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Synergistic Effects of *APOE* and *CLU* May Increase the Risk of Alzheimer's Disease: Acceleration of Atrophy in the Volumes and Shapes of the Hippocampus and Amygdala

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Abstract.
Background: The volume loss of the hippocampus and amygdala in non-demented individuals has been reported to increase the risk of developing Alzheimer's disease (AD). Many neuroimaging genetics studies mainly focused on the individual effects of *APOE* and *CLU* on neuroimaging to understand their neural mechanisms, whereas their synergistic effects have been rarely studied.
Objective: To assess whether *APOE* and *CLU* have synergetic effects, we investigated the epistatic interaction and combined effects of the two genetic variants on morphological degeneration of hippocampus and amygdala in the non-demented elderly at baseline and 2-year follow-up.
Methods: Besides the widely-used volume indicator, the surface-based morphometry method was also adopted in this study to evaluate shape alterations.
Results: Our results showed a synergistic effect of homozygosity for the *CLU* risk allele C in rs11136000 and *APOE* ε4 on the hippocampal and amygdalar volumes during a 2-year follow-up. Moreover, the combined effects of *APOE* ε4 and *CLU* C were stronger than either of the individual effects in the atrophy progress of the amygdala.
Conclusion: These findings indicate that brain morphological changes are caused by more than one gene variant, which may help us to better understand the complex endogenous mechanism of AD.

Keywords: *APOE*, *CLU*, morphometry, subcortical structures, synergistic

[1]Data used in preparation for 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 the 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

Alzheimer's disease (AD) is an irreversible neurodegenerative brain disorder caused by genetic and environmental factors [1, 2]. Due to its high mortality [3] and heritability [4] rates, genetic risk factors can serve as powerful markers to identify at-risk individuals for developing AD [5]. Previous reports have shown that gene-gene interactions play a critical role in modulating brain structure and cognitive performance [5–8]. Information about whether the major risk genes can synergistically accelerate AD-related atrophy of subcortical structures before the onset of dementia symptoms will likely contribute to the identification of at-risk populations [9].

Apolipoprotein E is the major lipid transporter in the brain [10]. It is encoded by the Apolipoprotein E gene (*APOE*), which has three major polymorphic alleles: $\varepsilon 2$, $\varepsilon 3$, and $\varepsilon 4$ [11] as well as six genotypes: $\varepsilon 2/\varepsilon 2$, $\varepsilon 2/\varepsilon 3$, $\varepsilon 2/\varepsilon 4$, $\varepsilon 3/\varepsilon 3$, $\varepsilon 3/\varepsilon 4$, and $\varepsilon 4/\varepsilon 4$ [12]. Clusterin (*CLU*) is associated with tissue injury and aging [13], which has two major polymorphic alleles: C and T, three genotypes: CC, CT, and TT. The $\varepsilon 4$ allele of *APOE* and the C allele of *CLU* is thought to increase the risk of developing AD [14, 15]. Numerous studies have found *APOE* $\varepsilon 4$ allele effects on entorhinal cortex [16, 17], hippocampal [18–20], and amygdalar volume [21], as well as temporal lobe volumes [22] in non-demented elderly. These structures are closely related to memory and cognitive performance, supporting them as effective markers during disease progression [23]. Interestingly, the *CLU* (rs11136000) is associated with worse episodic memory performance in non-demented elders [24]. The hippocampus and amygdala are brain areas crucial for episodic memory, suggesting that the integrity of the hippocampus and amygdala might be injured by *CLU* [25–27]. Yet, few studies have investigated the possible effects of the *CLU* gene on brain structure in non-demented elderly. Recent work has shown that the gene combinations of *CLU* and *APOE* additively modified the risk of association with AD [28]. Moreover, previous research has identified that they have additive effects on medial temporal lobe activity [29] and ventricular expansion [30]. *APOE* and *CLU* proteins share various major features: they cooperatively suppress amyloid-β (Aβ) deposition [31]; they interact with a shared set of cell-surface receptors [32]; and they promote neurite outgrowth [33, 34]. Because of these biological connections, *APOE* and *CLU* polymorphisms may lead to the development of AD by affecting similar pathways [29]. However, to the best of our knowledge, the interaction effects between *APOE* $\varepsilon 4$ and *CLU* C carrier status have been rarely studied [35], and no study has examined the synergistic adverse effects of *CLU* and *APOE* on AD-related brain alterations.

The histopathological changes in the early cases of AD are typically seen within the medial temporal lobe beginning in the preclinical phase [36, 37], in particular, the hippocampus and amygdala. Besides, the degeneration of the hippocampus and amygdala in non-demented individuals has been reported to increase the risk of developing AD [38–40]. Prior works on subcortical structures mainly focused on volumetric methods [5, 7, 41–44]. Recent studies [45–47] highlighted the importance of identifying the easily-affected subregions, since the pathological significance of these subregions may be more sensitive. To date, most studies on hippocampal subregions have concentrated on cross-sectional [20, 23, 48, 49] and a few studies have tried to explore the subregional deformations of the amygdala [48]. Cross-sectional investigations remain inherently limited, as they poorly outline whether abnormalities were dynamically altered over time [50]. In this case, we tried to determine the dynamic changes in subregions of hippocampus and amygdala during 2-year periods, and whether they were detectable in the preclinical phase of AD, which may provide new insights for the comprehension of the effects of multiple risk variants on the hippocampus and amygdala.

