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RESEARCH ARTICLE

Aging and *APOE-ε4* are determinative factors of plasma Aβ42 levels

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Abstract

Objective: The aim of this study was to confirm determinative factors for plasma Aβ and its association with cognitive function. **Methods:** Fasting plasma Aβ40 and Aβ42 levels were measured by ELISA in 1019 participants in the Iwaki Health Promotion Project. The relationships between plasma Aβ and health-related items, including physical characteristics, cognitive function tests, blood chemistry, and *APOE-ε4* genotype were analyzed. **Results:** The plasma levels of Aβ40 and Aβ42, and Aβ40/42 ratio were found to significantly increase with aging. The age-dependent increase in Aβ42 level was significantly suppressed by *APOE-ε4*. Renal function was an associated factor for the plasma Aβ40 level. The plasma Aβ42 level and Aβ40/42 ratio correlated with cognitive function. **Interpretation:** Age and *APOE-ε4* are major determinative factors of plasma levels of Aβ42 and the Aβ40/42 ratio. These factors are critical adjustment factors for the usage of plasma Aβ as a biomarker of central nervous system amyloidosis.

Introduction

Alzheimer's disease (AD) is observed at a critical rate due to the aging population. The latest research suggests that it is possible to prevent pathological processes in AD by developing disease-modifying therapies, such as anti-Aβ antibodies and BACE-1 inhibitors, against Aβ amyloidosis, which act on pathological cascades, including tauopathy. Prospective cohort studies have reported that the ratio of Aβ40/42 is significantly associated with late-life cognitive decline,[1] and risk of developing MCI and AD.[2–6] Systematic reviews and meta-analyses have also suggested that the plasma Aβ40/42 ratio can predict the development of AD and dementia.[7] However, these findings indicated significant heterogeneity,[7] and plasma levels of Aβ40 and Aβ42 alone were not significantly associated.[8,9]

The Alzheimer's Disease Neuroimaging Initiative (ADNI) and the Dominantly Inherited Alzheimer Network (DIAN) have confirmed the efficacy of neuropsychiatric tests and neuroimaging using cerebrospinal fluid (CSF) biomarkers, including amyloid PET, demonstrating that signatures of brain Aβ amyloidosis can be found approximately 30 years before the onset of dementia.[10,11]

Recent studies have clarified that the plasma Aβ42/40 ratio is inversely correlated with cortical amyloid burden in AD, which can be converted into MCI,[12,13] and that the plasma Aβ42/40 ratio is a useful screening marker for brain Aβ amyloidosis in normal individuals.[14,15] Approximately 30–50% of Aβ in the plasma originates from the brain.[15] Age, *APOE-ε4,* and AD pathology are specific determinants of Aβ turnover kinetics from the brain to CSF, and finally to plasma.[15,16]

We therefore focused on determinant factors of plasma Aβ levels. As Aβ amyloidosis initiates midlife, it is necessary to analyze these factors in large community-based studies on young adolescent to elderly subjects. Age and *APOE-ε4* are two major factors accelerating CNS amyloidosis leading to the onset of AD dementia.[17] The gene dose of *APOE-ε4* may decrease plasma Aβ42 levels with natural aging, or long-term preclinical stage of AD dementia.[10,17] For this reason, basic information on how plasma Aβ levels are regulated over time by blood biochemical factors, cognitive function, and lifestyle remains to be clarified in order to adjust plasma Aβ levels for CNS amyloidosis-specific markers.[18,19] Here, we analyzed definite factors of plasma Aβ of participants in The Iwaki Health Promotion Project (IHPP) in 2014, a community-based annual health checkup study designed to prevent and improve lifestyle-related diseases and quality of life.

