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Associations of Growth-Associated Protein 43 with Cerebral Microbleeds: A Longitudinal Study

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Abstract.
Background: Cerebral microbleeds (CMB) play an important role in neurodegenerative pathology.
Objective: The present study aims to test whether cerebrospinal fluid (CSF) growth-associated protein 43 (GAP-43) level is linked to CMBs in elderly people.
Methods: A total of 750 participants from the Alzheimer's Disease Neuroimaging Initiative (ADNI) who had measurements of GAP-43 and CMBs were included in the study. According to the presence and extent of CMBs, participants were stratified into different groups. Regression analyses were used to assess cross-sectional and longitudinal associations between GAP-43 and CMBs.
Results: Participants with CMB were slightly older and had higher concentrations of CSF GAP43. In multivariable adjusted analyses for age, gender, *APOE* ε4 status, and cognitive diagnoses, higher CSF GAP-43 concentrations were modestly associated with CMB presence (OR = 1.169, 95% CI = 1.001–1.365) and number ($\beta = 0.020$, SE = 0.009, $p = 0.027$). Similarly, higher CSF GAP43 concentrations were accrual of CMB lesions, associated with higher CMB progression (OR = 1.231, 95% CI = 1.044–1.448) and number ($\beta = 0.017$, SE = 0.005, $p = 0.001$) in the follow up scan. In stratified analyses, slightly stronger associations were noted in male participants, those 65 years and older, carriers of *APOE* ε4 alleles, and with more advanced cognitive disorders.
Conclusions: CSF GAP-43 was cross-sectionally associated with the presence and extent of CMBs. GAP-43 might be used as a biomarker to track the dynamic changes of CMBs in elderly persons.

Keywords: Alzheimer's disease, Alzheimer's disease Neuroimaging Initiative, cerebral microbleeds, CSF biomarker, GAP-43, synaptic dysfunction

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[2]Data used in preparation of this article were obtained from the Alzheimer's Disease Neuroimaging Initiative (ADNI) database (http://adni.loni.usc.edu). As such, the investigators within the ADNI contributed to the design and implementation of ADNI and/or provided data but did not participate in analysis or writing of this report. A complete listing of ADNI investigators can be found at: http://adni.loni.usc.edu/wp-content/uploads/how_to_apply/ADNI_Acknowledgement_List.pdf.

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INTRODUCTION

Cerebral small vessel disease (CSVD) is recognized as a major cause of functional impairment in the elderly [1, 2], and cerebral microbleeds (CMBs) are a surrogate marker of CSVD [3]. CMBs were defined as homogeneous hypointense lesions not exceeding 10 mm in the white or gray matter in diameter on T2*GRE images [4]. And they were associated with amyloid-β (Aβ) [5], total tau, phosphorylated tau [6], soluble E-selectin [7], and tumor necrosis factor receptor 2 [8]. In the general population, high microbleed counts were related to an increased risk of cognitive impairment and dementia [9, 10]. The number of CMBs can predict adverse cognitive outcomes. Therefore, more available and reliable markers are warranted to monitor the status of CMBs in the future.

Growth-associated protein 43 (GAP-43) is a presynaptic protein and a marker of synaptic dysfunction that is involved in neuronal growth and axonal development. It was related to increased amyloid and tangle burden in the amygdala, cortex, and hippocampus [11–15]. GAP-43 is considered to be a sensitive and early marker of post-ischemic brain injury [11, 16, 17] and traumatic brain injury [18]. There is evidence that cerebrospinal fluid (CSF) GAP-43 level correlates with the severity of stroke, white matter lesions, atrophy, as well as infarct size [19]. Previous studies exploring the associations between GAP-43 and CMBs were scarce. The purposes of the present study are to explore whether GAP-43 is associated with CMBs and explore the potential of GAP-43 as a reliable predictor of CMBs.

METHODS

ADNI study design

Data used in the preparation of this article were obtained from the Alzheimer's Disease Neuroimaging Initiative (ADNI) database (http://adni.loni.usc.edu). The ADNI was launched in 2003 as a public-private partnership, led by Principal Investigator Michael W. Weiner, MD. The primary goal of ADNI has been to test whether serial magnetic resonance imaging (MRI), positron emission tomography, other biological markers, and clinical and neuropsychological assessment can be combined to measure the progression of mild cognitive impairment (MCI) and early Alzheimer's disease (AD). For up-to-date information, see http://www.adni-info.org.