The purpose of the current study was to test the hypothesis that *APOE* $\varepsilon 4$ and *CLU* C have synergistic adverse effects on the shapes/volumes of bilateral hippocampi/amygdalae at the baseline and during 2-year periods in non-demented elders. Moreover, we also tested the relations between hippocampus/amygdala atrophy and the gene dose (0, 1, or 2) of *APOE* $\varepsilon 4$ or *CLU* C.

METHODS

Alzheimer's Disease Neuroimaging Initiative database

Longitudinal data used in this article were collected from the Alzheimer's disease Neuroimaging Initiative (ADNI) database (http://www.adni.loni.usc.edu). The ADNI, led by Principal Investigator Michael W. Weiner MD, was launched in 2003 as a public-private partnership. ADNI was mainly used to investigate the progression of mild cognitive impairment (MCI) and early AD by using serial magnetic

resonance imaging (sMRI), positron emission tomography (PET), and other biological markers. The participants have been recruited by ADNI from more than 50 sites across the United States and Canada. All participants gave written informed consent. For up-to-date information, visit http://www.adni-info.org.

Participants

The underlying pathology of AD preceded the onset of cognitive symptoms by many years [51]. Thus, censoring risk genetic effects may help early diagnosis and preventative interventions of dementia. Here, we limited the current analyses to normal control (NC) and MCI participants whose genotypes data of *CLU* at rs11136000 locus and *APOE* were available. Also, we took into consideration the protective effects of *APOE* ε2 allele against AD, and the *APOE* ε2 carriers were excluded. We included participants with genetic information and four longitudinal MRI scans at baseline, 6-month, 12-month, and 24-month. However, at a time point, if a sample did not obtain MR images or MR images were excluded during data preprocessing, the sample was excluded. Finally, 171 non-demented elders including 82 NC and 89 MCI were selected at all time points. The inclusion criteria for NC individuals were Mini-Mental State Examination (MMSE) scores ranging from 24 to 30 and a Clinical Dementia Rating score of zero. Subjects with MCI had MMSE scores ≥ 24, the Clinical Dementia Rating score of 0.5, and they preserved activities of daily living and the absence of dementia. The details of inclusion and exclusion criteria can be found in [45, 52].

Genetic data

In this article, the *APOE* and *CLU* genotypes of participants were obtained from the ADNI database. On the basis of *APOE* ε4 status, the *APOE* genotype was coded as 0, 1, and 2 for the presence of 0, 1, and 2 *APOE* ε4 allele [53]. Besides, subjects were further coded as 0, 1, and 2, representing the number of *CLU* C allele, since previous studies have suggested that C allele of *CLU* rs11136000 was a risk allele for AD [54].

To test the interaction effects, we created 9 gene-gene cohorts based carrying the status of *CLU* rs 11136000 and *APOE*. According to genotypes of *CLU* rs11136000, these subjects were divided into 3 groups (38 TT homozygotes; 72 CT heterozygotes; and 61 CC homozygotes). For each of 3 groups, subjects were further divided into ε3 homozygotes (ε3/ε3), ε3 heterozygotes (ε3/ε4), and ε4 homozygotes (ε4/ε4) based on *APOE* allele status.

To test for combined effects, we created 3 gene-gene cohorts based on the number of the risk alleles in *CLU* rs11136000 and *APOE*. Participants with neither *APOE* ε4 allele nor *CLU* C allele (zero risk allele) were classified into the "0" group (TT+ε3/ε3); participants with either C allele or ε4 allele were classified into the "1" group (TT+ε3/ε4 or ε4/ε4; and ε3/ε3 + CT or CC); participants with more than one risk allele at each locus were classified into the "2" group (ε3/ε4 + CT or CC; and ε4/ε4 + CT or CC).

MRI data acquisition

All subjects were scanned with the MR acquisition protocol as previously described in detail [55]. In brief, brain MR imaging was acquired at 58 sites, using 1.5T MRI scanners in GE Healthcare, Siemens Medical Solutions USA, or Philips Electronics. High-resolution T1-weighted MRI scans were collected using a sagittal 3-dimensional magnetization-prepared rapid gradient echo (3D MP-RAGE) sequence with repetition time = 2400 ms; echo time = 1000 ms; flip angle = 8°. Moreover, image correction procedures and post-acquisition correction of certain image artifacts were implemented at a single site to ensure the consistency of these preprocessing steps [53, 55]. The volumes of brain structures were processed using the FreeSurfer version 5.3 software (http://surfer.nmr.mgh.harvard.edu/) based on the 2010 De sikan-Killany atlas [56]. The quality of segmentation of all subcortical structures was manually checked and excluded the images of segmentation errors.

