Novel plasma biomarker surrogating cerebral amyloid deposition

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Abstract: Alzheimer’s disease (AD) is the most common and devastating dementia. Simple and practical biomarkers for AD are urgently required for accurate diagnosis and to facilitate the development of disease-modifying interventions. The subjects for the study were selected on the basis of PiB amyloid imaging by PET. Forty PiB-positive (PiB⁺) individuals, including cognitively healthy controls (HC), and mild cognitive impairment and AD individuals, and 22 PiB-negative (PiB⁻) HC participated. Employing our novel highly sensitive immunoprecipitation-mass spectrometry, we measured plasma amyloid β-proteins (Aβs; Aβ1-40 and Aβ1-42) and Aβ-approximate peptides (AβAPs), which were cleaved from amyloid precursor protein (APP). Among the AβAPs, APP669-711 appeared to be a good reference for deciphering pathological change of Aβ1-42. We evaluated the performance of the ratio of APP669-711 to Aβ1-42 (APP669-711/Aβ1-42) as a biomarker. APP669-711/Aβ1-42 significantly increased in the PiB⁺ groups. The sensitivity and specificity to discriminate PiB⁺ individuals from PiB⁻ individuals were 0.925 and 0.955, respectively. Our plasma biomarker precisely surrogates cerebral amyloid deposition.

Keywords: Alzheimer’s disease, amyloid β-protein, biomarker, mass spectrometry, immunoprecipitation, PiB amyloid imaging

Introduction

Alzheimer’s disease (AD) is the most common and devastating form of dementia that ultimately causes death. The searching for biomarkers to identify people at risk of AD development has been intensified because disease-modifying interventions are likely effective in the presymptomatic stage.¹,² The decreased levels of amyloid β-protein (Aβs; Aβ1-40 and Aβ1-42) and Aβ-approximate peptides (AβAPs), which were cleaved from amyloid precursor protein (APP). Among the AβAPs, APP669-711 appeared to be a good reference for deciphering pathological change of Aβ1-42. We evaluated the performance of the ratio of APP669-711 to Aβ1-42 (APP669-711/Aβ1-42) as a biomarker. APP669-711/Aβ1-42 significantly increased in the PiB⁺ groups. The sensitivity and specificity to discriminate PiB⁺ individuals from PiB⁻ individuals were 0.925 and 0.955, respectively. Our plasma biomarker precisely surrogates cerebral amyloid deposition.

reliable signatures of cerebral amyloid deposition, which occurs early in the pathophysiological development of AD³–⁸ or may accelerate the antecedent tauopathy⁹ before the clinical onset of dementia by 10 years or more in case of AD development. However, given that CSF examination is invasive and PET imaging is costly and hardly available, these biomarkers are not appropriate for screening people at risk of AD development. Thus, plasma Aβs have been extensively studied for their usefulness as alternative biomarkers.¹,² Although measuring plasma Aβs may be valuable in longitudinal and pharmacodynamic studies,¹⁰–¹³ so far it has not satisfactorily been informative on the state of cerebral amyloid deposition in cross-sectional studies.¹⁰,¹⁴,¹⁵

In the investigation of plasma Aβs in healthy individuals by our novel highly sensitive immunoprecipitation-mass spectrometry (IP-MS),¹⁶ we have recently detected various Aβ-approximate peptides (AβAPs), which were cleaved from the amyloid precursor protein (APP) close to the amino- and/or carboxyl-terminus of Aβs.¹⁷ In this study, we aim to examine whether plasma Aβs and/or AβAPs can be used as biomarkers surrogating cerebral amyloid...
deposition. Classifying study participants solely on the basis of clinical diagnosis can bias the performance of biomarkers\(^{18}\) because a large proportion of cognitively normal aged individuals exhibit AD pathologic features, including cerebral amyloid deposition.\(^{19}-21\) Thus, we carried out amyloid imaging by PET using Pittsburgh Compound B (PiB) on all the participants, and then carefully analyzed the performances of levels of A\(/\beta\) and/or A\(/\beta\)APs as biomarkers. Here, we report that the ratio of A\(/\beta\)AP (APP\textsubscript{699-711}) to A\(\beta\)\textsubscript{42} is a reliable plasma biomarker precisely surrogating cerebral amyloid deposition.