Participants in ADNI have been enrolled from over 50 sites across Canada and the United States. The data we used in this study were collected from December 16, 2005, to May 2, 2019. All the regional ethics committees have approved the ADNI study. Written informed consent was given by all study participants.

Participants

In the present cohort, a total of 750 participants, including cognitively normal (CN, $n = 236$) individuals, MCI ($n = 405$) subjects, and AD ($n = 109$) patients, provided baseline CSF GAP-43 data. And among them, 749 individuals had more than one follow-up scan (Fig. 1). The participants who simultaneously met the following criteria were included: 1) 55–90 years old, 2) a minimum of 6 years of education, 3) fluency in speaking English or Spanish, and 4) no obvious neurological disorders other than AD. The CN participants were defined as having a Mini-Mental State Examination (MMSE) score of 24 or higher and a Clinical Dementia Rating (CDR) of 0. Participants in the MCI group were defined as non-demented individuals with an MMSE score of 24 or higher, a CDR score of 0.5, preserved activities of daily living, and objective memory loss measured by the Wechsler Memory Scale (WMS) Logical Memory II test. Participants with AD dementia were defined as those met the Alzheimer's Disease and Related Disorders Association criteria for probable AD and the National Institute of Neurological and Communication Disorders and Stroke [20] with an MMSE score of 20 to 26 and a CDR score of 0.5 to 1.0 [21–23].

CSF GAP-43 quantification

CSF GAP-43 was analyzed using an in-house enzyme-linked immunoassay (ELISA) method described previously in detail [13]. The ELISA was implemented using a combination of the mouse monoclonal GAP-43 antibody NM4 (encapsulated antibody) and a polyclonal GAP-43 antibody (detector antibody) which recognizes the C-terminus of GAP-43. GAP-43 concentration in CSF samples was calculated via interpolation from the calibrator curve (4PL weighted 1/Y2). These analyses were performed by laboratory technicians certified by the committees. The assay range was 312–20,000 pg/mL. The repeatability coefficient of variation (CV) % of quality controls (QC1 and QC2) was 5.5% versus 11%, and the inter-assay CV% was 6.9% versus

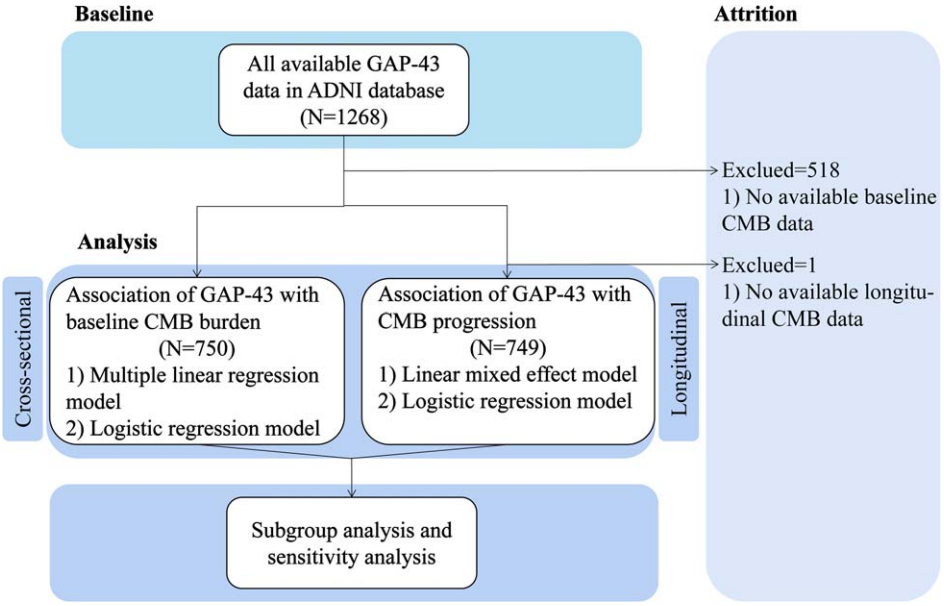

Fig. 1. A flow diagram of the study.

15.6% during sample runs in the clinical evaluation study.

