects who remained normal and in the progressor PD group 7 years later. The MI/Cr ratio at baseline was significantly increased in the progressor AD group compared with subjects who remained normal ( $*p = 0.046$ ) (A). The MI/Cr ratio increased in the progressor MCI, AD, and DLB groups compared with subjects who remained normal after 5 years. Moreover, the MI/Cr ratio in the progressor AD group was significantly elevated compared with the progressor MCI group 5 years after baseline (B). The NAA/MI ratio showed a significant decline in the progressor MCI, AD, and DLB groups compared with subjects who remained normal and those in the progressor PD group from baseline before the onset of each disease. Thus, the NAA/MI ratio could differentiate the progressor AD group from the progressor MCI group at baseline. A decreased NAA/MI ratio in the progressor MCI, AD, and DLB groups was found at all three data collection points: baseline, 5, and 7 years after baseline (C).

with subjects who remained cognitively normal:  $29.3 \pm 0.8$  (29.0) at 5 years after baseline. A significant decrease in MMSE scores was found in the MCI:  $26.7 \pm 1.5$  (27.0) ( $***p < 0.0001$ ), AD:  $24.3 \pm 1.1$  (24.0) ( $***p < 0.0001$ ) and DLB:  $24.9 \pm 1.3$  (25.0) ( $***p < 0.0001$ ) groups compared with subjects who remained cognitively normal:  $29.3 \pm 0.8$  (29.0) at

the final point (7 years from baseline) (Table 2 and Fig. 5A).

#### Hippocampal atrophy: VSRAD: Z-score

The VSRAD results indicated no apparent hippocampal atrophy during the asymptomatic stage

Table 1a  
NAA/Cr at baseline, and 5 and 7 years after baseline

MRS NAA/Cr	Baseline	5 years later	7 years later (final point)
Remained cognitively normal	1.86 (average) $\pm$ 0.29 (SD) (median: 1.86)	1.75 $\pm$ 0.22 (1.76)	1.70 $\pm$ 0.20 (1.71)
Progressor MCI	1.68 $\pm$ 0.28 (1.70)	1.60 $\pm$ 0.31 (1.60)	1.66 $\pm$ 0.31 (1.66)
Progressor AD	1.57 $\pm$ 0.18 (1.54)	1.51 $\pm$ 0.12 (1.55)	1.54 $\pm$ 0.18 (1.53)
Progressor PD	1.66 $\pm$ 0.12 (1.69)	1.69 $\pm$ 0.12 (1.70)	1.69 $\pm$ 0.20 (1.68)
Progressor DLB	1.63 $\pm$ 0.07 (1.67)	1.41 $\pm$ 0.58 (1.62)	1.31 $\pm$ 0.21 (1.30)

Table 1b  
MI/Cr at baseline, and 5 and 7 years after baseline

MRS MI/Cr	Baseline	5 years later	7 years later (final point)
Remained cognitively normal	0.92 (average) $\pm$ 0.09 (SD) (median: 0.91)	0.91 $\pm$ 0.07 (0.90)	0.86 $\pm$ 0.07 (0.85)
Progressor MCI	0.92 $\pm$ 0.11 (0.90)	0.95 $\pm$ 0.13 (0.94)	0.97 $\pm$ 0.13 (0.97)
Progressor AD	1.00 $\pm$ 0.09 (1.00)	1.07 $\pm$ 0.14 (1.09)	1.17 $\pm$ 0.13 (1.09)
Progressor PD	0.87 $\pm$ 0.07 (0.86)	0.85 $\pm$ 0.05 (0.86)	0.98 $\pm$ 0.09 (1.00)
Progressor DLB	1.04 $\pm$ 0.07 (1.04)	0.92 $\pm$ 0.10 (0.91)	1.02 $\pm$ 0.10 (1.01)

Table 1c  
NAA/MI at baseline, and 5 and 7 years after baseline

MRS NAA/MI	Baseline	5 years later	7 years later (final point)
Remained cognitively normal	2.00 $\pm$ 0.19 (mean $\pm$ SD) (median: 2.00)	1.93 $\pm$ 0.19 (1.93)	1.88 $\pm$ 0.19 (1.88)
Progressor MCI	1.79 $\pm$ 0.18 (1.77)	1.69 $\pm$ 0.18 (1.67)	1.64 $\pm$ 0.18 (1.62)
Progressor AD	1.58 $\pm$ 0.08 (1.58)	1.52 $\pm$ 0.08 (1.52)	1.51 $\pm$ 0.09 (1.51)
Progressor PD	1.92 $\pm$ 0.09 (1.90)	1.83 $\pm$ 0.10 (1.83)	1.78 $\pm$ 0.10 (1.78)
Progressor DLB	1.57 $\pm$ 0.10 (1.62)	1.51 $\pm$ 0.07 (1.51)	1.50 $\pm$ 0.07 (1.50)

until 5 years after baseline (Fig. 5B). However, VSRAD indicated significant hippocampal atrophy in subjects with progressor AD:  $1.76 \pm 0.11$  (1.72) (\*\* $p=0.009$ ), MCI:  $1.67 \pm 0.20$  (1.76) (\* $p=0.02$ ), and DLB:  $1.90 \pm 0.04$  (1.89) (\*\* $p<0.0001$ ) compared with subjects who remained cognitively normal:  $1.51 \pm 0.22$  (1.56) at 5 years after baseline (Fig. 5B, C). At 7 years after baseline, VSRAD Z-scores clearly showed hippocampal atrophy in MCI:  $1.76 \pm 0.08$  (1.79) (\*\* $p=0.001$ ), AD:  $2.03 \pm 0.08$  (2.00) (\*\* $p<0.0001$ ) and DLB:  $1.95 \pm 0.04$  (1.96) (\*\* $p<0.0001$ ) compared with subjects who remained cognitively normal:  $1.64 \pm 0.22$  (1.69).