**Materials and methods**

**Subject selection and clinical classification.** The subjects of the present study, between the ages of 65 and 85 years, were recruited from the community-dwelling aged individuals and outpatients of the National Center for Geriatrics and Gerontology (NCGG) Hospital. On the basis of results of PiB amyloid imaging by PET and a set of neuropsychological examinations, 40 PiB-positive (PiB\(+\)) and 22 PiB-negative (PiB\(\neg\)) individuals were selected. The PiB\(+\) group consisted of 11 cognitively healthy controls (HC\(+\)), 12 individuals with mild cognitive impairment (MCI), and 17 patients with AD. The PiB\(\neg\) group included only cognitively healthy controls (HC\(\neg\)). Comprehensive neuropsychological batteries, including Mini-Mental State Examination (MMSE), Alzheimer’s Disease Assessment Scale Cognitive Component Japanese version (ADAS-Jcog), Logical Memory II from the Wechsler Memory Scale, and Geriatric Depression Scale (GDS) were administered to all the subjects. The clinical diagnoses of AD and MCI were made in accordance with the criteria developed by NIA-AA.\(^{22},23\) PiB positivity on amyloid imaging by PET was visually determined as described below (PiB PET visual rating). All the sets of examinations were carried out within about one year. Individuals with any significant medical, neurologic or psychiatric diseases other than AD and MCI were excluded from the selection based on medical history, neurologic examination, appropriate laboratory tests, neuropsychological examinations, and MRI. This study was approved by the Ethics Committee of NCGG and Shimadzu Corporation. All the subjects, proxies of AD and MCI individuals as well, provided written informed consent prior to examination to participate in the clinical studies of cognitive impairment and AD at NCGG.

**Brain imaging.** MR image acquisition. Brain MR images were obtained using a Trio 3T scanner (Siemens, Germany), an Avanto 1.5T scanner (Siemens), or an Ingenia 1.5T scanner (Philips, Netherlands). The protocol consisted of 3D T1-weighted, T2-weighted, and FLAIR (fluid attenuated inversion recovery) imaging.

PiB PET image acquisition. 3D static PET imaging for 50–70 min after intravenous injection of 555+/−185 MBq PiB was carried out using a PET-CT camera, Biograph True V (Siemens). X-ray CT for attenuation correction was performed before PET imaging.

PiB PET visual rating. The visual rating of PiB PET images reported by Rabinovici et al.\(^{24}\) was slightly modified for the present study. PiB PET images were visually read by two experienced nuclear medicine physicians (K.I. and T.K.) who were blind to the clinical data. The obtained static images were displayed with a rainbow scale and an inverse gray scale. PiB images were rated as “PiB-positive (PiB\(+\))” when the tracer binding in the cortical gray matter was deemed equal to or greater than that in the white matter, and as “PiB-negative (PiB\(\neg\))” when only nonspecific tracer binding in the white matter was observed. The judgments for the PiB positivity should agree 100% between the two experts. The results of the visual rating were used for the grouping of the subjects (PiB\(\neg\) and PiB\(+\)), and also used as the gold standard for the receiver operating characteristics (ROC) analyses (see Statistical analyses).

PiB PET data analysis. The reconstructed static PET images (168 × 168 × 111 matrices, 2.036 × 2.036 × 2.036 mm voxel size) were spatially normalized in NMI stereotactic space with parameters obtained from individual 3D-T1 MR images coregistered to PiB PET images by Diffeomorphic Anatomical Registration Through Exponentiated Lie Algebra (DARTEL). The normalized PiB PET images were masked with the grey-matter-segmented MR images to exclude the white matter and regions outside the brain. The region of interest (ROI) values were obtained from the PiB images of the grey matter using the Automated Anatomical Labeling Atlas.\(^{25}\) All the ROI values were transformed into standardized uptake value ratio (SUVR) by dividing them by the average ROI value in the cerebellar hemispheres. Mean cortical SUVR (PiB-mcSUVR)\(^{26}\) was obtained by averaging the SUVRs of the frontal, parietal, and temporal ROIs. PiB SUVR images were generated by dividing the masked PiB images by the
average value in each of the cerebellar hemispheres on a pixel-by-pixel basis. The PiB SUV images were spatially smoothed using a Gaussian kernel filter of 8 mm at full width at half maximum. Using these smoothed SUV images, voxelwise regression analyses for plasma biomarkers as covariates were performed using Statistical Parametric Mapping (SPM8, Wellcome Trust Centre for Neuroimaging, University College, London, UK).

