Comparative analyses of plasma amyloid-β levels in heterogeneous and monomerized states by interdigitated microelectrode sensor system

INTRODUCTION

The controversy behind the utilization of plasma biomarkers in the diagnosis of Alzheimer disease (AD) is due to the unreliable results concerning amyloid-β (Aβ) concentrations, where some studies indicate a decrease (1), an increase (2), or no significant change (3–5). Despite the inconsistency, most agree that Aβ is the earliest existing biomarker in AD and is transported from the brain, where it exists as soluble and insoluble aggregates, to the blood by low-density lipoprotein receptor–related protein 1 (6). It is unclear whether these aggregates penetrate the blood-brain barrier, but studies have reported that oligomeric Aβ is a component of plasma Aβ (7). Thus, we believe that these oligomeric Aβ are in a heterogeneous mixture of diverse size and can affect the quantitative measurements of plasma Aβ. An objective of our study was to confirm the presence of Aβ as a heterogeneous mixture in patient plasma by monomerizing aggregates to measure the true concentrations of Aβ (7). It is also unclear whether the concentrations and conditions of Aβ aggregates are maintained at a consistent daily level. Contingent upon the possibility that homeostasis is not regularly maintained, it would become essential to establish a method aimed to normalize Aβ concentrations for a comparative analysis of intra- and interindividual variations.

For plasma Aβ to serve as a biomarker for AD, we hypothesized that two prerequisites must be satisfied. First, the method of quantifying and normalizing levels of heterogeneous Aβ species must minimize confounding variables, thereby enabling clinical differentiation of patients with AD from nondemented individuals. Second, since Aβ exists in the blood at a concentration of picogram per milliliter, a detection device should have a limit of detection (LOD) at a level of subpicogram per milliliter. In this study, we devised an interdigitated microelectrode (IME) sensor system and used it in conjunction with 4-(2-hydroxyethyl)-1-piperazinopropanesulfonic acid (EPPS), which is a small molecule that aids in converting aggregated Aβ into its monomeric form (8). EPPS was used to homogenize Aβ biomarkers in AD patient plasma samples; thus, the comparing levels of Aβ by standard (CLASS) method could be implemented to diminish inter- and intraindividual variations (Fig. 1). While in a heterogeneous state, immunochemical measurements of Aβ may not yield the true concentration of Aβ because of several obstructing factors: the epitope hidden within oligomerized structures and the steric hindrance caused by size differences between Aβ (~4.5 kDa) and its antibody (~150 kDa). In addition, previous clinical studies reported that concentrations of plasma Aβ fluctuate inter- and intraindividually, possibly because the absorption, metabolism, and excretion of plasma Aβ vary among and within individuals depending on different lifestyle choices, such as diet and physical activity (9, 10). On the basis of clinical evidence, we expected nondemented controls to have most of their Aβ in monomeric states, while patients with AD would have aggregated Aβ in their blood. Thus,
disaggregation of heterogeneous plasma Aβ would not only allow for the accurate measurement of Aβ concentrations but also serve to normalize individual variations and fluctuations within protein levels. The diagnostic device has a sensitive LOD that can detect plasma Aβ concentrations at 0.1 pg/ml. Through the alteration of the detection medium from plasma to a buffer solution and enhanced signal processing with amplification and cancellation, we optimized the device performance and sensitivity as high as tens to hundreds of picograms per milliliter. From two clinical institutes in the Republic of Korea, Asan Medical Center (AMC) and Korea Institute of Radiological & Medical Sciences (KIRAMS), we collected blood samples of patients with AD (n = 61) and individuals with normal cognition (NC) (n = 45), who underwent clinical interviews, neurological examinations, neuropsychological and laboratory tests, magnetic resonance imaging (MRI) scans, and amyloid–positron emission tomography (PET) scans to avoid the influence of potential bias from a single-center trial.

RESULTS

Plasma Aβ disaggregation

Previously, EPPS was found to serve as a disaggregating agent to remove Aβ plaques and oligomers in the brains of an APP/PS1 double transgenic (TG) Alzheimer mouse model (8). To confirm whether EPPS elicits the same effect on plasma Aβ aggregates, we spiked Aβ aggregates into citrated human plasma purchased at various concentrations, treated citrated-plasma aliquots with 200 mM EPPS for 2 hours at 4°C, and performed Aβ-targeted sandwich enzyme-linked immunosorbent assay (ELISA) (Fig. 2A). Aβ monomers were prepared in three different concentrations—100, 200, and, 400 pg/ml—in phosphate-buffered saline (PBS) and incubated for 72 hours at 37°C for aggregation before the addition of EPPS. The formation of oligomers was confirmed by electrophoresis with the photoinduced cross-linking of unmodified protein method, as previously reported (8). As a result, we observed a significant (Fig. 2B: P = 0.0003; Fig. 2C: P = 0.1465; Fig. 2D: P = 0.0146; two-tailed paired t test) increase in Aβ levels in citrated-plasma aliquots after EPPS treatment (Fig. 2, B to D). Results indicated that the measurement of total Aβ concentrations was inversely correlated with the aggregation state of the protein and that EPPS was capable of fulfilling its role in disaggregating plasma Aβ. Unfortunately, aggregated Aβ could not be detected in the concentration of 100 pg/ml when citrated-plasma samples were not treated with EPPS. These results suggest that the inability to detect any Aβ species in human plasma arose from other underlying causes, most likely due to very low concentrations of plasma Aβ, which required a more precise detection device with a smaller LOD.