In addition, VSRAD Z-scores showed significant differences among subjects with MCI, AD, PD:  $1.70 \pm 0.15$  (1.70), and DLB, as follows: AD > MCI (\*\* $p<0.0001$ ), AD > PD (\*\* $p<0.0001$ ), and DLB > PD (\*\* $p<0.0001$ ) (Table 3 and Fig. 5B).

*Serial changes of MMSE scores, Z-scores (VSRAD), and NAA/MI ratio on  $^1H$  MRS at baseline, 5 years, and 7 years after baseline in each group*

The change in each parameter (MMSE score, hippocampal atrophy shown by the Z-score of the

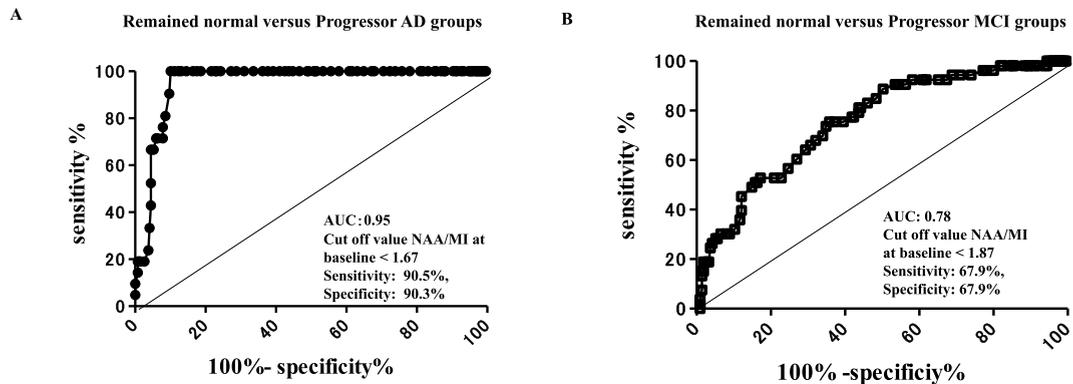


Fig. 4. ROC analysis of  $^1\text{H}$  MRS in the PCC at baseline. ROC analysis of the  $^1\text{H}$  MRS in the PCC at baseline, the ratio of NAA/MI showed an AUC of 0.95, sensitivity of 90.5%, and specificity of 90.3% at the cut-off value of  $<1.67$  in the remained normal versus progressor AD groups (A), and an AUC of 0.78, sensitivity of 67.9%, and specificity of 67.9% at the cut-off value of  $<1.87$  in the remained normal versus progressor MCI groups. The ROC analysis indicated that cognitively normal subjects with NAA/MI ratio values  $<1.67$  in the PCC may be at risk of AD in the future.

Table 2  
MMSE scores at baseline, and 5 and 7 years after baseline

MMSE score	Baseline	5 years later	7 years later (end point)
Remained cognitively normal	29.3 (average) $\pm$ 0.7 (SD) (median: 29.0)	29.3 $\pm$ 0.8 (29.0)	29.3 $\pm$ 0.8 (29.0)
Progressor MCI	29.1 $\pm$ 0.8 (29.0)	27.8 $\pm$ 1.3 (28.0)	26.7 $\pm$ 1.5 (27.0)
Progressor AD	29.1 $\pm$ 0.9 (29.0)	27.6 $\pm$ 0.9 (28.0)	24.3 $\pm$ 1.1 (24.0)
Progressor PD	29.8 $\pm$ 0.5 (30.0)	29.5 $\pm$ 0.8 (30.0)	29.6 $\pm$ 0.7 (30.0)
Progressor DLB	29.0 $\pm$ 0.8 (30.0)	25.1 $\pm$ 0.7 (25.0)	24.9 $\pm$ 1.3 (25.0)

VSRAD, and the NAA/MI ratio in the PCC) in each group was analyzed at three points: baseline, 5 years, and 7 years (final point) after baseline.

The results showed that MMSE scores at 7 years after baseline were significantly lower compared with those at 5 years and at baseline in subjects with progressor MCI and AD (Fig. 6A). Significantly increased VSRAD Z-scores at 7 and 5 years after baseline indicated that hippocampal atrophy was also increased in these groups. The NAA/MI ratio indicated that values decreased from baseline in subjects with MCI, subjects with progressor AD, and those with DLB. Although the NAA/MI ratio in subjects with AD, PD, and DLB clearly tended to decrease, the analysis did not reveal any significant changes in these groups. Instead, the NAA/MI ratio showed a significant decrease at 5 and 7 years after baseline in subjects who remained cognitively normal and at 7 years after baseline compared with baseline (Fig. 6A, B).