Materials and Methods

Subjects

A total of 1109 participants with complete data sets out of 1167 enrolled participants were analyzed. The age of 619 participants ranged from 19 to 59 years (mean age of 54 years; 365 females) and 490 participants were older than 60 years of age (mean age of 68 years; 323 females). The baseline characteristics of participants are presented in Table 1. Clinical diagnoses of dementia, Alzheimer dementia (AD), and mild cognitive impairment (MCI) were based on the NIA-AA clinical criteria.[20,21] A total of 200 medical and paramedical staff examined participants between 6:30 to 13:00 over 10 days at Iwaki culture center. After written informed consent, a mini-mental state examination (MMSE) for all participants, the logical memory II tests (delayed recall: LM-II) from the Wechsler Memory Scale-Revised (WMS-R), and a detailed questionnaire for memory disturbances and ADL conditions were performed for participants older than 60 years of age. During and after these items, medical and neurological examinations, motor performance, blood pressure, height, body weight, BMI, and body fatty ratio (BFR) were evaluated, and common laboratory tests were performed for complete blood cell count, nutrition, liver and renal function, diabetes mellitus, cholesterol and lipid metabolism, endocrine system, immunology, cardiovascular biomarkers, and urine analysis (details in Tables S1 and S2).

Aβ40 and Aβ42 Quantitation

Ten milliliters of morning fasting blood was taken into an EDTA-2Na tube and immediately centrifuged at 1400 g for 10 min, separated to plasma in a polypropylene tube, and stored frozen at −80°C until use. Sandwich ELISA was used to quantify plasma Aβx-40 and Aβx-42 levels using a Human/Rat β Amyloid (40) ELISA Kit Wako II and a Human/Rat β Amyloid (42) ELISA Kit Wako High-Sensitive (Wako Pure Chemical Industries, Ltd, Osaka, Japan).[22,23] Microplates were precoated with monoclonal BNT77 (IgA, anti-Aβ11-28, specific for Aβ11-16) and sequentially incubated with 25 μL of samples, followed by application of horseradish-peroxidase-conjugated BA27 (anti-Aβ1-40, specific for Aβ40) or BC05 (anti-Aβ35-43, specific for Aβ42/43). The sensitivity was 0.049 pmol/L (assay range 1.0–100 pmol/L) in the Aβ40 assay and 0.024 pmol/L (assay range 0.01–20.0 pmol/L) in the Aβ42 assay. Intra- and interassay coefficients of variation were less than 10% for both

Table 1. Baseline characteristics of participants in the IHPP.

Characteristics (average and SD)	Total population	19–59 y	60–92 y
Number of participants	1109	619	490
Age (y)	54.2 (15.3)	43.1 (10.4)	68.2 (6.4)
Gender (female/male)	688/421	365/254	323/167
Height (cm)	160.1 (9.3)	163.6 (8.6)	155.6 (8.1)
Weight (kg)	58.4 (11.3)	60.2 (12.4)	56.1 (9.2)
Education (years)	11.8 (1.8)	12.5 (1.5)	11.0 (1.8)
MMSE score	29.3 (1.3)	29.7 (0.7)	28.7 (1.7)
Aβ40 (pmol/L)	106.2 (15.5)	100.3 (12.9)	113.5 (15.3)
Aβ42 (pmol/L)	11.36 (1.70)	11.0 (1.55)	11.8 (1.80)
Aβ40/Aβ42 ratio	9.42 (1.10)	9.16 (0.98)	9.74 (1.16)
Number of *APOE-ε4* alleles			
0 (*ε2/ε3, ε3/ε3*)	878	478	400
1 (*ε2/ε4, ε3/ε4*)	225	135	90
2 (*ε4/ε4*)	6	6	0
Alzheimer's dementia	2	N.D.	2
Mild cognitive impairment	26	N.D.	26
Normal	1081	619	462

SD: standard deviation; MMSE: mini-mental state examination; y: years of age; N.D.: not determined.

Aβ40 and Aβ42. After excluding samples with mean values over +3 standard deviation by Grubbs' method,[24,25] 1091 assay values were analyzed.