Shape processing

All T1-weighted MR images were automatically segmented by FIRST, a model-based subcortical structure integration tool developed as part of FMR IB Software Library (FSL) (https://fmrib.ox.ac.uk/fslwiki/FIRST/). Based on the segmentation of the hippocampus and amygdala, we visually checked the segmented images. Images with segmentation errors were reprocessed and would be excluded if the error could not be corrected. After segmentation for the hippocampus and amygdala, the surface of the hippocampus and amygdala were reconstructed with the topology-preserving level set method [57]. Based on that, the marching cubes algorithm [58] was applied to generate the triangular surface meshes and then

the progressive meshes [59] and loop subdivision [60] were used to smooth surfaces and refine the meshes. We also manually checked all the meshes and excluded the images with rough meshes. Subsequently, the conformal grid for each surface was obtained through the holomorphic 1-form basis [61], and then the feature images of the surface were formed by scaling the range of the conformal representation [62]. Finally, the feature images with a chosen template image were aligned via an inverse-consistent surface fluid registration method [62, 63]. More details of the description can be found in [20].

After aligning hippocampus and amygdala surfaces for all subjects, we extracted the radial distance by computing their vertex-wise features. The isoparametric curve (see red curves in Fig. 1d) is perpendicular to the medial axis, on the computed conformal grid [64], after which RD value is easily found at each vertex. The radial distance value represents the distance between each surface point and the middle axis [65], as a method to quantify surface deformation. Some studies suggested that the different subregions of the hippocampus and amygdala have different specific functions [66–68]. To detect the effects of risk-allele on the subdivisions of the hippocampus and amygdala, the radial distance (RD) features were used to generate a distance map in 3D, and smaller RD values were taken as an index of atrophy [69]. Figure 1 gives an overview of processing in this paper.

Statistical associations of the two genotypes with subcortical atrophy

The Hardy-Weinberg equilibrium (HWE) between expected and observed genotypic distributions of both single nucleotide polymorphisms (SNPs) was tested by the HWE version 1.2 [70] program (Columbia University, New York). In cross-sectional studies, the multivariable linear regression models (MLR) describe the relationship between a set of independent variables and a dependent variable. In this study, the MLR was used to assess the associations between single-gene/interaction/combined effects and morphological changes in the hippocampus and amygdala, after adjusting for baseline age, gender, and total intracranial volume (ICV). Besides, the *APOE* ε4 carrier status was added as a covariate for

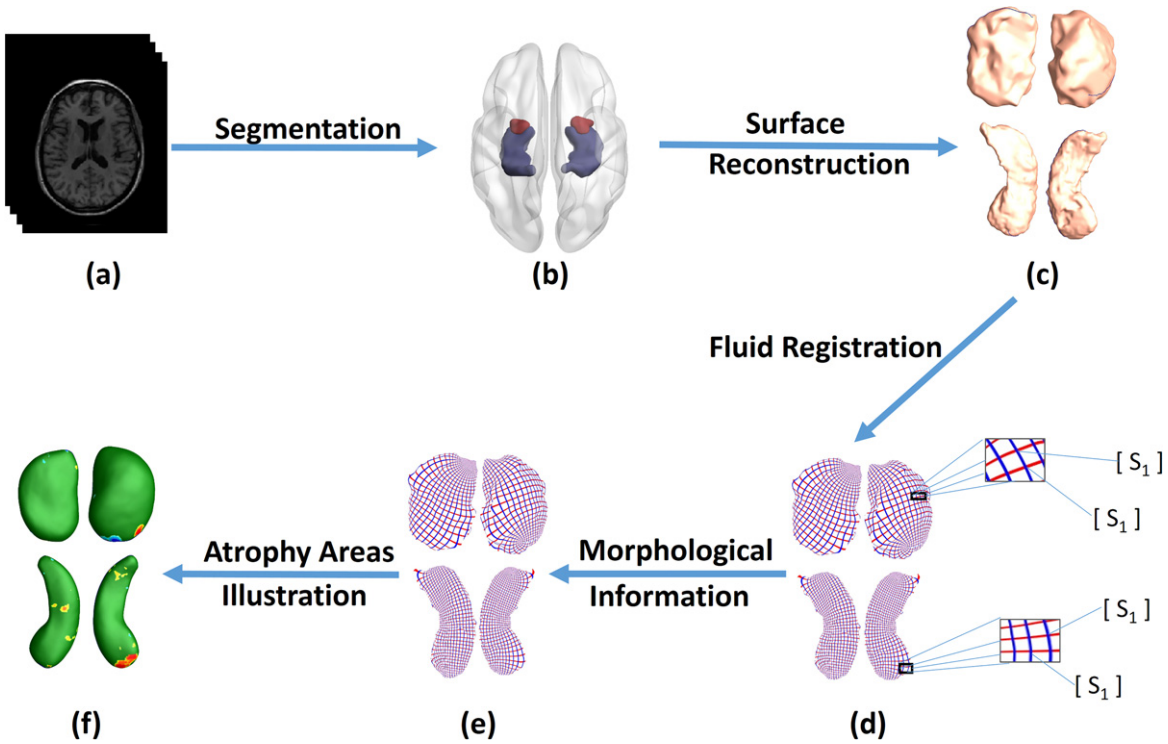

Fig. 1. The key steps of hippocampus/amygdala morphometry analysis: (a) T1-MR images; (b) MR images were segmented to obtain subcortical structures; (c) The 3D surfaces were reconstructed based on obtained subcortical structures; (d) 3D surface parameterization; (e) Obtaining morphological information; (f) Analyzing the effect of risk variants on the shapes of hippocampus and amygdala.

the main effects model of *CLU*; likewise, the *CLU* status was added to covariate for the main effects model of *APOE*.