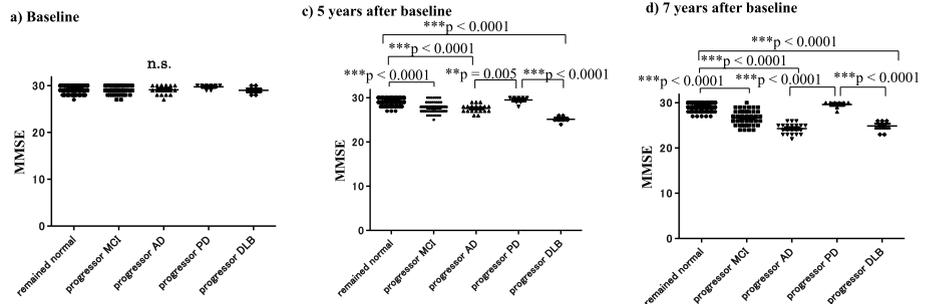
*CSF  $\text{A}\beta_{42}$  and CSF-p-tau obtained at the final point (7 years after baseline) in each group*

The CSF  $\text{A}\beta_{42}$  levels of subjects in the AD group and the MCI group were significantly lower compared with subjects who remained cognitively normal ( $***p < 0.0001$ ) (Fig. 7A). The CSF-p-tau levels of the AD subjects were significantly higher compared with subjects who remained cognitively normal, and compared with the other neurological disease groups. The levels of CSF p-tau in subjects with AD were significantly higher compared with the MCI ( $**p = 0.0071$ ) and DLB ( $*p = 0.0484$ ; Fig. 7B) groups. ROC analysis revealed that the level of CSF  $\text{A}\beta_{42}$  7 years after baseline enabled differentiation of subjects with AD from those who remained cognitively normal with an AUC of 0.91, sensitivity of 77.8%, and specificity of 86.9% at a cut-off value  $<621.2$  pg/ml. The level of CSF- $\text{A}\beta_{42}$  differentiated subjects with MCI from those

Table 3  
Z-scores (VSRAD) at baseline, and 5 and 7 years after baseline

Z-score (VSRAD)	Baseline	5 years later	7 years later (end point)
Remained cognitively normal	1.51 ± 0.22 (average ± SD) (median: 1.56)	1.61 ± 0.17 (1.62)	1.64 ± 0.22 (1.69)
Progressor MCI	1.58 ± 0.20 (1.69)	1.67 ± 0.20 (1.76)	1.76 ± 0.08 (1.79)
Progressor AD	1.58 ± 0.09 (1.53)	1.76 ± 0.11 (1.72)	2.03 ± 0.08 (2.00)
Progressor PD	1.41 ± 0.11 (1.40)	1.77 ± 0.07 (1.76)	1.70 ± 0.15 (1.70)
Progressor DLB	1.69 ± 0.05 (1.66)	1.90 ± 0.04 (1.89)	1.95 ± 0.04 (1.96)

## A) MMSE score



## B) Hippocampal atrophy: Z-score (VSRAD)

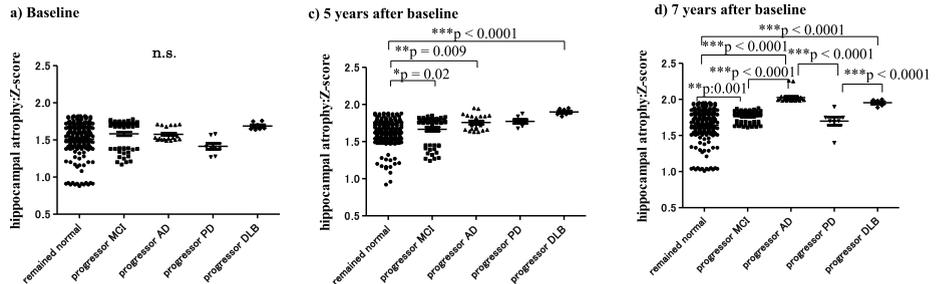


Fig. 5. MMSE scores and hippocampal atrophy at baseline, 5 years and 7 years after baseline. Decline of MMSE scores was not clear until 1 year prior to the 5 years after baseline. However, the decline of the MMSE in the progressor MCI, AD, and DLB groups was apparent compared with subjects who remained cognitively normal 5 years later (A). Hippocampal atrophy shown by VSRAD Z-scores became apparent in subjects with progressor MCI, AD, and DLB 5 years after baseline. At 7 years from baseline, hippocampal atrophy on VSRAD was significant in the progressor MCI, AD and DLB groups, compared with subjects who remained cognitively normal ( $***p < 0.0001$ ) (B).

who remained cognitively normal with an AUC of 0.86, sensitivity of 79.4%, and specificity of 82.6% at a cut-off value  $<692.3$  pg/ml. The CSF-p tau levels were able to differentiate subjects with AD from those who remained cognitively normal with an AUC of 0.97, sensitivity of 94.4%, and specificity of 91.3% at a cut-off value  $>57.9$  pg/ml. The CSF-p tau levels could also differentiate subjects with MCI from those who remained cognitively normal with an AUC of 0.79, sensitivity of 79.4%, and specificity of 82.6% at a cut-off value  $>41.7$  pg/ml (Fig. 7C). The results of the CSF- $A\beta_{42}$  and CSF p-tau analyses 7 years after baseline revealed

that subjects in the AD group showed AD-specific biomarker changes.