APOE genotyping

DNA of 1,151 Iwaki residents was purified from peripheral whole blood using the QIAamp® 96 DNA Blood Kit (QIAGEN, Hilden, Germany), and *APOE* genotype was determined by Toshiba corporation using the Japonica Array consisting of population-specific SNP markers designed from the 1070 whole genome reference panel.[26,27] Fifty-three samples that were not determined by the microarray analysis were genotyped by direct sequencing by the Greiner corporation using the following primer set: Forward primer; 5′ TGG ACG AGA CCA TGA AGG AGTT and reverse primer; CAC CTG CTC CTT CAC CTC GTCCA, except for 11 samples that we analyzed using the following primer set: Forward primer; 5′ TGG ACG AGA CCA TGA AGG AGT and reverse primer; CAC CTG CTC CTT CAC CTC GTCCA.

Statistical analysis

Plasma Aβ40, Aβ42, Aβ40/42 ratios did not deviate significantly from normal distribution according to the histograms. To clarify the relationships between plasma Aβ levels and other factors, including blood examination data, life style, and motor functions, correlation analysis was used. For comparison of normal distribution factors, Pearson's correlation coefficient analysis was applied. If normalization was not possible, Spearman's rank correlation coefficient analysis was used. To examine the effects on plasma Aβ by aging, linear regression models were used. To plot the age-dependent changes in plasma Aβ, the simple linear regression model was applied, and the linear regression line was drawn by the method of least squares. To compare the significance between the slopes of the linear regression models and to adjust for confounding factors, multiple regression analysis was applied. To examine whether Aβ and cognitive function are related, we compared the plasma Aβ levels between the high MMSE scores group (29 or 30) and low MMSE scores (less than 29) in subjects aged 60 years and over. In this group comparison, multiple logistic regression was used to adjust for age. Two-tailed *P*-values less than 0.05 were considered significant. These analyses were performed with IBM SPSS Statistics, version 24 (IBM Japan, Tokyo) and GraphPad Prism, version 7 (GraphPad Software, San Diego, CA). In this study, statistical analyses were conducted with all 1019 participants, including 991 normal, 26 MCI, and 2 AD dementia individuals.

Results

Plasma Aβ Levels and relationship with *APOE* genotype

The mean±SD of the Aβ40 plasma level was 106.2 ± 15.5 pmol/L, that of the Aβ42 level was 11.36 ± 1.7, and that of the Aβ40/42 ratio was 9.42 ± 1.1 in all participants. A significant linear increase with age was observed for Aβ40 levels ($Y = 0.4724X + 79.65$, $r^2 = 0.2208$, $P < 0.0001$), Aβ42 levels ($Y = 0.02466X + 10.04$, $r^2 = 0.04898$, $P < 0.0001$), and the Aβ40/42 ratio ($Y = 0.02234X + 8.113$, $r^2 = 0.09725$, $P < 0.0001$) (Fig. 1A–C).

To evaluate whether the *APOE-ε4* alleles affect plasma Aβ levels, age-dependent changes in plasma Aβ levels between *APOE-ε4* carriers and noncarriers were analyzed. Age-dependent increases in Aβ40 levels were observed in both non-*APOE-ε4* allele carriers ($Y = 0.4619X + 80.29$, $r^2 = 0.2163$, $P < 0.0001$) and *APOE-ε4* carriers ($Y = 0.5153X + 77.08$, $r^2 = 0.2389$, $P < 0.0001$). Aβ42 levels were increased in noncarriers ($Y = 0.02984X + 9.842$, $r^2 = 0.07497$, $P < 0.0001$) but not in *APOE-ε4* carriers ($Y = 0.0001912X + 10.92$, $r^2 = 0.00002616$, $P = 0.8068$) with aging. The Aβ40/42 ratios were increased both in noncarriers ($Y = 0.01701X + 8.327$, $r^2 = 0.066$, $P < 0.0001$) and carriers ($Y = 0.04561X + 7.159$, $r^2 = 0.2658$). Plasma Aβ40 and Aβ42 levels, and the Aβ40/42 ratio increased with aging, except for Aβ42 levels in *APOE-ε4* carriers by simple linear regression (Fig. 2A–F).