Fig. 1. Representative scheme of plasma Aβ detection and analysis procedure. (1) Blood samples are collected into a heparin vacutainer and centrifuged. (2) Isolated plasma is then aliquoted into two samples: (3) no chemical addition (sample A) and EPPS addition (sample B). (4) After disaggregation of heterogeneous Aβ in sample B, (5) both plasma samples A and B are applied to IME sensor chips, which contain immobilized 6E10 antibodies on the surface to detect Aβ. (6) Last, the concentration of Aβ in sample B is divided by the concentration of Aβ in sample A.
Fig. 2. Dissociation of Aβ aggregates and detection of Aβ on IME sensor. (A) Scheme of Aβ monomerization by EPPS treatment after spiking plasma samples with aggregated Aβ. (B to D) Changes of Aβ levels in plasma via EPPS treatment (before and after) when Aβ aggregates were spiked into human plasma. Concentrations of Aβ are 400 pg/ml (B), 200 pg/ml (C), and 100 pg/ml (D). Statistical comparisons were made with two-tailed t test: P = 0.0003 (B) and P = 0.0146 (D). Black squares, untreated samples; white squares, EPPS-treated samples. (E) IME chip and the enlarged image of one IME pair (scanning electron microscopic image). (F) Sequential process for surface modification of the IME sensor. The IME sensor was sequentially functionalized with APMES, PVP-CHO, glutaraldehyde, Aβ antibody (6E10), and BSA. (G) Image of IME sensor and PDMS microfluidic chip. Each sample was injected into two microfluidic channels on the IME unit chip. (H) IME unit chip with PDMS microfluidic chip photograph. Two channels were filled with colored solutions. (I) Impedance change (|ΔZ/Z₀|, %) of the IME by interaction between Aβ and its antibody. (J) Logarithmical linear sensitivity to Aβ levels (n = 5). Error bars indicate SDs. (K and L) Analysis of mouse plasma Aβ levels (black dot, WT (n = 9); red dot, TG (n = 9)). (K) WT mouse plasma without and with EPPS treatment. (L) TG mouse plasma without and with EPPS treatment. The dot data represent multiple (n = 5) independent experiments. Two-tailed t tests were performed in the statistical analyses (*P < 0.05 and ***P < 0.001; nonsignificant analysis is not indicated).
IME sensor preparation

According to results from recent clinical studies, Aβ concentrations in plasma are observable within a picogram per milliliter scale (11). To detect differences in concentration between plasma samples, the LOD should be lower than 10 pg/ml. Unfortunately, the LODs of conventional detection systems, such as ELISA, do not meet this standard. Therefore, we prepared an enhanced detection system based on our previously reported IME sensor chip (12).

The chip unit was fabricated using conventional microelectromechanical system techniques and contained four IME sensors that are composed of platinum electrodes (5 μm wide and 150 nm thick) (Fig. 2E). A silicon dioxide (SiO2) layer, the chip’s sensing zone, was exposed in 5-μm gaps between the platinum electrodes (Fig. 2E). The sensing zone was functionalized to detect low concentrations of Aβ in plasma by sequentially coating the silicon dioxide layer with 3-((ethoxydimethylsilyl)propylamine (APMES), polyvinyl pyrrolidone-aldehyde (PVP-CHO), anti-Aβ 6E10 antibody, and bovine serum albumin (BSA) (Fig. 2F). The APMES introduced primary amines to the silicon dioxide layer of the IME sensor chip to conjugate with PVP-CHO. To capture Aβ in the plasma samples, anti-Aβ 6E10 antibody was covalently bonded to the PVP-CHO, and then BSA was used to coat the chip surface to avoid nonspecific binding of anonymous plasma biomarkers. Two microchannels were incorporated on top of the chip to simultaneously load two different plasma samples (EPPS treated and nontreated) on the detection system (Fig. 2G). To ensure that the polydimethylsiloxane (PDMS) microchannels were well confined for loading liquid onto the unit chip, we conducted color-metric verification by injecting green and blue color dyes, diluted in deionized water (DW), into the microchannels (Fig. 2H).

Sensor optimization was characterized by injecting PBS solutions of synthetic Aβ42 onto the IME sensors and subsequently measuring the impedance changes (Fig. 2I). The interaction between Aβ and anti-Aβ antibodies on the sensors was measured using an impedance measurement system (PGSTAT302N, Metrohm Autolab and IME analyzer, Cantis Incorporation). The detection procedure was conducted within 30 min, which consists of a 5-min stabilization period with PBS buffer, 20-min binding period with Aβ in PBS, and 5-min washing period with PBS. The final concentration of Aβ bound to the IME sensors was determined by the impedance change equation below

$$\text{Impedance change (\%)} = \left| \frac{Z_{\text{after}} - Z_{\text{before}}}{Z_{\text{before}}} \right| \times 100$$