#### Correlation between $^1H$ MRS metabolites and MMSE and CSF biomarkers at the final point, 7 years after baseline

Correlation analyses between MMSE scores and  $^1H$  MRS metabolites (NAA/Cr, MI/Cr and NAA/MI) were conducted 7 years after baseline. MMSE scores exhibited a significant negative correlation with the MI/Cr ratio (Pearson  $r: -0.459$ ,  $***p < 0.0001$ ), a significant positive correlation with the NAA/MI ratio

Figure 6

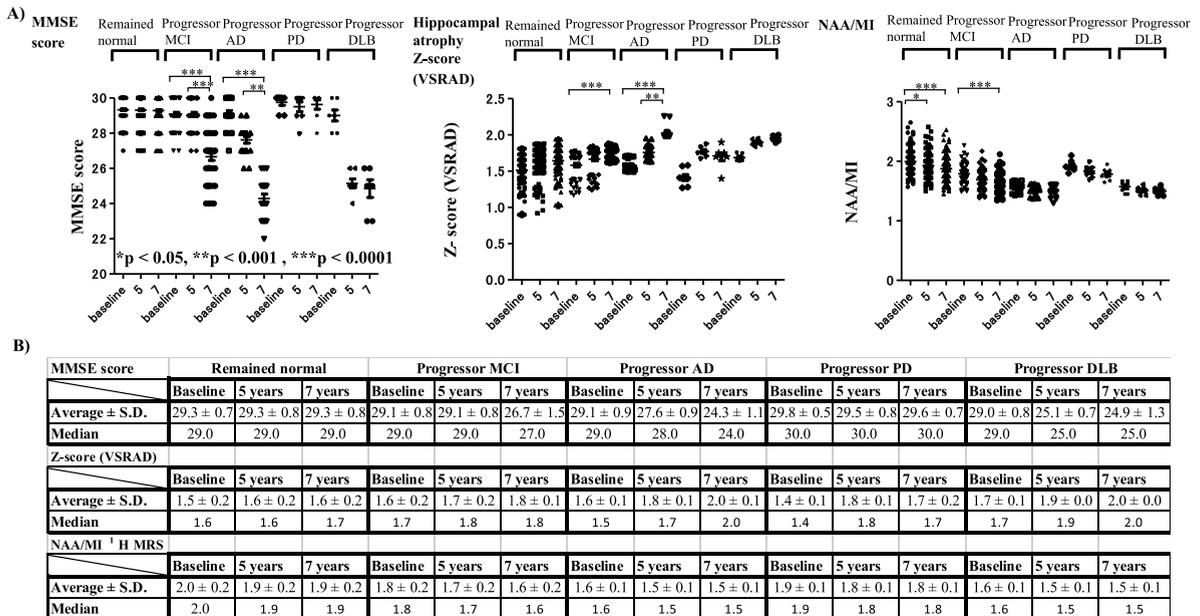


Fig. 6. Serial changes in MMSE scores, Z-scores (VSRAD) and NAA/MI ratio on <sup>1</sup>H MRS at baseline, 5 years, and 7 years after baseline in each group. Serial changes at baseline, 5 years, and 7 years later are shown in the Figure and Table. MMSE scores 7 years after baseline decreased significantly compared with 5 years after baseline in the progressor MCI and AD groups (A). The hippocampal atrophy shown by VSRAD Z-scores revealed significantly increased Z-scores at 7 years and 5 years compared with baseline. In the progressor MCI, AD and DLB groups, it is noted that NAA/MI ratio was already decreased from baseline. Although the NAA/MI ratio in the AD, PD, and DLB groups tended to decrease progressively, the results did not reveal significant serial changes in these groups. Instead, the NAA/MI ratio showed a significant decrease at 7 and 5 years after baseline in subjects who remained cognitively normal, and in the progressor MCI group 7 years after baseline compared with baseline (A, B).

(Pearson r: -0.459, \*\*\**p* < 0.0001), but no correlation with the NAA/Cr ratio (Fig. 8). Regarding the relationship between CSF- Aβ<sub>42</sub>, CSF-p-tau, and NAA/MI ratio, we examined the correlation between NAA/MI and NAA/MI in each subject 7 years after baseline. The results did not find any significant statistical correlation between the NAA/MI ratio and the levels of CSF-Aβ<sub>42</sub> and CSF-p-tau.

*Effect of apolipoprotein E ε4 (APOE4 allele) and NAA/Cr, MI/Cr, and NAA/MI ratio on <sup>1</sup>H MRS at baseline*

The MI/Cr ratio at baseline was significantly higher in subjects with two copies of the ε4 allele (1.01 ± 0.12 [1.00]) compared with subjects with no ε4 allele (0.95 ± 0.14 [0.95], *p* = 0.045). The NAA/MI ratio at baseline was significantly decreased in subjects with two copies of the ε4 allele 1.63 ± 0.27 (1.60) compared with subjects with one copy of the ε4 allele 1.73 ± 0.22. (1.73) (\*\**p* = 0.004)

and subjects without the ε4 allele: 1.73 ± 0.27 (1.70), (\*\**p* = 0.006). The NAA/Cr ratio showed no significant difference between subjects with and without the ε4 allele (Fig. 9).