**Immunoprecipitation-mass spectrometry.** The levels of plasma Aβs and AβAPs were measured by matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF MS) followed by immunoprecipitation (IP). We used F(ab') fragments of two monoclonal antibodies (6E10 and 4G8) that are specific to different Aβ epitopes, coupling to polyethylene glycol (PEG) on magnetic beads [hetero-F(ab')-(PEG)24 beads] for IP. The procedure was slightly modified from that described previously. In brief, 250 µL of plasma was mixed with an equivalent amount of binding buffer [100 mM Tris-HCl, 800 mM N-acetyl-D-glucosamine, 0.2% w/v n-dodecyl-β-D-maltoside (DDM), 0.2% w/v n-nonyl-β-D-thiomaltoside (NTM), 300 mM NaCl; pH adjusted to 7.4] with 10 pM stable-isotope-labeled (SIL) Aβ1-38. After filtration by centrifugation, the plasma sample was pretreated with 500 µL of Protein G Plus Agarose (50% slurry; Pierce, Rockford, IL) at 4 °C for 1 h. Then, the hetero-F(ab')-(PEG)24 beads were washed twice with 50 mM glycine-HCl buffer (pH 2.8) containing 1% OTG and then three times with washing buffer (50 mM Tris-HCl, 0.1% w/v DDM, 0.1% w/v NTM, and 150 mM NaCl; pH adjusted to 7.4). The beads were incubated with the plasma sample at 4 °C for 1 h, and then washed five times with washing buffer, then with 50 mM ammonium acetate (pH 7.4), and then with H2O. The bound Aβs and AβAPs were eluted with 2.5 µL of acetonitrile/H2O (7:3 v/v) containing 5 mM HCl. Each eluate (0.5 µL) was immediately applied onto four wells of a 900 µm µFocus MALDI plate™ (Hudson Surface Technology, Inc., Fort Lee, NJ) with an equal volume of the α-cyano-4-hydroxycinnamic acid (CHCA) solution containing methanediophosphonic acid (MDPNA). Mass spectra were acquired using a MALDI-linear TOF mass spectrometer (AXIMA Performance, Shimadzu/KRATOS, Manchester, UK) equipped with a 337 nm nitrogen laser in the positive ion mode. The limit of detection was established at an S/N of 3:1. The peptide mass tolerance for quantification was set within 2.5 Da of the theoretical mass. One immunoprecipitation preparation produced four mass spectra and consequently yielded four peak intensities per analyte peptide. The levels of plasma Aβ and AβAPs were obtained by averaging the four intensity ratios of each of the Aβ and AβAP peaks to an internal standard (SIL-Aβ1-38) peak. Through our exploratory assessments, we found that one of the AβAPs, APP669-711, could be a good reference for deciphering the individual change of Aβ1-42 level. Thus, we analyzed performances of the ratio of plasma Aβ1-42 to APP669-711 as a biomarker, comparing with those of Aβ1-42, and the ratio of Aβ1-42 to Aβ1-40. We verified the preciseness of the assay using human EDTA plasma from healthy individuals, purchased from Tennessee Blood Services (Memphis, TN). The intra- and inter-day assay coefficients of variants obtained were 9.93% (n = 4) and 19.41% (n = 4) for Aβ1-40, 1.67% and 9.77% for Aβ1-42, 8.48% and 12.13% for APP669-711, respectively.