For concentration determination, we only considered the signals within the 5-min (Z_before) and 25-min (Z_after) periods (Fig. 2I). The reaction time was set to 20 min since the impedance signal rapidly increased after the initial injection of the sample but stabilized after 20 min. Impedance changes of Aβ solutions were approximately 4.0, 5.5, 7.3, 9.2, and 8.9% for 100 fg/ml, 1 pg/ml, 10 pg/ml, 100 pg/ml, and 1 ng/ml, respectively (Fig. 2I). To confirm the reliability of the device, we established two negative controls, one of which used an antibody lacking cross-reactivity: prostate-specific antigen (PSA) antibody (Fig. S1). The other negative control consisted of using different antigens with the 6E10-functionalized IME: Aβ, PSA, and brain-derived neurotrophic factor (BDNF). Impedance changes of the PSA antibody–functionalized IME was detected after injecting Aβ samples (10 μg/ml), which showed a 1.4% change. Results using the 6E10-functionalized IME in conjunction with BDNF (10 ng/ml), PSA (10 ng/ml), and Aβ (10 pg/ml) showed that impedance changes were 1.2, 1.1, and 7.3%, respectively. Therefore, the LOD of the unit chip for Aβ detection is hundreds of femtograms per milliliter and exhibits selectivity in detecting the target sample.

**Aβ detection in the plasma of AD mouse model**

We applied the EPPS and IME detection method to plasma samples of AD TG mice before clinical investigation. Nine-month-old doubly female APP/PS1 TG mice (strain name: B6C3-Tg (APPswe, PSEN1De9) 85Dbo/J) and age-matched female wild-type (WT; B6C3F1) mice were prepared (n = 9 per group). To analyze concentrations of Aβ in prepared mouse blood, plasma samples were obtained and treated with or without EPPS and applied to the IME sensor. After the application of mouse plasma sample to the sensor, the impedance changes according to mouse plasma Aβ reaction with and without EPPS treatment were monitored, respectively. The impedance changes of WT mice samples were a median value of 4.5%, lower quartile of 4.1%, and upper quartile of 4.6% (Fig. 2, K and L). The impedance change of the TG mouse group without EPPS treatment exhibited a median value of 6.0%, lower quartile of 4.5%, and upper quartile of 6.7%. With EPPS treatment as in Fig. 2 (K and L), the WT mouse group yielded a median value of 4.9%, lower quartile of 4.4%, and upper quartile of 5.7%. The median value, lower quartile, and upper quartile of impedance change from the TG mouse group with EPPS treatment were 7.6, 5.3, and 9.2%, respectively. These results show that TG groups exhibited an increased signal in impedance changes compared with the WT after EPPS treatment, indicating that TG mouse plasma contained aggregated Aβ, which was dissociated by EPPS.

**Measurements of plasma Aβ levels in heterogeneous and monomerized states**

Demographics and clinical information of participants from two clinical institutes are described in Table 1. At AMC, a total of 53 plasma samples were obtained from patients with AD (n = 32) and individuals with NC (n = 21). Another 53 plasma samples were additionally obtained from 29 patients with AD and 24 individuals with NC at KIRAMS, Seoul, Korea. All individuals underwent clinical interviews, neurological examinations, detailed neuropsychological and laboratory tests, 3T MRI scans, and amyloid-PET scans. Each plasma sample collected from AMC and KIRAMS was divided into two aliquots: One was treated with 100 mM EPPS for Aβ monomerization and the other aliquot was left untreated, for use as a control containing heterogeneous Aβ species. Samples were then incubated at room temperature for 30 min on a rocker or shaker before they were subjected to analysis by the biosensor system for measurements. In both AD and NC groups, treatment of EPPS alone did not significantly differentiate one group from another (Fig. 3). Adding EPPS to samples did increase Aβ concentrations, similar to the results that we obtained from the mouse study, but it was not significant enough for us to confidently determine AD diagnosis. However, since the WT mice do not express human Aβ, it does not perfectly reflect human NC that showed altered concentrations of Aβ after EPPS treatment. Although the disaggregation of oligomeric Aβ into homogeneous monomers enabled us to measure the true concentration of plasma Aβ, we performed internal standardization of data to remove potential inter- and intraindividual variations.

**Comparative analysis of plasma Aβ levels in heterogeneous and monomerized states**

When we compared plasma Aβ levels before and after EPPS treatment per individual, we found a pattern where most individuals in the AD
Table 1. Demographics and clinical information of participants. Normally distributed variables were tested using a Student’s t test and presented with means and SD. Categorical variables were tested using a χ2 test and presented with relevant percentages of the variables. APOE, apolipoprotein; N.A., not applicable; HT, hypertension; DM, diabetes mellitus; HL, hyperlipidemia; MMSE, Mini-Mental State Examination; CDR, Clinical Dementia Rating; CDR-SB, Clinical Dementia Rating Scale–Sum of Boxes; GDS, Global Deterioration Scale; GDepS, Geriatric Depression Scale; SUVR, standardized uptake value ratio.