CMB quantification

The 3 Tesla MRI protocol consisted of 3D T1-weighted MPRAGE and T2-weighted GRE sequences, which has been previously described (http://adni.loni.usc.edu/methods/documents/mriprotocols/). CMBs are defined as homogeneous hypointense lesions in the white or gray matter not exceeding 10 mm in diameter on T2*GRE images. The CMBs are best seen in the gradient-echo T2 sequence (hypointense lesions); In the T2, T1, and FLAIR sequences, they are isointense. The severity of CMBs was characterized using the Standards for Reporting Vascular Changes on Neuroimaging (STRIVE). CMBs were quantified in minimum deformation template space according to every voxel (based on corresponding proton density, T1 and T2 intensities), prior probabilities of CMBs, and the conditional probabilities of CMBs based on the presence of CMBs at adjacent voxels. All available T2*GRE scans of a participant were used for the rating of individual CMBs. This study included participants with definite CMBs as cases and those without definite CMBs as controls at baseline. To examine the associations of CSF GAP-43 with CMB progression, we used a four-grade system for CMBs according to the CMB number, namely grade 0 (0, $n = 483$), grade 1 (1, $n = 143$), grade 2 (2–4, $n = 87$), and grade 3 (>4, $n = 37$) [9]. In addition, CMB progression was determined by the differences in counts between scans. In our study, the progression of CMBs was defined as an increase in CMB number from the baseline level during the follow-up period.

Statistical analysis

Pairwise comparisons were conducted to test for intergroup differences using the chi-square test for categorical variables and the Student *t*-test for continuous variables. CSF GAP-43 concentrations and CMB numbers were normalized using the "car" package in R software in appropriate situations. We used the Student *t*-test to investigate the association between CSF GAP-43 and CMB presence. One-way ANOVA is applied to test the association of CSF GAP-43 with CMB grades.

We conducted logistic regression using the presence of CMBs as a dependent variable and GAP-43 as an independent variable. Multiple linear regression was used to investigate the cross-sectional associations of GAP-43 with the grade and number of CMBs using GAP-43 level as the exposure factor and the number and grade of CMBs as the outcome. The logistic regression model was also used to assess the effect of GAP-43 on the CMB progression, with CAP-43 as the exposure and having/not having CMB progression as the outcome. In addition, we explored

Table 1
Demographic characteristics of study participants at baselines

Variables	Overall ($n=750$)	CMB absence ($n=483$)	CMB presence ($n=267$)		
			1 ($n=143$)	2–4 ($n=87$)	>4 ($n=37$)
Age, y	72.3	71.5	73.3	73.6	76.4
Gender male, no. (%)	395 (52.7)	243 (61.5)	75 (19.0)	49 (12.4)	28 (7.1)
Educational Level, y	16	16	16	16	16
APOE ε4 carriers, no. (%)	343 (45.7)	212 (61.8)	64 (18.7)	38 (11.1)	29 (8.5)
Baseline diagnosis (CN/MCI/AD)	236/405/109	161/259/63	49/73/21	20/53/14	6/20/11
hypertension	198 (41.0)	61 (42.7)	33 (37.9)	22 (59.5)	314 (41.9)
hyperlipidemia	242 (50.1)	70 (49.0)	50 (57.5)	24 (64.9)	386 (51.5)
hyperglycemia	53 (11.0)	14 (9.8)	12 (13.8)	6 (16.2)	85 (11.3)
coronary heart disease	21 (4.3)	9 (6.3)	9 (10.3)	3 (8.1)	42 (5.6)
smoking status	138 (28.6)	28 (19.6)	20 (23.0)	9 (24.3)	195 (26.0)
alcohol consumption	12 (2.5)	3 (2.2)	2 (2.3)	1 (2.7)	18 (2.4)
GAP.43 (ng/ml)	5236.4 ± 2846.0	5028.2 ± 2528.0	5407.5 ± 3341.5	5587.5 ± 3280.8	6467.1 ± 3263.6

Categorical variables are reported as numbers and percentages; continuous variables are reported as means ± SDs. CMB, Cerebral microbleeds; y, years; no., number; *APOE*, Apolipoprotein E; CN, cognitively normal; MCI, mild cognitive impairment; AD, Alzheimer's disease; GAP-43, growth-associated protein 43.

the effects of GAP-43 on the CMB progression in the four different grades of CMBs. A linear mixed effect model was used to evaluate the predictive value of baseline GAP-43 for the change in CMB number. The model includes the random intercept and slopes for time and an unstructured covariance matrix of the random effects, using the interaction between time (a year is the unit for time) and GAP-43 as the predictor, as well as cognitive diagnoses, *APOE* ε4 genotypes and demographic factors as other covariates. The primary analyses of the total participants were adjusted for gender, age (<65 years or ≥ 65 years), education level, *APOE* ε4 status (having no or one/two *APOE* ε4 alleles), and baseline cognitive diagnoses (CN, MCI, and AD). Then we conducted subgroup analyses stratified by age, gender, *APOE* ε4 status, and cognitive diagnoses.