**Statistical analyses.** We pre-assigned the sample size to be approximately 60 subjects. We considered that the sample size would be adequate because of the following reasons. The CSF Aβ1-42, which is one of the most promising biomarkers reflecting the cerebral Aβ deposition, significantly correlates with the quantitative measure of PiB-PET, and the reported correlation coefficients (r) were about −0.57 to −0.73. Because we aimed to establish a novel plasma biomarker with comparable clinical usefulness to the CSF biomarkers, we expected that the coefficient of determination (r²) of our biomarker should be more than 0.25. With this value, we would have 95% power to detect a useful biomarker at a significance level of 5% with a total sample size of 47. Statistical analyses were performed using SPSS ver. 21 (IBM, New York, USA) and JMP software ver. 8 (SAS Institute, Cary, USA). For the subjects’ demographics, categorical data such as gender and APOE ε4 carrier distributions were analyzed using the Chi-square test, and the Bonferroni correction was applied for multiple comparisons. Normally distributed continuous data were analyzed using the Student t-test or one-way ANOVA with Tukey-Kramer post-hoc comparisons. Not normally distributed data were analyzed using the Kruskal Wallis test followed by the Steel-Dwass post-hoc test. For the analyses of the biomarker and PiB-mcSUVR data, when the values were not normally distributed, the reciprocal transformation was applied and normality was ensured. Analysis of covariance (ANCOVA) was used to assess the group differences, and the Bonferroni correction was
applied for the post-hoc tests. Receiver operating characteristic (ROC) analyses were performed to estimate the capability of the plasma biomarkers to discriminate individuals with cortical Aβ deposition (PiB+) from those without deposition (PiB−). The area under the curve (AUC), sensitivity and specificity were calculated to assess the discriminative capability of a biomarker. The Pearson product-moment correlation coefficient analysis was conducted to evaluate the strength of the link between each biomarker and cortical Aβ deposition assessed using PiB-mcSUVR values. Multiple regression analysis and partial correlation analysis were also performed to assess the influence of possible confounders that may affect the correlation between each biomarker and PiB-mcSUVR. All the tests were two-tailed, and the significance level of difference was set at $P < 0.05$.

**Results**

**Demographics.** The demographics and clinical characteristics of the subjects are summarized in Table 1. In the PiB classification based on the visually rated PiB positivity, there were significant differences in age and allele frequency of $APOE \varepsilon 4$. Therefore, we adjusted for age in the statistical analyses of the biomarkers. In the detailed classification based on the clinical category, age, the scores of MMSE, ADAS-Jcog, LM2 and GDS, and the allele frequency of $APOE \varepsilon 4$ were significantly different among groups. No significant differences were observed in sex ratio and educational attainment among the groups.

**Measurement of Aßs and AßAPs.** Plasma samples obtained from the participants were spiked with SIL-Aß1-38 at 10 pM and subsequently subjected to IP-MS as described previously. The representative mass spectra of plasma Aßs and AßAPs from PiB− (HC−) and PiB+ (AD) subjects are shown in Fig. 1. Given that the signal intensity of hydrophobic peptides such as Aß1-42 decreases in MALDI-TOF MS, the Aß1-42/Aß1-40 ratio in IP-MS was smaller than that in previous studies. In addition to Aß1-38 and Aß1-40, the peaks of various AßAPs (Aß1-38, Aß1-42, Aß1-39, OxAß1-40, and APP669-711 indicated in Fig. 2) were generally detected in all subjects. There did not appear to be a distinct difference in the relative signal intensities of most of these peptides. However, note that the ratio of the signal intensity of Aß1-42 to that of APP669-711 in AD subjects was remarkably different from that in HC− subjects (Fig. 1, arrows).

| PiB classification | PiB− (n = 22) | PiB+ (n = 40) | Statistics (P-value) |
|--------------------|---------------|---------------|----------------------|
| sex (M : F)        | 8 : 14        | 19 : 21       | 0.397                |
| age (y)            | 72.1 ± 2.9    | 75.2 ± 4.7    | 0.006                |
| $APOE \varepsilon 4$ (%) | 3/22 (13.6) | 26/40 (65.0) | <0.001               |