**DISCUSSION**

We followed 289 cognitively normal subjects without neurological deficits or brain atrophy at baseline. These subjects underwent long-term observation of neurological conditions, assessment of cognitive status using MMSE scores, and analysis of metabolites of <sup>1</sup>H MRS in the PCC with MRI over 7 years. Of the 289 participants, 200 (69%) remained cognitively normal (“remained normal”); 53 (18.3 %) developed MCI (“progressor MCI”); 21 (7.3 %) developed AD (“progressor AD”); eight (2.8%) developed PD (“progressor PD”); and seven (2.4%) developed DLB (“progressor DLB”) within 7 years from baseline. The results revealed that the NAA/Cr ratio was significantly decreased in the progressor MCI group, the AD group, and the DLB group compared with

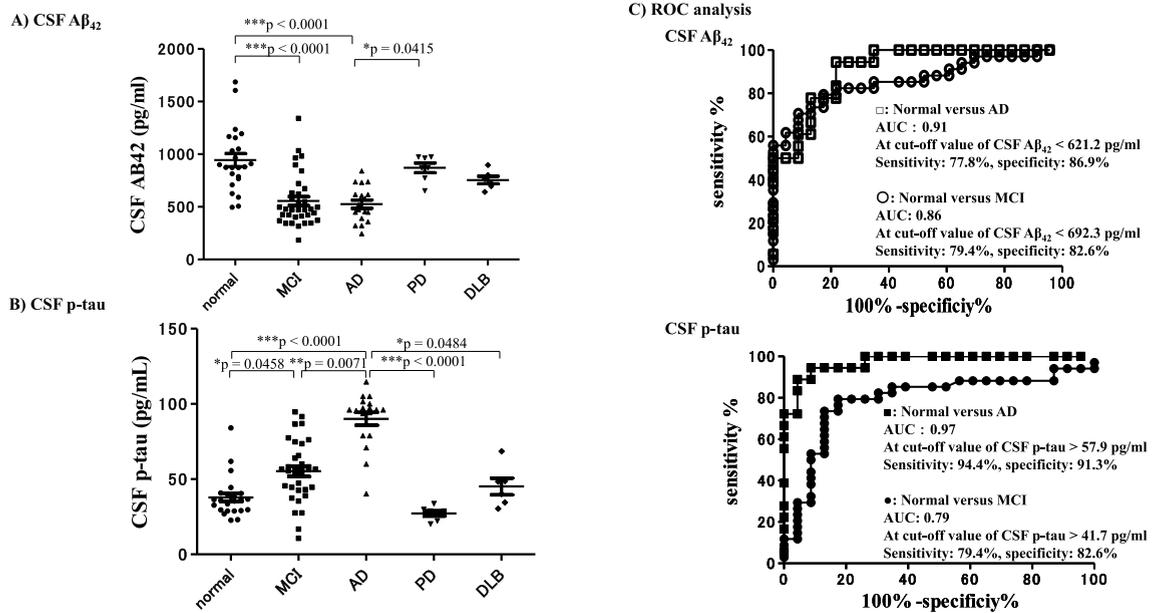


Fig. 7. CSF-Aβ<sub>42</sub> and CSF-p-tau 7 years after baseline. CSF-Aβ<sub>42</sub> and CSF-p-tau measured at the final point (7 years after baseline) in each group. CSF-Aβ<sub>42</sub> levels of subjects in the AD and MCI groups were significantly lower compared with subjects who remained cognitively normal (A). CSF-p-tau levels were significantly higher in the AD group compared with subjects who remained cognitively normal and with the other neurological disease groups. CSF p-tau levels in subjects with AD were significantly higher than those in subjects with MCI and subjects with DLB (B). ROC analysis revealed that the level of CSF-Aβ<sub>42</sub> 7 years after baseline could differentiate subjects with AD from those who remained cognitively normal with an AUC of 0.91, sensitivity of 77.8%, and specificity of 86.9%, with a cut-off value of <621.2 pg/ml. The level of CSF-Aβ<sub>42</sub> differentiated subjects with MCI from those who remained cognitively normal with an AUC of 0.86, sensitivity of 79.4%, and specificity of 82.6% with a cut-off value of <692.3 pg/ml. CSF p-tau levels could differentiate AD subjects from those who remained cognitively normal with an AUC of 0.97, sensitivity of 94.4%, and specificity of 91.3% with a cut-off value of >57.9 pg/ml, and differentiate MCI subjects from normal subjects with an AUC of 0.79, sensitivity of 79.4%, and specificity of 82.6% with a cut-off value of >41.7 pg/ml (C). Thus, the CSF-Aβ<sub>42</sub> and CSF p-tau results revealed that subjects in the AD group exhibited AD-specific biomarker changes.