After adjusting for total protein, platelet count, and creatinine levels, which were previously reported as confounding factors for plasma Aβ levels,[18,19] the multiple linear regression model was used to clarify whether the age-dependent increases in Aβ levels were affected by *APOE-ε4*. There were significant differences between carriers and noncarriers in regression lines of Aβ42 ($P < 0.0001$) and Aβ40/42 ($P < 0.0001$) but not Aβ40 ($P = 0.76$) (Fig. 3A–B, details in Table S3). To further validate these results, multiple linear regression model analyses were performed after adjustments for hemoglobin, platelet count, albumin, creatinine, blood urea nitrogen, fasting plasma glucose (FPG), free fatty acid, hemoglobin A1c, and cystatin C, which were all found to be correlated with both plasma Aβ40 and Aβ42 levels in our study. There were also significant differences between carriers and noncarriers in regression lines of Aβ42 ($P = 0.001$) and Aβ40/42 ($P < 0.0001$) but not Aβ40 ($P = 0.923$) (details in Table S4). Thus, the age-dependent increases in Aβ42 levels were suppressed by *APOE-ε4*, whereas age-dependent increases in the Aβ40/42 ratio were enhanced by *APOE-ε4*.

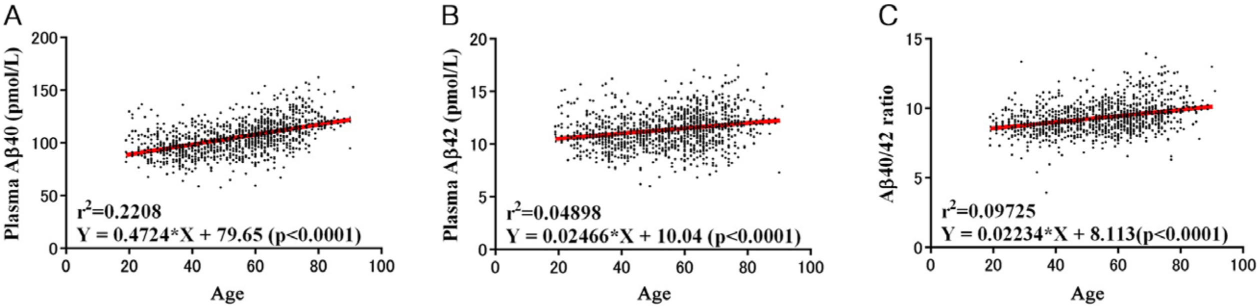

Figure 1. Age-related plasma Aβ changes. The relationship between age and plasma levels of Aβ or the Aβ40/42 ratio analyzed by linear regression. Determination coefficients (r^2) and regression equations are shown (N = 1109). Significant linear increases with age were observed for plasma Aβ40 and Aβ42 levels, and Aβ40/42 ratio (A-C).