For PiB classification, the PiB− group is the same as HC− group, and the PiB+ group includes all of the HC+, MCI and AD individuals. Values are presented as mean ± SD. Statistical analyses were performed using the chi square test (sex, $APOE \varepsilon 4$), Student t-test or one-way ANOVA (age, ADAS), and the Kruskal Wallis test (education, MMSE, LM2, GDS). Post-hoc results were as follows: HC− vs AD ($P < 0.001$); HC−, HC+ and MCI vs AD ($P < 0.001$); HC− vs MCI ($P < 0.05$); HC− and HC+ vs AD ($P < 0.001$); HC− and HC+ vs AD ($P < 0.001$); HC− and HC+ vs MCI ($P < 0.01$); HC− and HC+ vs AD ($P < 0.001$); HC− vs AD ($P < 0.001$) and HC− vs MCI ($P < 0.01$), MCI vs AD ($P < 0.01$) and HC− vs MCI ($P < 0.05$); HC− and HC+ vs AD ($P < 0.001$), HC− and HC+ vs MCI ($P < 0.01$), HC− and HC+ vs AD ($P < 0.001$); HC− vs AD ($P < 0.001$) and HC− vs MCI ($P < 0.01$).

MMSE = Mini-Mental State Examination; ADAS-Jcog = Alzheimer’s Disease Assessment Scale-Cognitive Component-Japanese version; LM2 = Logical Memory II from the Wechsler Memory Scale–Revised (paragraph A only); GDS = Geriatric Depression Scale; $APOE \varepsilon 4$ = positive for apolipoprotein E $\varepsilon 4$. 

![Image](image_url)
Fig. 1. MALDI-TOF mass spectra of plasma Aβs and AβAPs. Representative mass spectra obtained by IP-MS of plasma samples from the HC− (A) and AD (B) subjects are shown. In addition to Aβ1-40 and Aβ1-42, AβAPs including Aβ1-38, Aβ3-40, Aβ1-39 and APP669-711 were simultaneously measured by MALDI-TOF MS. Four mass spectra (represented in red, blue, green and orange) were obtained from one immunoprecipitation. The levels of Aβs and AβAPs were calculated by averaging the four intensity ratios of Aβs and AβAPs peak to SIL-Aβ1-38 peak. The arrows represent the difference in signal intensity between Aβ1-42 and APP669-711.

Fig. 2. Overview of Aβs and AβAPs detected by IP-MS. Seven Aβs and AβAPs were detected in plasma samples. The arrows above the sequence indicate the proteolytic processing sites of β- and γ-secretases. OxAβ1-40 represents Aβ1-40 with the oxidized methionine. sAPPβ = soluble APP; AICD = APP intracellular domain.
The upper part of Table 2 demonstrates since not Aβ1-42/APP669-711 but APP669-711/Aβ1-42 was normally distributed, the latter values were used for the statistical analyses. Therefore performances of the APP669-711/Aβ1-42 were compared with those of Aβ1-42 level and Aβ1-42/Aβ1-40. PiB-mcSUVR values were also analyzed as the ideal reference. All of the statistical results are summarized in Table 2.

**Permanences of the plasma biomarkers.** Since not Aβ1-42/APP669-711 but APP669-711/Aβ1-42 was normally distributed, the latter values were used for the statistical analyses. Therefore performances of the APP669-711/Aβ1-42 were compared with those of Aβ1-42 level and Aβ1-42/Aβ1-40. PiB-mcSUVR values were also analyzed as the ideal reference. All of the statistical results are summarized in Table 2.

**Group comparisons between PiB− and PiB+.** The upper part of Table 2 demonstrates the results of ANCOVA adjusted for age. Using our newly established method, the Aβ1-42 level showed a highly significant difference between the groups, Aβ1-42/Aβ1-40 did not improve the significant level; however, APP669-711/Aβ1-42 markedly enhanced the group-separation capability. The F-value and the effect size ($\eta^2$) of APP669-711/Aβ1-42 were comparable to those of PiB-mcSUVR.