|                | AMC (n = 32) | NC (n = 21) | P   | KIRAMS (n = 29) | NC (n = 24) | P   |
|----------------|--------------|-------------|-----|-----------------|-------------|-----|
| Age, mean (SD), year | 70.3 (10.0) | 69.5 (9.4) | 0.7611 | 75.8 (6.6) | 60.7 (9.5) | <0.0001 |
| Female sex, no. (%) | 21 (65.6) | 16 (76.1) | 0.413 | 18 (62.1) | 19 (79.2) | 0.177 |
| Education, mean (SD), year | 9.6 (5.0) | 9.9 (4.8) | 0.832 | 8.6 (4.6) | 15.1 (3.2) | <0.0001 |
| APOE ε4 allele, no. (%) | 14/28 (50.0) | 4/17 (23.5) | 0.026 | N.A. | N.A. | N.A. |
| DM, no. (%) | 7 (21.9) | 3 (14.3) | 0.490 | 8 (27.6) | 2 (8.3) | 0.075 |
| HL, no. (%) | 12 (37.5) | 11 (52.4) | 0.285 | 8 (27.6) | 5 (20.8) | 0.570 |
| MMSE, mean (SD) | 18.7 (4.5) | 27.5 (2.0) | 0.0001 | 13.0 (7.5) | 29.4 (1.0) | <0.0001 |
| CDR | 0.5, no. (%) | 9 (28.1) | 9 (42.9) | 5 (17.2) | 0 (0.0) | 0.0021 |
| ≥1, no. (%) | 23 (71.9) | 0 (0.0) | 0.0001 | 24 (82.8) | 0 (0.0) | 0.0001 |
| GDS, mean (SD) | 4.4 (0.7) | 2.0 (0.2) | <0.0001 | 4.7 (1.3) | 1.0 (0.2) | <0.0001 |
| GDepS, mean (SD) | 14.2 (8.7) | 13.7 (8.7) | 0.820 | N.A. | N.A. | N.A. |
| Amyloid-PET SUVR, mean (SD) | 1.649 ± 0.232 | 1.240 ± 0.214 | <0.0001 | 1.445 ± 0.140 | 1.176 ± 0.064 | <0.0001 |

Fig. 3. Aβ measurements in plasma sample levels in heterogeneous and monomerized states. (A) AMC plasma samples: AD (n = 32) and NC (n = 21). (B) KIRAMS plasma samples: AD (n = 29) and NC (n = 24). Left-handed graphs of (A) and (B) indicate EPPS treatment. Right-handed graphs of (A) and (B) indicate EPPS treatment: impedance changes of Aβ in plasma without EPPS treatment. Red dots, patients with AD; black dots, individuals with NC. Two-tailed t tests were performed in statistical analyses (nonsignificant analysis is not indicated).

Fig. 3. Aβ measurements in plasma sample levels in heterogeneous and monomerized states. (A) AMC plasma samples: AD (n = 32) and NC (n = 21). (B) KIRAMS plasma samples: AD (n = 29) and NC (n = 24). Left-handed graphs of (A) and (B) indicate no treatment (Nontreat); impedance changes (|ΔZ|/Z0, %) of Aβ in plasma without EPPS treatment. Right-handed graphs of (A) and (B) indicate EPPS treatment (EPPS-treat): impedance changes of Aβ in EPPS-treated plasma. Red dots, patients with AD; black dots, individuals with NC. Two-tailed t tests were performed in statistical analyses (nonsignificant analysis is not indicated).

group showed increased Aβ levels, while those in the NC group did not exhibit altered Aβ levels (Fig. 4, A and B). To address the possibility that EPPS may dissociate Aβ from other proteins, we examined the effect of EPPS on Aβ and human serum albumin, which is the most abundant protein in blood (fig. S2) (13). The results indicated that EPPS did not uncover Aβ species bound to human serum albumin. Given that internal standards are difficult to apply in regard to plasma samples, we divided the concentration of homogeneous Aβ monomers in EPPS-treated plasma samples by that of heterogeneous Aβ in nontreated plasma samples to calculate a self-standard ratio. As hypothesized, patients with AD were significantly distinguished from individuals with NC in both institutes using the self-standard ratio (P < 0.0001) (Fig. 4, C and D). With minimum overlaps between the two groups, results maintained a sensitivity of 93% and a specificity of 97%. The cutoff values of self-standard ratio for the diagnosis of AD were comparable between the AMC (1.278 with a sensitivity of 90.5% and a specificity of 96.9%) and the KIRAMS (1.215 with a sensitivity of 91.7% and a specificity of 89.7%) (fig. S3). The clinical implication of the CLASS method for plasma Aβ was assessed with objective measurements of fibrillar forms of Aβ in the brain and cognitive function tests. The global standardized uptake value ratio (SUVR) of the amyloid-PET scans correlated with the self-standard ratio of plasma Aβ in both institutions (r = 0.5511, P < 0.0001 and r = 0.4141, P = 0.0021, respectively) (Fig. 4, E and F). The self-standard ratio of plasma Aβ also strongly correlated with the Korean version of the mini-mental state examination (K-MMSE) scores in both clinical settings (r = -0.5456, P < 0.0001 and r = -0.6011, P < 0.0001, respectively) (Fig. 4, G and H). Although a positive correlation was noted between the SUVR and CLASS analyses regarding the entire participant group (Fig. 4, E and F), the AD subgroup analysis in the KIRAMS dataset
(Fig. 4F) showed an inverse correlation. Use of different amyloid-PET tracers could have affected the results, and a follow-up study assessing longitudinal changes of the blood Aβ and brain plaques will enable further understanding of the findings. These results, in relation to those of the amyloid-PET and MMSE, suggest that our diagnostic method parallels the progression of cognitive impairment and amyloid plaque deposition in AD brains.