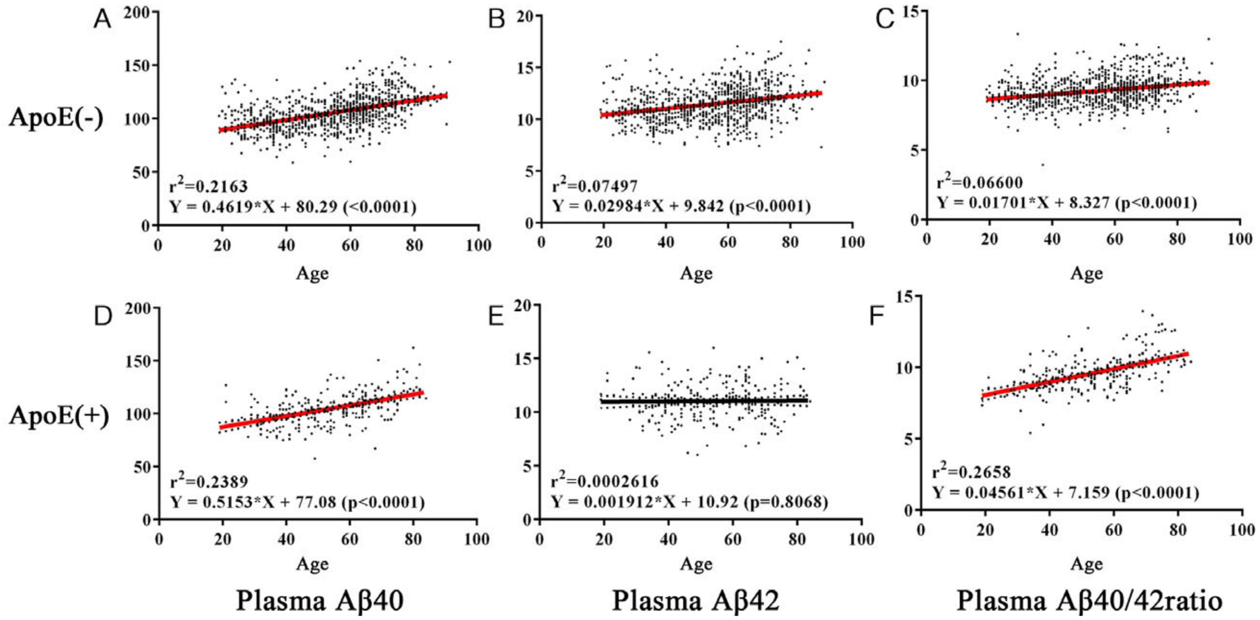

Figure 2. *APOE-ε4* suppresses age-dependent plasma Aβ increases. Analyses of the age-related plasma Aβ changes were performed for *APOE-ε4* carriers and noncarriers separately. Age-dependent increases in Aβ40 levels and the Aβ40/42 levels were observed in both noncarriers (A, C) and *APOE-ε4* carriers (D, F). Levels of Aβ42 were increased in noncarriers but not in *APOE-ε4* carriers with aging (B, E).

Association between MMSE scores and plasma Aβ levels

Subjects aged 60 years old and over were separated into high MMSE score (30, 29 points; n = 340) or low MMSE score (less than 28 points; n = 150) groups. Plasma Aβ40, Aβ42, and Aβ40/42 ratio levels were plotted, and an asterisk was plotted when there were significant differences between the two groups on multiple logistic regression analyses after adjusting for age (Fig. 4A–C). There was no significant difference in variables for Aβ40 levels (P = 0.25). However, significant differences in variables for both age and Aβ42 were observed for Aβ42 (P < 0.0001 and P = 0.04), and also by the model chi-squared test (P < 0.0001). The Hosmer-Lemeshow test demonstrated good predictability (P = 0.502), with a discrimination predictive value of 69.0%. On analysis of the plasma Aβ40/42 ratio, there were significant differences in both age and Aβ ratio (<0.0001 and P = 0.046), and by the model chi-squared test (P < 0.0001). Predictability was good (P = 0.502), with a discrimination predictive value of 70.2% (details in Table S5). There were no significant differences in Aβ concentrations between "AD and MCI group" and "randomly selected age and APOE genotype-matched high MMSE score group (28 participants)". Each P value was