**Capability to discriminate PiB− individuals from PiB+ individuals.** To evaluate the capability of APP669-711/Aβ1-42 to discriminate PiB+ individuals from PiB− ones, ROC analysis was performed. The results are shown in the middle part of Table 2 and Fig. 3. APP669-711/Aβ1-42 demonstrated an extremely high AUC (0.969). The sensitivity and specificity for the discriminative capability of APP669-711/Aβ1-42 were 0.925 (3 out of 40 were false negative) and 0.955 (1 out of 22 was false positive), respectively, with a cutoff value of 0.914. The performance indices of APP669-711/Aβ1-42 were also comparable to those of PiB-mcSUVR.

**Correlation between our plasma biomarkers and PiB-mcSUVR.** To evaluate the strength of the link between our plasma biomarkers and PiB-mcSUVR, we performed correlation and regression analyses. The results are shown in the lower part of Table 2 and Figs. 4A and 4B. All of the plasma biomarkers

| Table 2. Summary of statistical results for biomarkers |
|-----------------------------------------------------|
| ANCOVA group comparisons | Aβ1-42 | Aβ1-42/Aβ1-40 | APP669-711/Aβ1-42 | PiB-mcSUVR |
|--------------------------|--------|---------------|------------------|------------|
| PiB− (n = 22) mean (95% CI) | 0.21 (0.18–0.24) | 0.011 (0.009–0.012) | 0.72 (0.63–0.82) | 1.10 (0.98–1.21) |
| PiB+ (n = 40) mean (95% CI) | 0.14 (0.12–0.15) | 0.007 (0.006–0.008) | 1.13 (1.07–1.19) | 1.65 (1.57–1.72) |
| F-value | 8.19 | 7.89 | 24.98 | 31.62 |
| P-value | <0.001 | 0.001 | <0.001 | <0.001 |
| coefficient of determination (R²) | 0.298 | 0.290 | 0.564 | 0.621 |

**Upper part:** Results of analysis of covariance (ANCOVAs) for comparison between PiB− and PiB+ groups in biomarkers and PiB-mcSUVR. Values in parentheses represent 95% confidence interval (CI). All results are adjusted for age.

**Middle part:** Results of the receiver operating characteristic (ROC) analysis of biomarkers and PiB-mcSUVR to discriminate visually classified PiB− and PiB+ individuals (Fig. 3). The sensitivity indicates the true positive rate as calculated by (true positive)/(true positive) + (false negative)), and the specificity indicates the true negative rate as calculated by (true negative)/(false positive) + (true negative)). The AUC (the area under the ROC curve) express a kind of overall diagnostic accuracy. The cutoff values for Aβ1-42, Aβ1-42/Aβ1-40, APP669-711/Aβ1-42, and PiB-mcSUVR are 0.183, 0.009, 0.914, and 1.271, respectively.

**Lower part:** Results of the multiple regression analysis of biomarkers using PiB-mcSUVR and age as predictors. Also, results of the simple analysis of correlation (single correlation) to both PiB-mcSUVR and age, and results of partial correlation analysis adjusted for age or PiB-mcSUVR are shown.

*The values represent by the intensity of Aβ1-42 peak relative to that of SIL-Aβ1-38 peak as an internal standard.

*Note that the Aβ1-42/Aβ1-40 are markedly different from the reported values in other studies, because of methodological differences (see Results, Measurements of Aβ's and AβAPs).
showed statistically significant correlation with PiB-mcSUVR. In particular, APP669-711/\(A\beta1-42\) demonstrated high correlation coefficients both in the simple correlation (single \(r = 0.687, P < 0.001\)) and the correlation adjusted for age (partial \(r = 0.668, P < 0.001\)) (Fig. 4A). Multiple regression analysis showed there were no significant ageing effects. Moreover, the regression analysis of PiB-SUVR images using biomarker levels as covariate vectors demonstrated that plasma APP669-711/\(A\beta1-42\) significantly correlated with regional PiB retention (FWE corrected \(P < 0.05, T = 5.18\), extent threshold \(k = 200\) voxels). The visualized areas, robustly involving the frontal, precuneus, and posterior cingulate, and the parietotemporal cortices, appeared to correspond to the typical pattern of \(A\beta\) accumulation in AD (Fig. 4B, right).29,30 Although no significant clusters were found with the same threshold as the APP669-711/\(A\beta1-42\) threshold, the results with a lowered threshold (uncorrected \(P < 0.0001, T = 3.97\)) suggest that the plasma \(A\beta1-42\) level and \(A\beta1-42/A\beta1-40\) also reflect the cortical \(A\beta\) accumulation (Fig. 4B, left and middle).