Since vascular risk factors are frequently confounders, we should further take common vascular risk factors into consideration, such as hypertension, hyperglycemia, hyperlipidemia, and smoking [24]. To examine whether the association of GAP-43 with CMB burden was influenced by other health-related factors, sensitivity analyses after excluding each variable (hypertension, hyperlipidemia, hyperglycemia, smoking status, alcohol consumption, and coronary heart disease) were conducted. (Supplementary Tables 1–3). Loss of follow-up often occurs due to the longer observation time in cohort studies. If there were missing values, we adopted the direct deletion method based on the R language software. All the statistical analyses were performed using R (version 3.5.1) and IBM SPSS Statistics 26. Statistical significance was defined as $p < 0.05$ for all analyses.

RESULTS

A total of 750 participants (267 with CMBs and 483 without CMBs at baseline) from the ADNI study were included in our study. After the CMB examination at baseline, 749 participants had one or more measurements of CMBs during the follow-up (Fig. 1). The demographics and GAP-43 data of the participants were presented in Table 1. The total study population had a female proportion of 47.3%, an age range of 40 to 90 years old (mean = 72.3), a mean number of educational years of 16, and an *APOE* ε4 positive percentage of 45.7%.

CSF GAP-43 concentration and CMBs at baseline

Our Student *t*-test revealed a non-significant positive trend for higher CSF GAP-43 concentration in participants with CMBs (Cohen's $d = 0.147$, $p = 0.058$). In the stratified analyses, the association between CSF GAP-43 concentration and CMB presence was significant among the *APOE* ε4 carriers (Cohen's $d = 0.427$, $p < 0.001$) (Fig. 2). The results of one-way ANOVA showed higher GAP-43 concentration was associated with a higher CMB grade. In the stratified analyses, the associations remained significant in the elderly, *APOE* ε4 carriers, females, and males (Fig. 3).

Using the logistic regression model, we found that higher CSF GAP-43 concentrations were associated with CMB presence (OR = 1.169, 95% CI = 1.001–1.365, $p = 0.048$) (Fig. 4). In the stratified analyses, the association was still significant

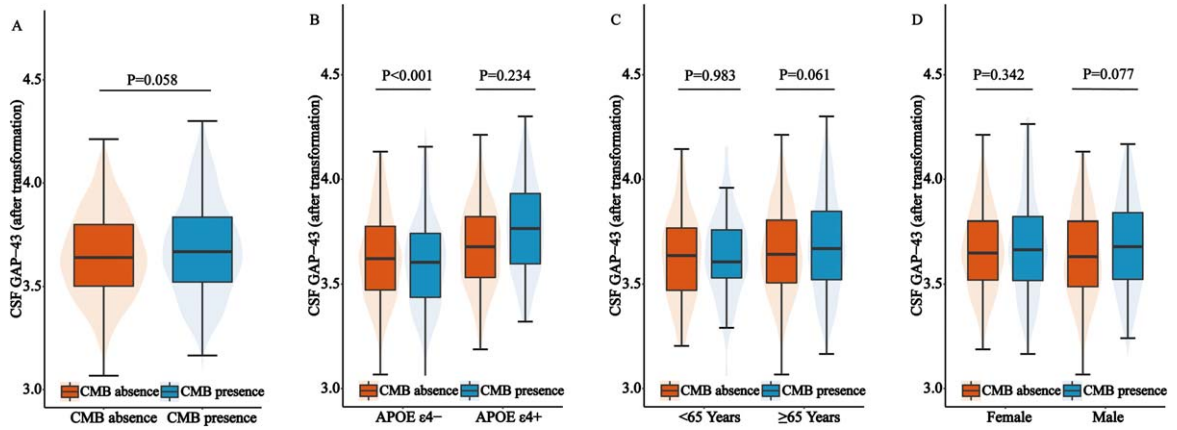

Fig. 2. GAP-43 in the presence/absence of CMBs. Values represent differences in CSF GAP-43 concentration in those with CMBs compared to those without CMBs. *p*-values were assessed by student's *t* test.