There are two clinical implications of the novel CLASS method. First, it showed significant correlations with the objective measurement of amyloid accumulation in the brain regardless of the tracers used for PET imaging ([18F]-florbetaben PET at AMC and [18F]-FC119S at KIRAMS). This suggests that the CLASS method can be applied to different amyloid-PET tracers available in clinical settings. Second, the new CLASS method showed correlations with diverse measurements of cognition, including MMSE. The correlations, however, were not maintained within each diagnostic group (patients with AD, red dots; individuals with NC, black dots; Fig. 4). As Aβ in the brain begins to accumulate and reach its plateau before clinical symptoms emerge, the information from numerous amyloid-PET scans has been used to differentiate disease status, but not to evaluate symptom progression in AD. Often, the correlation between Aβ and cognition has been reported in normal elderly individuals, but not in patients with AD in various clinical trials (14). Studies, including individuals with NC and patients with AD, have shown statistically significant, but not strong correlations between Aβ measurements and cognition, probably due to the saturated levels of Aβ in patients with AD who may still have worsening of symptoms with the disease progression (e.g., further aggregation of Aβ, neuronal loss, or tau accumulation) (15). Different from previous studies, strong correlations were noted between the self-standard ratio of plasma Aβ and cognitive measurement across the individuals with NC and patients with AD in our study. We interpreted the strong correlations between the self-standard ratio and cognition in the entire groups, including NC and AD, but not in each group as evidence that the CLASS method may capture active changes associated with the advancement from NC to AD. Longitudinal studies will provide further possible clinical applications for our findings.

**DISCUSSION**

In this study, we devised a biosensor system that sensitively and selectively measured the plasma Aβ of patients with AD who were previously diagnosed through conventional diagnostic methods: MMSE, MRI, and detective...
amyloid-PET. In addition, implementing the CLASS method in analyzing plasma treated with EPPS allowed us to not only reliably differentiate patients with AD from non-demented individuals but also propose a potential qualitative method to assess the severity of AD progression. It is noteworthy that the overall sensitivity and specificity remained unchanged in age- and gender-matched AD and NC subgroups. The present data further suggest that pathological progression of AD may be characterized by an increased concentration in soluble Aβ aggregates due to the fact that patients with AD exhibited increased concentrations of Aβ after EPPS-induced homogenization. Previous endeavors to establish correlations between Aβ levels and AD progression may have elicited inconsistencies due to the analysis of amyloid aggregates under heterogeneous conditions that indicated perplexing intra-individual fluctuations within patients on a weekly or monthly basis, which instigated the misinterpretation of Aβ as an unreliable biomarker (1, 3–5, 7).

The four common neural imaging methods consist of structural and functional MRI, [18F]-fludeoxyglucose (FDG)–PET, and amyloid-PET. Brain [18F]-FDG-PET analyzes the intracerebral glucose metabolism to characterize synaptic activity, while amyloid-PET imaging reflects the pathogenic progression of AD by physically depicting Aβ plaques (16). Through the comparison of results obtained from various PET techniques, researchers found that [18F]-FDG–PET exhibited higher specificity, whereas amyloid-PET had heightened sensitivity and enhanced capabilities in the differential diagnosis of varying denominations of dementia (17). Thus, these modalities are used to characterize Aβ, which is one of the select few neuropathology-related proteins that can penetrate the blood-brain barrier, in an attempt to diagnose possible patients in the prodromal stages of AD. Our method of analyzing levels of amyloid oligomers has the potential to enhance current evaluative approaches when used in tandem with modern diagnostic criteria. The integration of these two diagnostic devices can deliver the means necessary for enhanced screening to provide accurate prognosis for those with increased risk for AD.

Although the implications of our research on current diagnostic models are positive, our results could be enhanced through the application of different Aβ antibodies. In this experiment, we used the monoclonal 6E10 antibody, which is ineffective in distinguishing between full-length amyloid derivatives because of the antibody’s tendency to interact with the N terminus of Aβ. The fluctuations of amyloid isoforms in cerebrospinal fluid depend on their classification as either Aβ40 or Aβ42, since the former does not fluctuate as AD advances. However, conflicting reports state that the latter increases (18), decreases (19), or experiences an insignificant change (20, 21) as cognition is impaired. Thus, cohort studies designed to differentiate amyloid isoforms would allow for enhanced specificity in disease diagnosis, since distinguishing aggregation patterns between Aβ40 and Aβ42 may aid in delineating AD progression. In addition, monomer-specific antibodies, such as m266, may refine the results of CLASS methods (22).

Since Aβ biomarkers are found in a variety of biofluids, such as blood, tears, and saliva, the analytical methods implemented in this research may also be applied to the quantification of amyloid oligomers located in other regions (23). However, it still remains to be further investigated whether blood biomarkers can be used to undoubtably and consistently aid in the differential diagnosis of AD or predict the progression of AD symptoms in at-risk patients and patients with mild cognitive impairment by cohort studies. Additional studies are warranted to determine whether this diagnostic method targeting soluble Aβ aggregates in plasma is solely capable of differentiating patients with AD from non-demented individuals with AD neuropathology who manifest similar pathology indicators without symptoms of cognitive decline (24, 25).