The multivariable linear mixed-effects models (MLME) takes into account the group level structure in the data by simultaneously assessing effects within and across groups [71]. We applied the MLME [72–75] to study the associations between longitudinal change in volumes of hippocampus and amygdala and single-gene/interaction/combined effects at the 2-year follow-up. We denoted the baseline age for the subjects with B_{i}, the follow-up time for subject i and visit j with X_{ij} [76], *APOE* ε4 with D_{i}, *CLU* with C_{i}, the ICV with T_{i}, the interaction with $C_{\mathrm{i}}D_i$, and the volume Y_{ij} as the dependent variable. Then the linear mixed model can be constructed by:

$$Y_{\mathrm{ij}} = \beta_0 + \beta_1 B_1 + \beta_2 X_{ij} + \beta_3 T_i + \beta_4 C_i + \beta_5 D_i + \beta_6 C_i D_i + b_{0i} + b_{1i} X_{ij} \quad (1)$$

where β_0,β_1,...,$\beta_6$are fixed effects regression coefficients and b_{0i}, b_{1i} are random effects regression coefficients [76]. We also considered a simplified model without the interaction term [5, 77]. The variables in both regression models and linear mixed models were converted into a normalized Z-score. In agreement with previous studies [78], we used the *p*-value to assess the effect degree of combined/interaction/main effects. Because a smaller *p*-value indicated the independent variables had a more significant regression coefficient and more contributor towards the dependent variables. All the statistical analyses were conducted in R v3.6.1 software (The R Foundation).

Mapping genotype effects across the surfaces of hippocampus and amygdala

The surface features were used to generate distance maps that estimated relationships between the genotypes and regional shape morphometrics of hippocampus and amygdala. These analyses may help to reveal the vulnerable subregions of the hippocampus and amygdala and can observe the trend of deformation during 2-year follow-up. Similar to the volumetric analyses, the MLR and MLME were chosen for main effects analyses of genotype on surface maps at baseline and longitudinal study, respectively. The surface maps indicated that associations between the number of risk alleles (0, 1, or 2) of the genotype and regional shape morphometrics of hippocampus and amygdala. We also examined the epistatic interactions between *APOE* status and *CLU* genotypes on the shape morphometrics of the hippocampus and amygdala.

We thereafter evaluated the combined effects of *APOE* and *CLU* on the shape morphometrics of the hippocampus and amygdala. Vertex-based surface maps were corrected for multiple comparisons by the false discovery rate (FDR) ($q < 0.05$) method which was developed by Hochberg and Benjamini [79]. Moreover, baseline age, gender, and ICV were included as nuisance covariates in all surface-based analyses.

In consideration of the population structures, the same experiments and statistical analysis were performed. Most of these effects remained significant even after adjusting population structures. The detailed information is shown in the Supplementary Material.

RESULTS

Demographic and genetic characteristics

In general, 171 non-demented elderly adults with high-quality imaging data and *APOE* and *CLU* genotypic information were included in this article. The observed distributions of both *APOE* and *CLU* genotypes were in HWE ($p = 0.422$ for *APOE* and $p = 0.063$ for *CLU*). The Chi-square test was used to calculate differences for gender, and the one-way analysis of variance (ANOVA) was performed for

Table 1
Demographics characteristics of the genotypic subgroups

	Genotypic groups of *APOE*			*p*	Genotypic groups of *CLU*			*p*
	ε3/ε3	ε3/ε4	ε4/ε4		TT	CT	CC	
Age (s)	75.05 ± 5.36	74.1 ± 5.46	69.92 ± 5.75	**0.004***	74.03 ± 7.16	74.75 ± 4.79	74.1 ± 5.29	0.716
Female/Male	42/58	24/35	4/8	0.908	14/24	36/36	20/41	0.109
ICV (cm^3)	1561.1 ± 165.4	1559.78 ± 162.6	1524.2 ± 127.5	0.908	1591.69 ± 167.17	1531.54 ± 148.16	1568.36 ± 170.35	0.109
MMSE	28.69 ± 1.58	28.08 ± 1.63	27.17 ± 1.7	**0.004***	28.19 ± 1.53	28.74 ± 1.48	28.05 ± 1.85	0.172

Data expressed as mean ± SD (standard deviation). *APOE* ε4, apolipoprotein E ε4; *CLU*, clusterin protein gene; ICV, total intracranial volume; MMSE, Mini-Mental State Examination. Bold font denotes significant results ($p < 0.05$).