**Performances across clinical categories.** Additionally, the performances of the biomarkers were tested across the clinical categories. The results of ANCOVA (adjusted for age) demonstrate that APP669-711/\(A\beta1-42\) showed highly significant group differences with a large effect size (Table 3, upper part), which were almost comparable to those of PiB-mcSUVR. The post-hoc group comparisons demonstrated that APP669-711/\(A\beta1-42\) is sensitive in distinguishing between HC− and any other PiB+ groups (HC+, MCI and AD) (Fig. 5). On the other hand, APP669-711/\(A\beta1-42\) appeared not so sensitive in distinguishing groups classified according to the clinical severity of AD. Within the PiB+ group, only a comparison between HC+ and AD showed a significant difference. We also conducted ROC analysis to evaluate the discriminative capability of the plasma biomarkers across the clinical categories (Table 3, lower part, and Fig. 6). APP669-711/\(A\beta1-42\) showed very high sensitivity and specificity in discriminating HC-individuals from AD and MCI individuals. Importantly, the results demonstrated that APP669-711/\(A\beta1-42\) could identify PiB+ individuals within a cognitively healthy group (HC− vs HC+) with 90.9% sensitivity and 90.9% specificity with a cut off value of 0.863.

**Discussion**

This study shows that APP669-711/\(A\beta1-42\) in plasma has a big potential as a biomarker precisely surrogating cerebral amyloid deposition. Our biomarker clearly discriminated between PiB− and PiB+ groups with a large effect size (\(\eta^2 = 0.56\)), and the sensitivity and specificity in discriminating PiB+ individuals from PiB− individuals were very high (0.925 and 0.955, respectively). Furthermore, APP669-711/\(A\beta1-42\) significantly correlated with cortical PiB retention with a high correlation coefficient (age-adjusted partial \(r = 0.668\)). As a surrogate marker for cerebral amyloid deposition, the performances of APP669-711/\(A\beta1-42\) were far beyond those of reported plasma biomarkers31,32 and were comparable to those of CSF biomarkers.27,28,33 Considering invasiveness and cost, the clinical, as well as social, impact of our novel plasma biomarker would be very significant.

A great deal of effort has been made to determine whether plasma \(A\beta\)'s can be diagnostic and/or predictive biomarkers for AD; however, so far the results were contradictory and, in most studies, there was a broad overlap in the levels of plasma \(A\beta\) between controls and patients. This may be due to the difficulties in \(A\beta\) measurement in plasma, which are likely caused by various factors,14 including low...
Aβ concentrations and unavoidable Aβ binding to other proteins, which may mask the antibody epitope of Aβ. The utility of the plasma Aβ as AD biomarkers may also be complicated by the fact that plasma Aβs can originate from peripheral organs. In addition to these technical and biological aspects of plasma Aβs, the development of biomarkers for AD is generally hampered by a difficulty in correctly recruiting “control subjects” because a large proportion of cognitively normal aged individuals exhibit AD pathologic features, including cerebral amyloid deposition, which has recently been corroborated by amyloid PET studies.

To overcome these obstacles, we designed this study as follows. First, to increase detection sensitivity and specificity, we employed our novel IP-MS system. In this system, we used hetero-F(ab′) fragments of two monoclonal antibodies (6E10 and 4G8), which are specific to different Aβ epitopes, that were coupled to PEG on magnetic beads and then, the molecular species of the captured Aβs were precisely and simultaneously determined by MALDI-TOF MS. Compared with the performance of conventional sandwich ELISA measurements, the efficiency and accuracy of Aβ detection in our IP-MS system were markedly high. Indeed, the performances of Aβ1-42 in the present study (Table 2, Figs. 3 and 4) were much higher than those in previous studies.