Our results can be further validated by implementing multicenter studies, in which the CLASS method and EPPS can be simultaneously used. Current research methods detect variations of Aβ in patient plasma and cerebrospinal fluid by using ultrasensitive digital ELISA (23, 26, 27). Recent endeavors to enhance the sensitivity of single-molecule ELISA have successfully detected proteins in blood at concentrations less than a femtomolar (28). However, studies conducted with this technology resulted in contradictory findings regarding the concentration of amyloid biomarkers in patient biofluids (26, 27). Although ELISA already provides a high-throughput method that is both highly sensitive and specific to Aβ42, its faults lie in the fact that the antibodies used to heighten ELISA’s sensitivity might bind to other amyloid isoforms, provide no information regarding the size of the aggregates, and inadequately calculate oligomeric forms of Aβ in comparison to monomers (29–31). Through the successful integration of modern ELISA methods with CLASS, researchers may be capable of better conceptualizing the role of Aβ aggregates in the pathogenesis of AD. Future studies should further explore the implementation of CLASS as a method to distinguish between diseases analogous through the presence of amyloid variants and the applications of the IME chip in characterizing other fluid biomarkers that require higher sensitivity and specificity during detection by modifying the antibody within the device.

**MATERIALS AND METHODS**

**Plasma Aβ disaggregation**

In-house synthetic Aβ42 peptides (32, 33) were dissolved in dimethyl sulfoxide to obtain a 10 mM stock. The stock was diluted with PBS to make a 10 μM Aβ42 solution. The Aβ42 solution was incubated at 37°C for 72 hours to obtain aggregates including oligomers and then confirmed via silver staining with photoinduced cross-linking of unmodified protein chemistry method (34). Prepared Aβ42 aggregates were spiked into citrated human plasma (P9523, Sigma-Aldrich) to make final concentrations of 400, 200, and 100 pg/ml. Each Aβ42 plasma solution was aliquoted into two samples and was incubated with or without EPPS (200 mM) at 4°C for 2 hours to disaggregate Aβ42 oligomers. Prepared samples were seeded to the Aβ42 sandwich ELISA kit (KHB3442, Invitrogen), and then concentrations of Aβ42 were measured.

**Ethical regulations**

All animal experiments were carried out in accordance with the National Institutes of Health (NIH) Guide for the Care and Use of Laboratory Animals and the Animal Institutional Animal Care and Use Committee of Korea Institute of Science and Technology (KIST) (Seoul, Korea). Institutional Review Board (IRB) approval for studies with human participants was obtained for this project.

**Animal preparation and plasma collection**

Doubly mutated APP/PS1 TG mice [strain name: B6C3-Tg (APPswe, PSEN1De9) 85DBoi/+] and WT (B6C3F1) mice were obtained from the Jackson laboratory. Nine-month-old WT or TG male mice were prepared for blood collections (n = 9 per group). Mouse blood samples (approximately 1 ml per mouse) were collected into heparin vacutainer tubes (BD Vacutainer, BD), inverted gently to mix, and then centrifuged.
for 15 min at 3000 rpm in 4°C. Approximately 0.5 ml of plasma over the
buffy coat was gently obtained and aliquoted into 1.5-ml polypropylene
tubes. Protease inhibitor (PI; Complete, Mini, Roche) solution was
added to each aliquot. Samples were immediately frozen and stored
in a −80°C deep freezer for further analysis.

Device fabrication
The IME chip is composed of microfluidic channels that were fabricated
with micro/nanofabrication techniques. First, a 10.16 cm P-type silicon
wafer was prepared for IME chip fabrication. A 300-nm silicon dioxide
(SiO2) layer acts as an insulating layer, which was grown by thermal
oxidation. The 100-nm platinum (Pt) layer acts as an electrode and is
sequentially deposited by sputtering onto a 30-nm titanium (Ti) adhe-
sion layer. For micropatterning, the photoresist (PR; AZ GXR-601, AZ
Electronic materials) was covered on the Pt layers with a spin coater at
3000 rpm for 30 s and baked on a hot plate at 95°C for 150 s. The PR-
covered wafer was exposed to ultraviolet (UV) rays of G-line using an
aligner (MA6, Karl Suss). After UV exposure, the wafer was developed
with the developer (CD30, AZ Electronic Materials). The developed
wafer was baked on a hot plate at 115°C for 180 s. To build the Pt
electrode pattern, the Pt/Ti layers were etched using an inductively
coupled plasma–reactive ion etcher (Oxford Instruments). The PR
was removed using an oxygen plasma asher (microwave plasma ash,
Plasma Finish).

For delivering samples to the IME sensors, the PDMS-based micro-
fluidic channel chip mold was fabricated with micro/nanofabrication.
The cleaned silicon wafer was covered with epoxy-negative PR (SU-8
2050, MicroChem) using a spin coater at 4000 rpm. For soft baking,
PR-covered wafers were baked on a hot plate at 95°C for 6 min. The
spin-coated wafer was exposed to UV rays of G-line for 14 s using an
aligner. For exposure baking, the wafer was heated on a hot plate at 95°C
for 6 min. To build the microfluidic channel pattern, the wafer was
developed and rinsed with a developer (SU-8 developer, MicroChem)
and isopropyl alcohol (IPA) sequentially. The PDMS complex
(PDMS:elastomer, 10:1) with a silicon elastomer (Sylgard 184, Dow
Corning) was poured into the microfluidic channel mold and baked
in an oven at 80°C for 1 hour. The PDMS channel was aligned and
attached onto the IME chip.