baseline age, ICV, and MMSE. Among the 3 subgroups (Table 1), there were no significant differences in gender ($p = 0.908$ for *APOE* and $p = 0.109$ for *CLU*), ICV ($p = 0.908$ for *APOE* and $p = 0.109$ for *CLU*), MMSE ($p = 0.172$ for *CLU*), and age ($p = 0.716$ for *CLU*). However, age ($p = 0.004$) and MMSE ($p = 0.004$) significantly differed among the *APOE* subgroups. *Post hoc* analysis suggested that carrying with ε3/ε4 ($p = 0.024$) and ε4/ε4 ($p = 0.002$) genotype had lower MMSE score compared with those with ε3/ε3 genotype. Additionally, subjects with 2 *APOE* ε4 alleles were younger than those with 0 *APOE* ε4 alleles ($p = 0.002$) and those with 1 *APOE* ε4 allele ($p = 0.016$). The demographic information is shown in Table 1.

Volumetric analyses

Main effects of APOE and CLU genotype on hippocampal and amygdalar volumes

To track the atrophy trajectory of hippocampal and amygdalar volumes, we examined whether increasing numbers of risk alleles of *APOE* or *CLU* were related to the volume atrophy of bilateral hippocampi and amygdalae. As expected, carrying more *APOE* ε4 alleles was significantly associated with greater atrophy of the hippocampal (left hippocampus: $p = 0.029$; right hippocampus: $p = 0.009$, Table 2) and amygdalar (left amygdala: $p = 0.018$; right amygdala: $p = 0.022$, Table 2) volumes at baseline; these correlations were enhanced over time as well (Table 2). The main effects of *CLU* were found in the left amygdala ($p = 0.002$, Table 3) at baseline. Besides, right amygdalar volume was also affected over time (right amygdala: $p = 0.003$, Table 3). However, the *CLU* at rs11136000 locus was not significantly associated with volumes of bilateral hippocampi at the baseline or two-year follow-up (Table 3).

Table 2
Results of multiple linear analyses: Associations between *APOE* genotype and volume atrophy of hippocampus and amygdala

	Effects of *APOE* genotype (covariates: Intracranial volume, age, gender, and *CLU* status)	
	Hippocampus	Amygdala
BL		
Left hemisphere	$\beta = -0.266$	$\beta = -0.280$
	$p =$ **0.029**	$p =$ **0.018**
Right hemisphere	$\beta = -0.309$	$\beta = -0.272$
	$p =$ **0.009**	$p =$ **0.022**
24 m		
Left hemisphere	$\beta = -0.218$	$\beta = -0.252$
	$p =$ **8.4×10^{-5}**	$p =$ **1.76×10^{-4}**
Right hemisphere	$\beta = -0.299$	$\beta = -0.232$
	$p =$ **2.95×10^{-6}**	$p =$ **5.85×10^{-4}**

BL: Baseline; 24 m: at 24-month follow-up. [a] β: standardized coefficient estimate. The negative (–) coefficient estimate signifies that the volume decrease with the number of risk-allele, while the positive (+) coefficient estimate means the increase of volume. [b] p-values are rounded to three decimal places. Bold font denotes significant results ($p < 0.05$).

Table 3
Results of multiple linear analyses: Associations between *CLU* genotype and volume atrophy of hippocampus and amygdala

	Effects of *CLU* genotype (covariates: Intracranial volume, age, gender, and *APOE* status)	
	Hippocampus	Amygdala
BL		
Left hemisphere	$\beta = -0.059$	$\beta = -0.301$
	$p = 0.544$	$p =$ **0.002**
Right hemisphere	$\beta = 0.012$	$\beta = -0.272$
	$p = 0.904$	$p = 0.077$
24 m		
Left hemisphere	$\beta = -0.052$	$\beta = -0.243$
	$p = 0.330$	$p =$ **9.93×10^{-6}**
Right hemisphere	$\beta = 0.009$	$\beta = -0.165$
	$p = 0.854$	$p =$ **0.003**

BL: Baseline; 24 m: at 24-month follow-up. [a] β: standardized coefficient estimate. The negative (–) coefficient estimate signifies that the volume decrease with the number of risk-allele, while the positive (+) coefficient estimate means the increase of volume. [b] p-values are rounded to three decimal places. Bold font denotes significant results ($p < 0.05$).

Interactions of APOE and CLU genotypes in the volumes of hippocampus and amygdala

To determine whether *APOE* variants differently affected hippocampal and amygdalar atrophy in participants with zero, one, or two of *CLU* C risk alleles, we introduced the epistatic interaction terms of *APOE* and *CLU* in primary models of MLR and MLME containing the main effects of *APOE* and *CLU*. The analyses revealed that there were no

Table 4
The results of *CLU* subgroup research: The associations between *APOE* ε4 genotype and bilateral amygdalae in each subgroup of *CLU* at 2-year follow-up

	Left_amygdala		Right_amygdala	
	β	p	β	p
CLU TT	–1.089	0.522	–0.08	0.617
CLU CT	–0.129	0.222	–1.043	0.306
CLU CC	–0.515	**3.33×10^{-6}**	–0.498	**1.44×10^{-5}**

[a] β, standardized coefficient estimate. The negative (–) coefficient estimate signifies that the volume decrease with the number of risk-allele, while the positive (+) coefficient estimate means the increase of volume. [b] p-values are rounded to three decimal places. Bold font denotes significant results ($p < 0.05$).