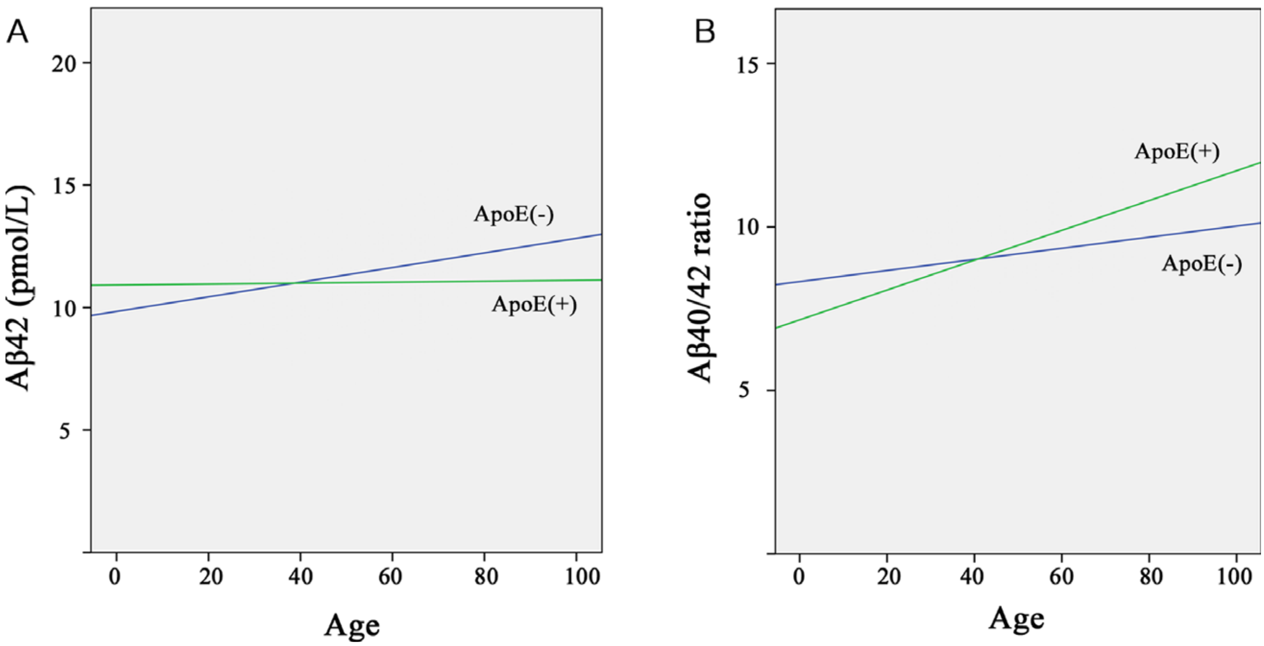

Figure 3. *APOE-ε4* alters age-dependent Aβ42 levels and Aβ40/42 ratio. The regression lines for age-related plasma Aβ42 and the Aβ40/42 ratio in *APOE-ε4* carriers and noncarriers were merged. There were significant differences between carriers and noncarriers in regression lines for Aβ42 (A) and Aβ40/42 (B) after adjusting for total protein, platelet count, and creatinine levels.

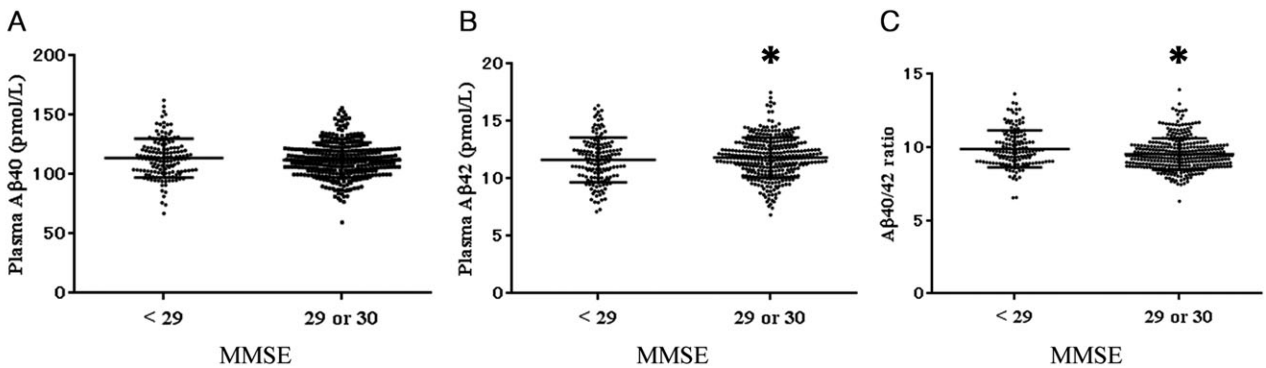

Figure 4. Correlation between MMSE scores and plasma Aβ levels. Comparison of plasma Aβ levels between high MMSE score and low MMSE score groups of subjects aged 60 years and over. There were significant differences (*) between the two groups in Aβ42 levels and the Aβ40/42 ratio on multiple logistic regression analyses after adjusting for age (A-C).