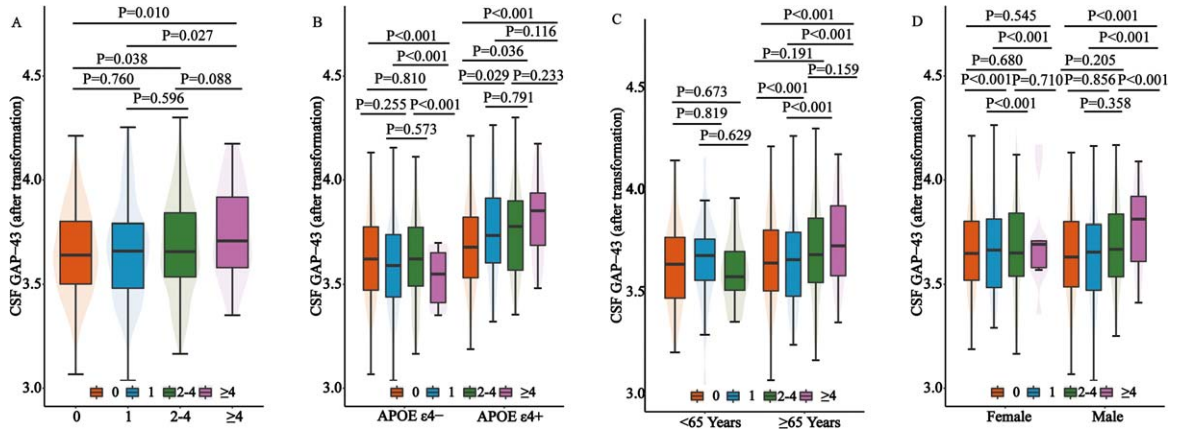

Fig. 3. GAP-43 in the CMB grades. Boxplots depicting the levels of CSF GAP-43 for each of the four CMB grades (grade 0, grade 1, grade 2, and grade 3). Values represent differences in GAP-43 among the four grades of CMBs. *p* values in pairwise comparisons were assessed by student's *t* test.

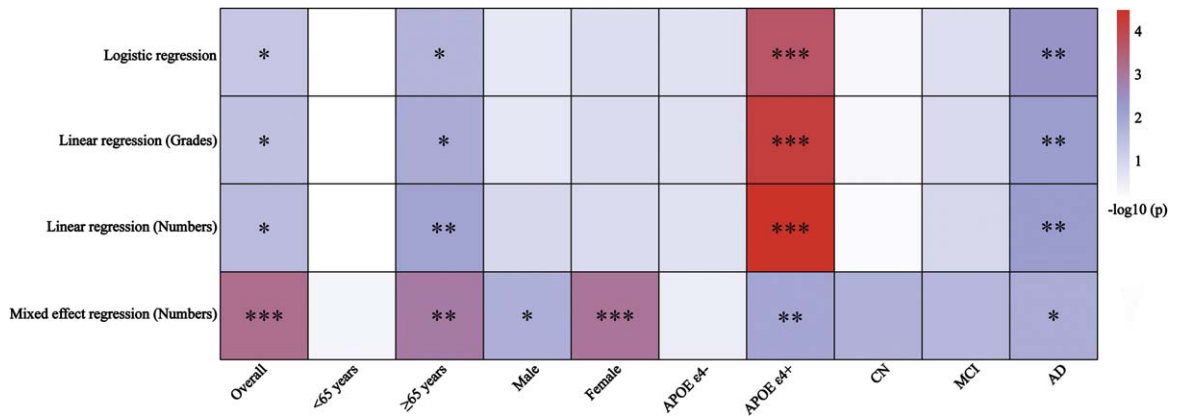

Fig. 4. Results of the associations between GAP-43 and CMBs. Regression analyses were used to assess cross-sectional and longitudinal associations between GAP-43 and CMBs. $^{*}p<0.05$, $^{**}p<0.01$, $^{***}p<0.001$.