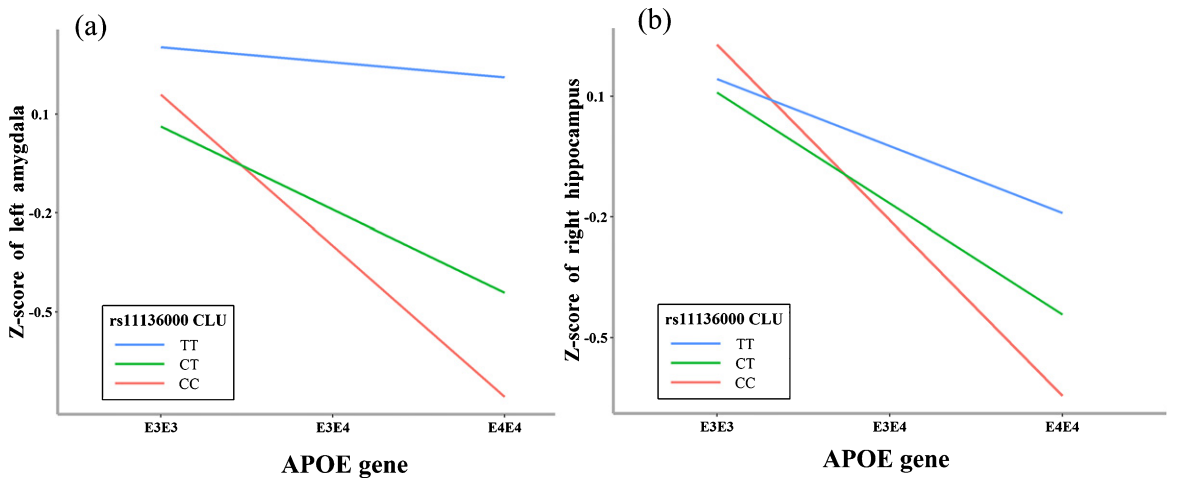

Fig. 2. The associations of *APOE* ε4 allele with greater atrophy of left amygdala (a) and right hippocampus (b) in the *CLU* subgroups at 2-year follow-up.

significant interaction on the hippocampal (left hippocampus: $p = 0.328$; right hippocampus: $p = 0.084$, Table 7) and amygdalar (left amygdala: $p = 0.118$; right amygdala: $p = 0.321$, Table 7) volumes at baseline. In the follow-up studies, as shown in Fig. 2, the epistatic interactions were present in the left amygdala ($p = 0.01$, Fig. 2a) and right hippocampus ($p = 0.012$, Fig. 2b), after controlling for baseline age, gender, and ICV.

To further explore the basis of the interactions in volumes of bilateral amygdalae and hippocampi over time, we performed subgroup research. Within the subgroup of *CLU* CC homozygotes, higher *APOE* ε4 loading was associated with greater atrophy in the volumes of the bilateral amygdalae (Table 4) and hippocampi (Table 5), whereas these associations were not found in the subgroup of *CLU* TT carriers. Moreover, carrying more ε4 allele was connected with only hippocampal volumes in the subgroup of CT heterozygotes (Table 4). All these results demonstrated that the *APOE* ε4 allele and *CLU* C homozygosity had a synergistic adverse effect in the volumes of bilateral hippocampi and amygdalae.

Table 5
The results of *CLU* subgroup research: the associations between *APOE* ε4 genotype and volumes of bilateral hippocampi in each subgroup of *CLU* at 2-year follow-up

	Left_hippocampus		Right_hippocampus	
	β	p	β	p
CLU TT	0.164	0.332	0.001	0.994
CLU CT	–0.380	**4.61×10^{-4}**	–0.449	**1.47×10^{-4}**
CLU CC	–0.329	**0.004**	–0.511	**3.07×10^{-6}**

[a] β, standardized coefficient estimate. The negative (–) coefficient estimate signifies that the volume decrease with the number of risk-allele, while the positive (+) coefficient estimate means the increase of volume. [b] p-values are rounded to three decimal places. Bold font denotes significant results ($p < 0.05$).

Combined effects of APOE and CLU in the volumes of hippocampus and amygdala

Results of the combined effects of *APOE* and *CLU* in the volumes of bilateral hippocampi and amygdalae are presented in Table 6. Within the volume analyses, we observed the positive relations between

Table 6
Results of multiple linear analyses: Combined effects of *APOE* status and *CLU* genotypes on the volume atrophy of hippocampus and amygdala

	Combined effects of *APOE* and *CLU* genotypes (covariates: Intracranial volume, age, gender)	
	Hippocampus	Amygdala
BL		
Left hemisphere	$\beta = -0.173$	$\beta = -0.332$
	$p = 0.115$	$p =$ **0.002**
Right hemisphere	$\beta = -0.175$	$\beta = -0.285$
	$p = 0.102$	$p =$ **0.008**
24 m		
Left hemisphere	$\beta = -0.132$	$\beta = -0.277$
	$p =$ **0.025**	$p =$ **5.83×10^{-6}**
Right hemisphere	$\beta = -0.163$	$\beta = -0.242$
	$p =$ **0.005**	$p =$ **7.32×10^{-5}**

BL: Baseline; 24 m: at 24-month follow-up. [a] β: standardized coefficient estimate. The negative (–) coefficient estimate signifies that the volume decrease with the number of risk-allele, while the positive (+) coefficient estimate means the increase of volume. [b] p-values are rounded to three decimal places. Bold font denotes significant results ($p < 0.05$).