Second, to decipher the pathological significance of unpredictable changes of Aβ1-42 in plasma, we used APP669-711 as a reference against Aβ1-42. Using the APP669-711 to Aβ1-42 ratio, the performances were extremely high compared with those obtained from Aβ1-42 and Aβ1-40 (Table 2, Figs. 3 and 4). Third, to correctly classify participants in terms of cerebral amyloid deposition, we carried out PiB amyloid imaging by PET of all the subjects. By objective
Table 3. Summary of statistical results for biomarkers for discriminating across clinical categories

| ANCOVA group comparisons          | Aβ1-42       | Aβ1-42/Aβ1-40 | APP669-711/Aβ1-42 | PiB-mcSUVR |
|-----------------------------------|--------------|---------------|-------------------|------------|
| HC− (n = 22) mean (95% CI)        | 0.21 (0.18–0.24) | 0.011 (0.009–0.012) | 0.72 (0.64–0.81) | 1.10 (0.99–1.20) |
| HC+ (n = 11) mean (95% CI)        | 0.14 (0.11–0.17) | 0.007 (0.005–0.009) | 0.99 (0.88–1.09) | 1.45 (1.33–1.57) |
| MCI (n = 12) mean (95% CI)        | 0.14 (0.10–0.17) | 0.008 (0.006–0.010) | 1.11 (1.01–1.21) | 1.74 (1.61–1.86) |
| AD (n = 17) mean (95% CI)         | 0.14 (0.11–0.17) | 0.008 (0.006–0.009) | 1.24 (1.15–1.33) | 1.74 (1.63–1.84) |
| F-value                           | 5.21         | 3.52          | 15.22             | 19.23      |
| P-value                           | <0.001       | 0.003         | <0.001            | <0.001     |
| coefficient of determination ($\eta^2$) | 0.403         | 0.313         | 0.664             | 0.714      |

Upper part: Results of the ANCOVA for comparisons among classified groups for biomarkers and PiB-mcSUVR. Values in the parentheses represent 95% confidence interval (CI). All results are adjusted for age. The post-hoc results are displayed in Fig. 5.

Lower part: Results of the receiver operating characteristics (ROC) analyses for each biomarker and PiB-mcSUVR to discriminate across the clinical categories; HC− vs. HC+, MCI, and AD. The cutoff value for each analysis was determined by the nearest point in the curve from the left upper corner. ROC curves are shown in Fig. 6.

Fig. 5. Group comparisons of the plasma biomarkers and PiB-mcSUVR across clinical categories. Distribution of each value is shown by a box-whisker plot. The boxes represent the 25th, 50th (median) and 75th percentiles of the data. The ends of whiskers represent the lowest (or highest) datum within 1.5-times interquartile range from the 25th (or 75th) percentile. The plotted values were original, but the results of the multiple comparisons were adjusted for age. All $p$-values were Bonferroni corrected, and the significance levels are represented by the number of asterisks: $^* P < 0.05$, $^{**} P < 0.01$, $^{***} P < 0.001$. 
determination of the state of cerebral amyloid deposition in each participant, we were able to confirm the usefulness of our biomarkers.

It remains to be elucidated why the APP669-711/Aβ1-42 ratio in the plasma showed the highest significance in correlation with the presence of cerebral amyloid deposition. One possible explanation is as follows. APP669-711 has almost the same size and amino acid sequence as Aβ1-42; thus, these two peptides may show the same metabolism rate, binding tendency to other molecules in the brain and plasma, and also penetration capability through the blood-brain barrier. In contrast, the aggregation tendency of APP669-711 may be extremely lower than that of Aβ1-42 because of the difference in both amino- and carboxyl-termini. Collectively, the coexistence of similar and dissimilar characteristics of Aβ1-42 likely allows APP669-711 in plasma to function as a good reference against Aβ1-42.

In conclusion, we found that APP669-711/Aβ1-42 is a highly sensitive plasma biomarker that precisely surrogates cerebral amyloid deposition. This simple and minimally invasive biomarker should be beneficial in the clinical diagnoses of AD, possibly substituting the CSF examination. In addition, our biomarker can be a very powerful screening tool to identify people at risk of AD development from a community, and thereby likely facilitates development of disease-modifying clinical trials for AD.

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