Functionalization process
To remove contamination and form hydroxyl groups, the IME chip was
dipped in a piranha solution (H2SO4:H2O2, 5:1) for 30 min. The chip
was rinsed and dried with DI water and nitrogen (N2) gas sequentially.
After the surface of the IME chip was cleaned, it was immersed in APMES
solution (1% in IPA, Sigma-Aldrich) for 3 hours and rinsed with IPA
and DIW. To chemically modify the surface of the chip, it was treated
using PVP-CHO solution (10 mM in 100 mM NaHCO3 buffer solution)
for 6 hours and sodium borohydride solution (NaBH4; 10 mM
in 100 mM NaHCO3 buffer solution) for 1 hour. The IME chip was
treated with a glutaraldehydesolution (1% in 100 mM NaHCO3 buffer
solution) for 30 min. The incubated samples were injected onto the IME unit
chip, and impedance changes were monitored.

Signal process of the sensor’s output
An impedance measurement system, with signal processing, was
prepared to measure the impedance of interactions between Aβ and
the 6E10 antibody. Low-level signals were measured by preventing elec-
trical noise signals from parasitic capacitance of IME. This was achieved
by embedding a signal cancellation and amplification module into the
impedance measurement system. Most of the noise resulted from the
biomolecule-containing buffer solution and relatively long electrode
length of the IME. In the IME chip, Zbiomolecule (functional IME) and
Zliquid (reference IME) represent the biomolecule impedance and buffer
solution impedance, respectively. The impedance change was attributed
to Zbiomolecule, while Zliquid is dominant in comparison with other
impedance components. After the measuring voltage was applied onto the
IME chip, the sample was loaded onto the surface of the chip. The func-
tional IME detected the impedance of biomolecules and buffer solutions
in the sample, whereas the reference IME only detected the buffer so-
lution impedance within the same sample. The cancellation process-
ing assisted in removing Zliquid and other impedance components.
For cancellation of the parasitic capacitance, the measured impedance
of biomolecules and buffer solutions was compared and calculated.
After the cancellation process, low-level signals were amplified with a
differential amplifier and digital signal processing. This output signal
was used to measure the interaction between Aβ and the 6E10 antibody.

Data analysis procedure and sample treatment
For data analysis, specific data processing was used to increase the re-
liability and reproducibility. First, the impedance of each fabricated IME
was measured to identify faulty IME (error range, >5%). The plasma
samples were prepared for the clinical test. Each real Aβ sample was
injected into eight IME sensors, and the impedance of eight IME sensors
was monitored independently. The rate of impedance change of the
eight IME sensors was calculated to minimize the deviation of IME im-
pedance. Using the average of impedance changes as a standard, the
impedance changes of five IME sensors were sorted out. Last, the aver-
age of impedance changes of five IME sensors, where the impedance
changes were close to the average of impedance by the truncated mean,
was used as the data for the clinical test.

For sample storage and delivery, the human blood samples were
dep-frozen and stored at −80°C. To prepare the samples for treatment,
they were defrosted at 4°C for 1 hour. Then, the plasma sample was
separated in half. EPPS solutions were prepared at 500 mM by dissolv-
ing the chemical powder with PBS, which can be replaced with citrated
human plasma (P9523, Sigma-Aldrich). EPPS (E9502, Sigma-Aldrich)
or PBS was added into either half of the plasma samples at 1:4 (v/v),
respectively. The mixed samples were incubated at room temperature
for 30 min. The incubated samples were injected onto the IME unit
chip, and impedance changes were monitored.

Demographics and clinical information
Demographics and clinical information of participants from two clinical
institutes are described in Table 1. All 53 participants, 32 patients
with AD and 21 individuals with NC, underwent clinical interviews,
neurological examinations, detailed neuropsychological and labora-
tory tests, 3T MRI scans, and [18F]-Florbetaben–PET scans at AMC,
Seoul, Korea. The following information was obtained from each par-
ticipant: age; gender; years of education; history of hypertension, dia-
etes mellitus, and hyperlipidemia; and apolipoprotein E4 allele status.
Scores or grades for K-MMSE, Clinical Dementia Rating (CDR), Global
Deterioration Scale (GDS), and Geriatric Depression Scale (GDepS)
were compared between the groups. Each patient with AD had a positive amyloid-PET scan and fulfilled the diagnostic criteria for probable AD proposed by the National Institute of Neurological and Communicative Disorders and the Alzheimer’s Disease and Related Disorders Association (35). Age- and gender-matched individuals with NC met the following criteria: (i) no history of neurological or psychiatric disorders except for memory complaint, (ii) NC measured by neuropsychological tests, (iii) normal activities of daily living, (iv) no brain structural lesions, and (v) negative amyloid-PET scans (36). Individuals with NC from the AMC cohort included individuals with CDR 0.5 who had subjective, but not objective, evidence of memory impairment (fig. S4). The IRB of AMC approved the study protocol, and informed consent was obtained from each participant. There was no difference in demographics between the groups. The AD group had more patients with CDR >0.5, higher CDR Scale–Sum of Boxes (CDR-SB) and GDS scores, and lower MMSE scores compared with the NC group. The global SUVRs measured in diverse methods demonstrated higher amyloid uptake in the AD group compared with the NC group (Table 1). Another 53 plasma samples were additionally obtained from 29 patients with AD and 24 individuals with NC at KIRAMS, Seoul, Korea. All the individuals from KIRAMS underwent a [18F]-FC119S amyloid-PET scan (12). The Korea Food and Drug Administration and the IRB of KIRAMS approved the study protocol. The AD group was older, and the NC group had a shorter education period. There was no difference in other demographics between the groups. The AD group had higher CDR, CDR-SB, and GDS scores and lower MMSE scores compared with the NC group (Table 1).