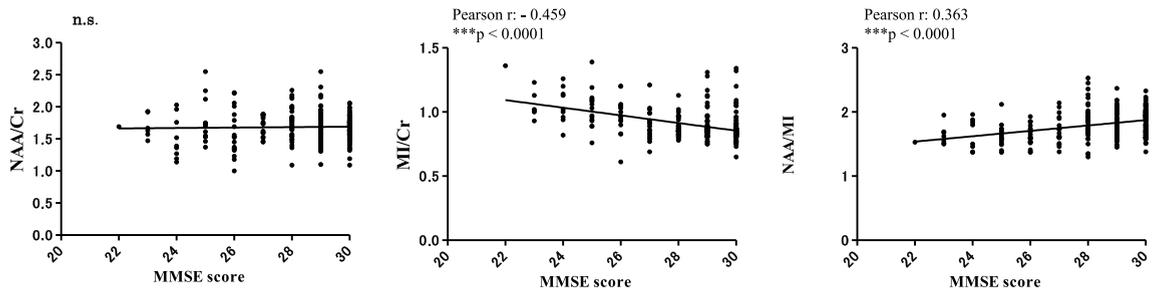


Fig. 8. Correlations between MRS metabolites (NAA/Cr, MI/Cr and NAA/MI) and MMSE scores 7 years after baseline. There were no significant correlations between MMSE scores and the NAA/Cr ratio. The MI/Cr ratio was negatively correlated with MMSE scores (Pearson r: -0.459, \*\*\*p < 0.0001). The NAA/MI ratio was positively correlated with MMSE scores (Pearson r: 0.363, \*\*\*p < 0.0001). The results did not reveal a significant correlation between the NAA/MI ratio and CSF-Aβ<sub>42</sub> or CSF-p-tau levels at 7 years from baseline (data not shown).

baseline (Table 1A and Fig. 3A). The MI/Cr ratio was significantly elevated in the progressor AD and DLB groups compared with baseline (Table 1B and Fig. 3B). The NAA/MI ratio in the progressor AD and DLB groups was significantly lower (approximately <1.7) compared with subjects who

remained cognitively normal from the asymptomatic baseline (approximately >1.8) (Table 1C and Fig. 3C). The results of the ROC analysis of cognitively normal compared with AD subjects indicated that cognitively normal older subjects with an NAA/MI ratio lower than 1.67 in the PCC may

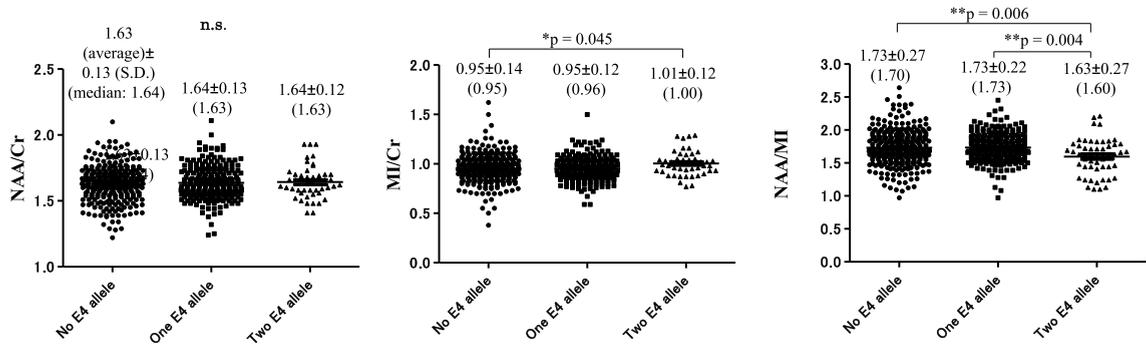


Fig. 9. Number of apolipoprotein E  $\epsilon 4$  alleles and NAA/Cr, MI/Cr, and NAA/MI ratio at baseline. There was no significant difference between subjects with the  $\epsilon 4$  allele and subjects without the  $\epsilon 4$  allele in NAA/Cr ratio. MI/Cr ratio at baseline was significantly increased in subjects with two copies of the  $\epsilon 4$  allele:  $1.01 \pm 0.12$  (1.00) compared with subjects without the  $\epsilon 4$  allele:  $0.95 \pm 0.14$  (0.95) ( $*p = 0.045$ ). The NAA/MI ratio at baseline was significantly decreased in subjects with two copies of the  $\epsilon 4$  allele  $1.63 \pm 0.27$  (1.60) compared with subjects with one copy of the  $\epsilon 4$  allele  $1.73 \pm 0.22$  (1.73) ( $**p = 0.004$ ) and subjects without the  $\epsilon 4$  allele:  $1.73 \pm 0.27$  (1.70), ( $**p = 0.006$ ).

be at risk of progressing to AD (progressor AD) within 7 years. The ROC analysis revealed that this measure had a sensitivity of 90.5%, and specificity of 90.3% (Fig. 4A). In contrast, the ROC analysis of the NAA/MI ratio revealed that the measure was relatively inaccurate for distinguishing the progressor MCI group from subjects who remained asymptomatic. However, it is possible that the asymptomatic subjects whose NAA/MI ratios were more or less than 1.7 may have been at risk of progressor MCI (Table 1C and Fig. 4B). MMSE scores and hippocampal atrophy on the VSRAD (Fig. 5A, B) did not show any apparent abnormalities in the pre-symptomatic stage until 5 years after baseline, compared with the  $^1\text{H}$  MRS results. These findings suggest that cognitive decline, as demonstrated by MMSE scores, and hippocampal atrophy (reflected by VSRAD) in the progressor MCI, AD, and DLB groups emerged 5–7 years after the appearance of abnormalities in  $^1\text{H}$  MRS. Thus, the current results suggest that  $^1\text{H}$  MRS alterations precede cognitive decline and hippocampal atrophy in subjects with progressor MCI, AD, and DLB.