Table 2
Results of the associations between GAP-43 and longitudinal CMB progression

Model	OR (95%CI)	*p*
Logistic		
Overall	1.2307 (1.0440–1.4484)	0.0126
<65 y	1.1467 (0.7103–1.7949)	0.5560
≥65 y	1.2598 (1.0565–1.5007)	0.0097
Male	1.2683 (1.0183–1.5804)	0.0334
Female	1.1993 (0.9284–1.5385)	0.1550
APOE ε4 noncarriers	1.0677 (0.8462–1.3371)	0.5729
APOE ε4 carriers	1.4133 (1.1132–1.7994)	0.0045
CN	1.1246 (0.8035–1.5411)	0.4754
MCI	1.2197 (0.9823–1.5132)	0.0701
AD	1.4249 (0.9299–2.2074)	0.1040

The model was used to assess the effect of GAP-43 on the probability of CMB progression, adjusted for gender, age, *APOE* ε4 status, and baseline cognitive diagnoses. Logistic, Logistic regression; OR, Odd ratio; *APOE*, Apolipoprotein E; CN, cognitively normal; MCI, mild cognitive impairment; AD, Alzheimer's disease.

in three subgroups of the elderly (OR = 1.217, 95% CI = 1.032–1.437, $p = 0.020$), *APOE* ε4 carriers (OR = 1.585, 95% CI = 1.252–2.031, $p < 0.001$), and AD patients (OR = 2.015, 95% CI = 1.284–3.341, $p = 0.004$) (Fig. 4). We used a linear regression model to verify the association and found that GAP-43 was positively correlated with the grade ($\beta = 0.016$, SE = 0.007, $p = 0.036$) and number ($\beta = 0.083$, SE = 0.009, $p = 0.027$) of CMBs (Supplementary Figure 1). In the stratified analyses, these positive correlations were still significant in the elderly, *APOE* ε4 carriers, and AD patients (Fig. 4).

Longitudinal associations between CSF GAP-43 and CMBs

The longitudinal data of CMBs in pooled samples were recorded during a median 4-year follow-up (range, 0.25–9). The logistic regression model showed that higher GAP-43 concentrations were associated with higher CMB progression (OR = 1.231, 95% CI = 1.044–1.448, $p = 0.0147$) in the follow up scan. In the stratified analyses, the association was still significant in the elderly (OR = 1.260, 95% CI = 1.057–1.501, $p = 0.010$), males (OR = 1.269, 95% CI = 1.018–1.580, $p = 0.033$), and the *APOE* ε4 carriers (OR = 1.413, 95% CI = 1.113–1.799, $p = 0.005$) (Table 2). When we explored the association in the four different grades of CMBs, we found a significant association in grade 3 (OR = 1.001, 95% CI = 1.000–1.002, $p = 0.040$).

The pooled results showed that there was an association between higher GAP-43 concentration and an increased CMB number ($\beta = 0.017$, SE = 0.005, $p < 0.001$). In the stratified analyses, the association remained significant in the subgroups of the elderly ($\beta = 0.017$, SE = 0.005, $p = 0.001$), *APOE* ε4 carriers ($\beta = 0.022$, SE = 0.009, $p = 0.009$), females ($\beta = 0.018$, SE = 0.004, $p < 0.001$), males ($\beta = 0.019$, SE = 0.007, $p = 0.011$), CN individuals ($\beta = 0.047$, SE = 0.020, $p = 0.017$), MCI individuals ($\beta = 0.015$, SE = 0.007, $p = 0.022$), and AD individuals ($\beta = 0.089$, SE = 0.035, $p = 0.014$) (Fig. 4).

DISCUSSION

Our study showed that GAP-43 had a positive association with baseline CMB burden. As the GAP-43 increased, the probability of CMB progression was greater. In cross-sectional stratified analyses, the association between GAP-43 and baseline CMB burden was still significant in the elderly (≥65 years), *APOE* ε4 carriers, and AD individuals. The logistic regression model showed the association between higher GAP-43 concentration and the increased probability of CMB progression was significant in the elderly, males, and the *APOE* ε4 carriers. A linear mixed effect model showed that the association between higher GAP-43 concentration and an increased CMB number was significant in the elderly, *APOE* ε4 carriers, females, males, CN individuals, MCI individuals, and AD individuals.