0.8838 in Aβ40 level, 0.4647 in Aβ42 level, and 0.2158 in Aβ40/42 ratio.

Factors affecting plasma levels of Aβ

Although the other blood chemistry test items were found to have significant linear correlations with Aβ levels, the correlation coefficients were very low. A strong correlation was only noted between cystatin C levels and Aβ40 levels ($r = 0.5276$). These results are shown in Tables S1 and S2. We additionally analyzed the correlation between plasma Aβ levels and habits or physical conditions. Weak correlations between both Aβ40 and Aβ42 levels, and alcohol intake, smoking amount, body fat ratio, and muscle mass were observed. Measurements of four major complex motor reaction tests, including the ruler drop test, timed up and go test, 10 m walk test, and whole-body reaction time test, were more associated with plasma Aβ40 and Aβ42 levels than simple muscle strength, but the correlation coefficients were low.

Discussion

Our results demonstrated the following: (1) Fasting plasma levels of Aβ40 and Aβ42, and the Aβ40/42 ratio age-dependently increased from 20 years old. (2) The presence of *APOE-ε4* suppressed these age-dependent increases in plasma Aβ42 levels. (3) Age and *APOE-ε4* were most significant factors for plasma Aβ42 levels and Aβ40/42 ratios after adjusting for previously indicated and newly examined factors, including blood chemistry, life style, and activity. (4) Only renal function was a definitive factor for plasma Aβ40 levels. (5) Plasma Aβ42 levels and Aβ40/42 ratios were correlated with lower MMSE scores in subjects aged over 60 years.

With a longer follow-up, repeated measurement of plasma Aβ may be useful as a simple and minimally invasive screening procedure to detect brain Aβ amyloidosis.[14–16] Aβ in plasma does not only originate in the brain because it is also involved in amyloid precursor protein (APP) metabolism in peripheral organs, it binds to several proteins and lipoproteins, is partially released from activated platelets, and is metabolized in the liver and cleared through the kidneys.[19] However, a recent study suggested that 30–50% of plasma Aβ originates from the CNS.[15] *APOE-ε4* is the strongest genetic risk factor for sporadic late onset AD, and markedly accelerates Aβ amyloid deposition in the brain and the onset age of dementia by approximately 10 years.[10,17] Recent studies have revealed that CNS-derived Aβ is cleared into the CSF[28] and peripheral blood,[29] and that the clearance rate is decreased in late onset AD,[30] and is differently regulated by age and presence of Aβ amyloidosis.[15,31] Association of plasma Aβ levels and cortical amyloid burden is also modulated by *APOE* isoforms.[32] Together with these data, our findings that aging and *APOE-ε4* are critical factors for plasma Aβ42 levels from 20 years of age are consistent with Aβ42 clearance from the brain to peripheral plasma. For this reason, adjustments of the plasma Aβ42 level and Aβ40/42 ratio for age, and *APOE-ε4* allele at any age are essential for evaluating plasma Aβ levels as biomarkers of the progress of brain Aβ amyloidosis or clinical trials of disease modifying drugs.