Regarding the serial changes in MMSE scores, hippocampal atrophy, and the NAA/MI ratio in the PCC (Fig. 6), the results indicated that cognitive decline and hippocampal atrophy occurred later, from 5 to 7 years after baseline (Fig. 6A, B). In contrast, the serial change in each group was not statistically significant except in the group of subjects who remained cognitively normal, and in the progressor MCI group. The NAA/MI ratio in subjects with progressor AD, PD, and DLB tended to decrease over time, but the changes were not statistically significant. There

are several potential explanations for this pattern of results. First, it is possible that the NAA/MI ratio in the AD and DLB groups was defined at baseline (i.e., pre-symptomatic stage of the disease), and remained relatively stable subsequently. Alternatively, because we had limited data, the statistical power may have been insufficient to reveal significant results. Thus, further studies will be needed to clarify this issue.

The results revealed a significant correlation between MMSE scores and MI/Cr and NAA/MI ratios (Fig. 8). However, significant correlations were not found between the NAA/MI ratio and CSF-A $\beta$ 42 or CSF-p-tau at 7 years from baseline (data not shown). Thus, our  $^1\text{H}$  MRS results suggest that cognitive function might be associated with the MI/Cr and NAA/MI ratios in the PCC; however, it is unclear whether decreased NAA/MI is directly associated with the molecular pathology of AD, such as abnormal deposition of amyloid and tau in the brain. The results of the  $^1\text{H}$  MRS analysis of metabolites and those of amyloid or tau PET should be compared with validate whether abnormalities in  $^1\text{H}$  MRS are associated with abnormal amyloid deposition and the presence of tau.

Regarding the effect of the number of APOE4 alleles in the 289 cognitively normal participants, the MI/Cr ratio was significantly higher in subjects with two  $\epsilon 4$  alleles, compared with subjects with no  $\epsilon 4$  alleles. However, the NAA/MI ratio was significantly lower in subjects with two  $\epsilon 4$  alleles or one  $\epsilon 4$  allele compared with subjects with no  $\epsilon 4$  alleles (Fig. 9). These results suggest that altered NAA/Cr, MI/Cr, and NAA/MI ratios in the PCC, along with the APOE4 allele, may be involved in the

pathophysiology of AD. These results are compatible with previous studies demonstrating that alterations of the metabolites in  $^1\text{H}$  MRS in the PCC are useful for predicting the onset of AD at an asymptomatic stage in sporadic AD. Moreover,  $^1\text{H}$  MRS studies of familial AD with genetic mutations have produced convincing results, and the method may also be applied to sporadic cases [44–46]. Godbolt et al. [44] reported  $^1\text{H}$  MRS in the PCC in pre-symptomatic mutation carriers of presenilin 1 or amyloid- $\beta$  protein precursor mutations. They showed a significant decrease in NAA/Cr and NAA/MI ratios in pre-symptomatic mutation carriers compared with controls, concluding that metabolic changes can be detected in pre-symptomatic mutation carriers years before the expected onset of AD [44]. Kantarci et al. [45] examined subjects with microtubule-associated protein (MAPT) mutations. They reported that symptomatic MAPT mutation carriers were characterized by decreased NAA/Cr and NAA/MI ratios, hippocampal atrophy, and an increased MI/Cr ratio, whereas presymptomatic MAPT mutation carriers exhibited increased MI/Cr and decreased NAA/MI ratios, with an unchanged NAA/Cr ratio and hippocampal volume compared with controls. These results indicate that the MI/Cr ratio, a possible index of glial activation, precedes the decrease of the NAA/Cr ratio, a measure of neuronal integrity and hippocampal atrophy [45].  $^1\text{H}$  MRS abnormalities in the PCC can be observed in the genetic background for the screening of prodromal AD. In addition, the decreased NAA/Cr and NAA/MI ratios and increased MI levels in the PCC could be characteristic findings in pre-symptomatic and symptomatic AD.

Although the pathomechanism of the alteration of metabolites in  $^1\text{H}$  MRS in the PCC is unknown,  $^1\text{H}$  MRS abnormalities in the PCC are likely to be an important finding reflecting the pathophysiology of AD. The PCC forms a central node in the default mode network of the brain, and the connections between the PCC and the hippocampus are altered at a very early stage of AD. It has also reported that amyloid deposition is prominent in the PCC, and reduced metabolism is recognized in the presymptomatic stages of AD. Metabolic abnormalities in the PCC could be linked to amyloid deposition in early AD [55–58]. Previous studies have proposed that a decreased NAA/Cr ratio reflects decreased neuronal integrity, while increased MI/Cr may reflect glial activation or neuroinflammation associated with