The association between GAP-43 and CMB burden may be explained as follows. Microglia, an innate immune cell, participate in the pathological changes of CMBs, which can remove amyloid [25] and infiltrating components from blood [26] and promote the development of inflammation and axons [27]. Pro-inflammatory microglia that produce pro-inflammatory cytokines, reactive oxygen species, and matrix metalloproteinases may be potential targets of CMBs [28]. CMBs mark the presence of diffuse vascular and neurodegenerative brain damage [9]. When the nervous system is damaged, GAP-43 expression increases until complete synaptic connections are established [29]. GAP-43, which is involved in axonal growth has been reported to have a possible relationship with microglia [30–32]. In addition, greater CMB burden and increased GAP-43 were often accompanied by inflammation [33–35]. Aβ load is a predictor of CMB burden [36, 37]. GAP-43 increased with the increased Aβ load, even in the earliest stages of Aβ deposition [38]. Previous studies suggested that GAP-43 and CMB burden were

both candidate biomarkers for cognitive decline [9, 39], which might contribute to the potential association between GAP-43 and CMBs. Our Student *t*-test found no significant association between CSF GAP-43 concentration and CMB presence. However, when we used the four-grade CMB system, we found significant differences in CSF GAP-43 concentration between the four grades of CMBs, suggesting that more precise grading of CMBs is needed for investigation into the associations between GAP-43 and CMBs. The association between GAP-43 and CMB burden is still significant in the elderly, *APOE* ε4 carriers, and AD patients. Here are some possible explanations. CMB burden was significantly associated with Aβ pathology in AD patients [40]. The prevalence of CMBs was found to increase with age [41, 42], especially among *APOE* ε4 carriers [41, 43]. Higher CSF GAP-43 levels were observed in *APOE* ε4 carriers [44], the elderly [38], and AD patients [13]. GAP-43 may have a better ability to track the progression of CMBs in the elderly, *APOE* ε4 carriers, and AD patients. Increased CSF GAP-43 concentration was longitudinally associated with an elevated probability of CMB progression in the total population, suggesting GAP-43 could be used to track the progression of CMBs. Further, in the four-grade system for CMBs, the association was significant in grade 3, which might be explained by the fact that the baseline burden of CSVD is closely related to the progression of CSVD [45, 46]. GAP-43 plays a predictive role. GAP-43 may have a better ability to track progression in grade 3 CMBs than in other grades.

Our study demonstrated the associations between GAP-43 and CMB burden. Previous findings showed that CMB was a surrogate marker of cognitive decline and it marked the presence of both diffuse vascular and neurodegenerative brain damage [9]. Besides, prior studies provided evidence that intracranial carotid artery calcification was associated with CMBs [47]. Previous studies also showed that CMBs could be an alternative marker of cerebrovascular diseases and a prognostic marker for hemorrhagic and ischemic risks [48, 49], which suggested that CMBs should be considered a marker for the severity of the underlying small vessel injuries rather than a specific marker of only future hemorrhagic risk [50]. Therefore, predicting the further trends of CMBs can help us identify the cerebrovascular risk. This study showed that GAP-43 was associated with the probability of CMBs presence, as well as the number and grade of CMBs. GAP-43 monitors the severity of CMBs, which helps people to raise early alertness, inhibit/prolong the progression of CMBs [51], and curb the progression of adverse health states.

The novelty of our study was to explore the cross-sectional and longitudinal associations of GAP-43 and CMBs. However, our study also had some limitations. The findings of our study cannot be used to prove the role of GAP-43 in CSVD. Relationships between GAP-43 and other CSVD MRI measures should be further investigated to explore the possible role of GAP-43 in CSVD. There is a lack of consideration of CMB topography, which is a relevant factor when considering the underlying arteriopathy. In the future, the relevant aspects will need to be supplemented. This study mainly discusses the relationship between CAP-43 and CMB pathology. In the future, different CMB lesions can be further studied with larger samples.

Conclusion

Our study showed that CSF GAP-43 was associated with the dynamic changes of CMBs in the elderly. Therefore, GAP-43 might be considered as a candidate biomarker to monitor the progression of CMBs.

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CONFLICT OF INTEREST

Jin-Tai Yu and Lan Tan are Editorial Board Members of this journal but were not involved in the peer-review process of this article nor had access to any information regarding its peer-review.

The authors have no conflict of interest to report.

DATA AVAILABILITY

The data used in preparation for this article were obtained from the publicly available ADNI database. The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

SUPPLEMENTARY MATERIAL

The supplementary material is available in the electronic version of this article: https://dx.doi.org/10.3233/JAD-230508.

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