Table 7
Results of multiple linear analyses: Interaction effects of *APOE* status and *CLU* genotypes on the volume atrophy of hippocampus and amygdala

	Baseline		24-month follow-up	
	β	p	β	p
Left_hippocampus	–0.162	0.328	–0.143	0.161
Right_hippocampus	–0.278	0.084	–0.217	**0.012**
Left_amygdala	–0.251	0.118	–0.234	**0.01**
Right_amygdala	–0.16	0.321	–0.17	0.063

[a] β, standardized coefficient estimate. The negative (–) coefficient estimate signifies that the volume decrease with the number of risk-allele, while the positive (+) coefficient estimate means the increase of volume. [b] p-values are rounded to three decimal places. Bold font denotes significant results ($p < 0.05$).

the number of risk alleles and atrophy of bilateral hippocampi/amygdalae. The baseline age, gender, and ICV were included as covariates in MLR and MLME. What is more, these associations became more obvious over time from the longitudinal analysis.

Surface-based analyses

Main effects of APOE and CLU genotype on surface morphology

The significant atrophy (cold colors) and expansion (warm colors) subregions were captured by the surface-based statistic maps. The surfaces of the left hippocampus and left amygdala (shown in the left sides of Fig. 3) were divided into multiple distinct areas based on previous reports [67, 68, 80]. At baseline, carrying more $\varepsilon 4$ alleles was associated with greater morphological alterations in the left hippocampus (Fig. 3b) and left amygdala (Fig. 3d). The significant atrophy subregions in the left hippocampus were in Cornu Ammonis (CA) 1 and subiculum, while in left amygdala were located in Amygdalostriate Transition Area (ASTR), Lateral (LA), and Anterior Amygdaloid Area (AAA). Additionally, these relationships also appeared in the subregions of the right hippocampus (Fig. 4a) and right amygdala (Fig. 4c) at 2-year follow-up. The atrophy areas of the right hippocampus were in CA 1 and subiculum, while the atrophy subregions of the left hippocampus (Fig. 4a) were gradually extended from CA 1 and subiculum to CA 2-3 over time. As for the amygdala, the significant atrophy areas of the right amygdala were extensively presented in Basolateral Ventromedial Part (BLVM), Basolateral (BL), ASTR, Central (CE), and AAA subregions, whereas the atrophy subregions of the left amygdala (Fig. 4c) were started in the ASTR, LA, and AAA and extended toward the whole surface gradually over time.

However, the effects of *CLU* genotype on regional atrophy of bilateral hippocampi and amygdalae at baseline and 2-year follow-up after controlling *APOE* status, baseline age, gender, and ICV, did not pass FDR correction. This may be due to the heavy burden of multiple tests, which were underpowered to detect small genetic effects (15,000 vertexes for each side of the hippocampus).

Interactions of APOE and CLU genotype on surface morphology

APOE status and *CLU* genotype showed epistatic interactions in regional atrophy of bilateral hippocampi and amygdalae at baseline. However, these influences both disappeared after FDR correction. Moreover, in a 2-year follow-up study, the interactions produced influences on the regional atrophy of bilateral hippocampi and amygdalae, but only the left amygdala (Fig. 4d) survived after FDR correction. The epistatic interaction effects of *APOE* and *CLU* on the regional morphological atrophy of the left amygdala were mainly found in the Posterior cortical (PCO), Anterior Cortical (ACO), CE, ASTR, BL, and AAA subregions.

Combined effects of APOE and CLU on surface morphology

The surface-based statistics revealed the combined effects of *APOE* and *CLU* genotypes on subregional atrophy of bilateral hippocampi and amygdalae at baseline and 2-year follow-up. At the 2-year follow-up, only bilateral amygdalae (Fig. 4b) remained significant after FDR correction. At the baseline, the bilateral amygdalae (Fig. 3c) and hippocampi (Fig. 3a) maps did not pass correction for multiple comparisons using an FDR of 5%, so we presented uncorrected maps. Moreover, we mapped contrast results with uncorrected bilateral hippocampi (Fig. 5) over time. Not all the maps survived correction for multiple comparisons, but interesting trends were still identified. The most atrophy clusters of bilateral amygdalae were overlapped at baseline and 2-year follow-up, and the areas of significant atrophy are mainly included BLVM, BL, AAA, and Medial (ME). Bilateral hippocampi also showed atrophy and expansion subregions which were located in the subiculum and CA 1 subregions. Furthermore, the areas of significant atrophy in the bilateral hippocampus mainly located in the subiculum, CA 1, and CA 2-3 subregions, according to the uncorrected maps at 2-year follow-up.