neuronal degeneration, and decreased NAA/MI is a sensitive marker reflecting pathological changes of MCI and AD [33–36, 43]. Wang et al (2015) reported a systematic review and meta-analysis of altered MRS metabolites in AD. They concluded that the NAA/Cr ratio decreased markedly in the PCC, and a significantly elevated MI/Cr ratio was found not only in the PCC but also in the parietal gray matter [43]. Lin et al. (2016) reported that NAA and the NAA/MI ratio were sensitive to the functional status of adults with Down syndrome progressing to AD, including prior to dementia [46]. Our results also showed that the MI/Cr ratio increased and the NAA/MI ratio decreased with an increase in the number of APOE  $\epsilon 4$  alleles (Fig. 9). The results suggest that low NAA/Cr and high MI/Cr ratios in the PCC may be involved in APOE  $\epsilon 4$  allele, and could be risk factors for the onset of AD. Importantly, Voevodskaya et al. [47] reported systematic results showing the altered metabolites in the PCC on  $^1\text{H}$  MRS in relation to cognitive status, amyloid PET findings, and APOE4 genotype. They reported that amyloid-positive cognitively healthy participants exhibited a significant increase in MI/Cr and MI/NAA levels compared with amyloid-negative healthy older subjects. In addition, MI/Cr and MI/NAA ratios were correlated with the amount of cortical deposition of amyloid evaluated in amyloid-positive healthy older subjects, and levels were found to be already elevated at the asymptomatic stages of AD. Moreover, MI/Cr concentrations were increased in healthy APOE  $\epsilon 4$  carriers with normal CSF A $\beta_{42}$  levels, suggesting that MI levels may reveal the regional neural effects of APOE  $\epsilon 4$  before amyloid pathology is detectable. They concluded that MI levels were elevated preceding amyloid pathology, and MI/Cr levels of APOE  $\epsilon 4$  carriers were elevated compared with those of APOE  $\epsilon 4$  non-carriers, independently of amyloid pathology. Interestingly, Voevodskaya et al. (2016) reported that MI/Cr and NAA/MI levels, but not NAA/Cr levels were altered by the APOE  $\epsilon 4$  allele in a dose-dependent manner [47]. These results suggest that increased MI levels, which could reflect glial activation and inflammatory changes linked to the APOE  $\epsilon 4$  allele may precede to amyloid and tau pathology in AD. Thus, the findings reported by Voevodskaya et al. (2016) are compatible with the current results (Fig. 9) [47]. In addition, our results also revealed a significantly decreased NAA/MI ratio and elevated MI/Cr ratio in the PCC using  $^1\text{H}$  MRS in subjects with progressor DLB, similar to previous

findings in AD subjects [59–62]. Although the pathomechanism underlying  $^1\text{H}$  MRS abnormalities in DLB is unknown, our  $^1\text{H}$  MRS results in subjects who developed DLB may reflect reduced metabolism and abundant amyloid deposition of the PCC, which could be characteristic findings in DLB, as previously reported [59–62]. These results suggest that the NAA/MI ratio in the PCC could be used for differential diagnosis among typical PD, PD with dementia, and DLB, and that  $^1\text{H}$  MRS should be used to examine patients with parkinsonism.

The current data were obtained at three time points (baseline, and 5 and 7 years after baseline), including MMSE scores, Z-scores (VSRAD) (showing brain atrophy), and metabolites of  $^1\text{H}$  MRS in the PCC. The results suggest that  $^1\text{H}$  MRS changes preceded changes in MMSE scores and the VSRAD, and that abnormalities in  $^1\text{H}$  MRS had already appeared in the asymptomatic stages at baseline. However, declining MMSE scores and hippocampal atrophy, shown by the VSRAD, appeared 5 years after baseline, but were not apparent during the asymptomatic period until 5 years after baseline. We speculated that the NAA/MI ratio in the PCC, shown by  $^1\text{H}$  MRS, might insidiously decrease for 7 years followed by cognitive decline and brain atrophy. Taken together with previous reports, our results indicate that subjects exhibiting decreased NAA/MI and increased MI/Cr ratios in the PCC with  $^1\text{H}$  MRS, in combination with carrying APOE  $\epsilon 4$ , may be at risk of developing AD or DLB. Based on these findings, we propose that the evaluation of the NAA/MI ratio in the PCC by  $^1\text{H}$  MRS may provide a useful method for screening preclinical AD in clinical settings.

However, the current study involved several limitations that should be considered. First, we were unable to analyze changes in CSF biomarkers because we could only examine the CSF biomarkers at one point, 7 years after baseline, because of ethical constraints. In addition, the three time points (baseline, 5 and 7 years later) of data collection for  $^1\text{H}$  MRS and MMSE and the single time point for the CSF biomarkers were insufficient for rigorous longitudinal analysis. In the future, longitudinal studies over a longer period from the asymptomatic stage through the symptomatic stage to worsening of the condition, with greater sampling frequency, should be performed to validate whether the NAA/MI ratio in the PCC could be used as a screening marker for the prediction of AD, as suggested by our results. It may be beneficial to perform  $^1\text{H}$  MRS together

with  $^{18}\text{F}$ -fluorodeoxyglucose, amyloid, and tau-PET to investigate whether the  $^1\text{H}$  MRS abnormalities are associated with the molecular pathology of AD. Thus, despite these limitations, to our knowledge the current study is the first demonstration of the clinical utility of  $^1\text{H}$  MRS in the PCC for screening preclinical or prodromal AD. Further extensive multicenter prospective studies involving serial  $^1\text{H}$  MRS should be performed to validate whether decreased NAA/MI ratio in the PCC detected with  $^1\text{H}$  MRS could be a risk marker for preclinical AD.

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