Neuropsychological test

All patients underwent neuropsychological tests using the Seoul Neuropsychological Screening Battery (37). This included the following cognitive domain–specific functions: digit span forward and backward, for attention; the Korean version of the Boston Naming Test (38), for language; the Rey-Osterrieth Complex Figure Test (RCFT) copy test, for visuospatial function; the Seoul Verbal Learning Test immediate recall, delayed task, and a recognition task, for verbal memory; RCFT immediate recall, delayed recall task, and a recognition task, for assessing visual memory function; and the phonemic and semantic Controlled Oral Word Association Test and a Stroop test (color reading), for frontal/executive function.

MRI and amyloid-PET acquisition

MRI images were obtained using the protocol described in Table 2 and previous reports (39, 40). [18F]-florbetaben and [18F]-FC119S amyloid-PET images were collected using the acquisition methods described in Table 2. Briefly, a single dose of 300 MBq ± 20% [18F]-florbetaben was intravenously administered in a maximum volume of 10 ml at AMC. PET images were acquired from 90 to 110 min after injection with a three-dimensional (3D) list-mode acquisition setting on the scanners. The 5 min × four frames of PET scans were reviewed for any motion artifacts, and motion-free frames were selected and summed. Last, 20-min static PET images were used to obtain SUVR for further analysis.

Quantitative amyloid-PET analysis

FreeSurfer 5.1 was used to generate automatic volumes of interest (VOIs) for the quantitative PET analysis. First, the PET image of each patient was co-registered to the corresponding 3D T1 MR image using a rigid-body transform-based registration method using Statistical Parametric Mapping 8 (SPM8; www.fil.ion.ucl.ac.uk/spm/software/spm8/). After automated segmentation, correction was made when necessary in accordance with the FreeSurfer manual (http://surfer.nmr.mgh.harvard.edu/fswiki/). The global SUVR was calculated from lobar VOIs in AD signature areas (occipital, parietal, temporal, frontal, medial frontal, central, and posterior cingulate cortices, precuneus, and hippocampus/amygdala) using the cerebellar cortex as a reference (41, 42).

Plasma collection from AMC

AMC blood samples (5 ml per individual) were collected into heparin vacutainer tubes (BD Vacutainer, BD), inverted gently to mix, and then centrifuged for 15 min at 3000 rpm in room temperature. Approximately 2.5 ml of plasma over the buffy coat was obtained and aliquoted into 500-μl polypropylene tubes with a minimum amount of 375 μl per tube (>75% of the tube volume). Samples were immediately frozen, stored in a −80°C deep freezer, and delivered to the plasma biomarker detection team on dry ice for further analysis. Frozen plasma samples were thawed before adding the PI (Complete, Mini, Roche).

Plasma collection from KIRAMS

KIRAMS blood samples (10 ml per individual) were collected into heparin vacutainer tubes (BD Vacutainer, BD) and immediately delivered...
to the plasma biomarker detection team on ice. Blood samples were inverted gently to mix and then centrifuged for 15 min at 3000 rpm in 4°C. Approximately 5 ml of plasma over the buffy coat was obtained and aliquoted into 1.5-mL polypropylene tubes with a minimum amount of 500 µL per tube. PI solution (cOmplete, Mini, Roche) was added to each aliquot. Samples were immediately frozen and stored in a −80°C deep freezer for further analysis.

**Statistical analysis**

Statistical analyses were conducted with GraphPad Prism 7 using Student’s two-tailed t test comparisons, repeated-measures analysis of variance (ANOVA) or one-way ANOVA, followed by Bonferroni’s post hoc comparisons (**P < 0.05, ***P < 0.01, ****P < 0.001); other comparisons were not significant). Error bars represent SEMs.

**SUPPLEMENTARY MATERIALS**

Supplemental material for this article is available at https://advances.sciencemag.org/cgi/content/full/5/4/eaav1388/DC1

**Fig. S1.** Antibody-antigen responses on IME sensor.

**Fig. S2.** Gel electrophoresis of samples obtained through immunoprecipitation.

**Fig. S3.** Sensitivity and specificity.

**Fig. S4.** CDR scores of AMC participants.

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Competing interests: K.S.H., J.K., and Y.K.Y. are inventors on a patent related to this work issued by the KIST (no. KR101754239, registered on 6 July 2017). Y.K., H.Y.K., S.L., K.S.H., J.H.R., and Y.K.Y. are inventors on another patent related to this work issued by the KIST (no. US10006920, registered on 26 June 2018). The authors declare that they have no other competing interests.

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