Technical problems, including storage tubes, temperature, periods, buffers, and pipetting, during the assay procedure affect plasma Aβ levels.[27] Sleep-wake cycles of Aβ production and clearance also affect CNS Aβ levels.[33] We carefully managed fasting morning blood sampling, storage, and assay procedures, and obtained intra- and interassay coefficients with a variation of less than 10% in both Aβ40 and Aβ42 assays. We then analyzed the correlations among plasma Aβ and other blood factors. In the ADNI cohort, platelet count, creatinine, and total protein affected plasma Aβ levels.[18,19] However, the IHPP cohort comprising a wide range of age did not report similar findings. Creatinine levels were correlated with plasma Aβ40 and Aβ42 as well as previous study.[18,34] The present study demonstrated a strong correlation between plasma Aβ40 and cystatin C levels. Cystatin C may respond to plasma Aβ and renal function more sensitively than creatinine. Higher LDL-C and Lower HDL-C levels were both associated with cerebral amyloidosis[35] but not with late life cholesterol or AD neuropathology.[36] Our results suggested that serum cholesterol levels are not directly corrected with plasma Aβ levels. Type 2 diabetes mellitus is a well-known risk factor for AD. Type 2 diabetes is positively associated with CSF Aβ42, but negatively associated with cerebral cortical Aβ burden.[37] Although a few large scale-studies have reported an association between glucose metabolism and plasma Aβ by strict sampling of morning fasting blood, we found no correlation among plasma Aβ levels, FPG, hemoglobin A1c, and glycoalbumin, indicating no direct relationship between plasma Aβ and blood glucose levels. In conclusion, there were no strong determinant factors directly related with plasma Aβ levels, except Cystatin C for Aβ40 level, in the IHPP cohort.

Regarding the relationship between plasma Aβ and lifestyle, no direct association was found with systolic or diastolic blood pressure,[38,39] nor with alcohol intake, hours of sleep or smoking amount by questionnaire survey. Physical and motor activity, including 10MWT, RDT, TUG, and WBRT as candidates for integrated cognitive processes that require attention, planning, visuospatial, and motor processes, demonstrated linear associations with the plasma Aβ40/42 ratio. However, these correlation coefficients were weak, suggesting that plasma Aβ40/42 is not a predictor for complex motor activity related with cognitive function.[40]

Prior major cohort studies have reported that plasma Aβ is a risk factor or predictive marker for AD onset in healthy older community members aged at least 55 years.[1–12] In contrast, after analyzing fasting blood samples from healthy individuals of a wide age range, we observed the natural course of and factors affecting plasma Aβ40 and Aβ42. The period from mid-life to elderly is critical for preclinical progression of Aβ amyloidosis. Consistent with other reports, we found that decreased plasma Aβ42 levels and increased Aβ40/42 ratio were associated with low cognitive ability in participants aged over 60 years. Furthermore, plasma Aβ42 levels were stably regulated mainly by age and *APOE-ε4*. As this study was cross-sectional, we were unable to validate plasma Aβ42 and Aβ40/42 ratio as a predictive biomarker for the onset of AD. This is one limitation of our study. Furthermore, we were also unable to analyze the association between Aβ and vascular factors by MRI. To resolve these limitations, longitudinal confirmation is necessary. To confirm this basic data from the 2014 study, we are repeating the same annual surveys from 2015 to 2017, to clarify the factors of plasma Aβ and evaluate plasma Aβ40 and Aβ42 as biomarkers of onset of Aβ amyloidosis in the brain.

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Author Contributions

T.N., S.N., and M.S. conceptualized and designed the study. T.N., N.N., S.N., and K.I. acquired and analyzed the data. T.N., T.K., Y.S., M.H., K.I., S.N., and M.S. drafted the text and prepared the figures.

Conflicts of Interest

The authors declare that there are no conflicts of interest.

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Supporting Information

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Table S1. Correlation between plasma levels of Aβ and other blood tests 1.
Table S2. Correlation between plasma levels of Aβ and other blood tests 2.
Table S3. Result of multiple linear regression model analysis about whether age-dependent increases in Aβ levels are affected by presence of *APOE-ε4* adjusting for total protein, platelet count and creatinine levels.
Table S4. Result of multiple linear regression model analysis about whether age-dependent increases in Aβ levels are affected by presence of *APOE-ε4* after adjustments for hemoglobin, platelet count, albumin, creatinine, blood urea nitrogen, fasting plasma glucose, free fatty acid, hemoglobin A1c, and cystatin C.
Table S5. Result of multiple logistic regression analyses between plasma Aβ and MMSE scores after adjusting for age.