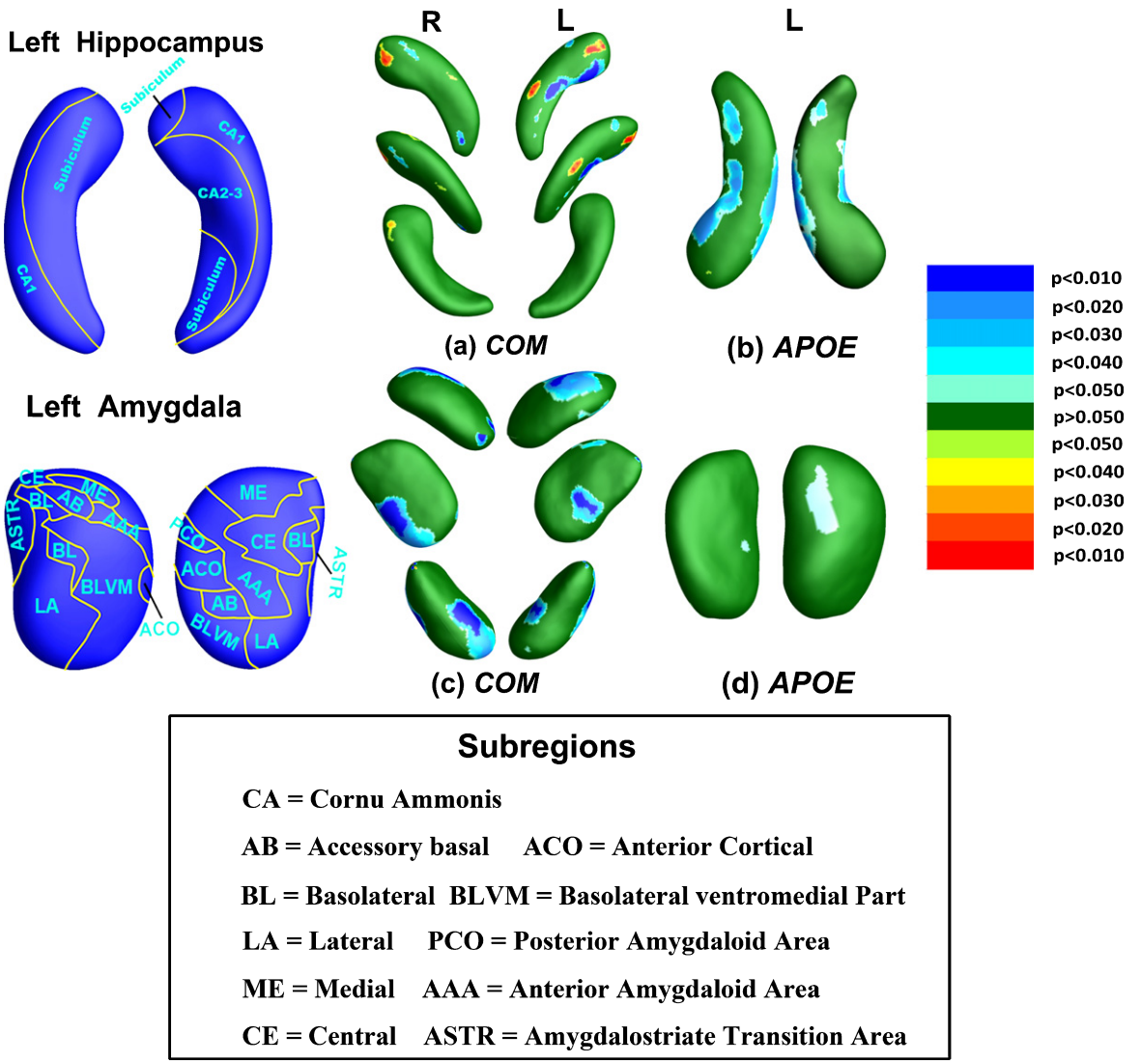

Fig. 3. Surface statistical maps. Top row: (a): the combined effects of *APOE* and *CLU* genotypes on shape deformation of the bilateral hippocampi at baseline (uncorrected). (b): the effects of main *APOE* genotype on shape atrophy of left hippocampus at baseline. Bottom row: (c): the combined effects of *APOE* and *CLU* genotypes on shape deformations of bilateral amygdalae at baseline (uncorrected). (d): the effects of main *APOE* genotype on shape atrophy of left amygdala at baseline. DarkGreen-to-Blue hues indicate subregions in which higher risk-allele loading is associated with atrophy of the surfaces, DarkGreen-to-Red hues indicate regions in which higher risk-allele loading is associated with greater expansion of the surfaces.

DISCUSSION

The main finding of this study is that *CLU* genotype modulates the effects of *APOE* ε4 on the subcortical structures in both hemispheres at 2-year follow-up. In the subgroup of subjects with C allele homozygotes, with the increasing number of ε4 allele, *APOE* showed additive effects in the volumes of the bilateral amygdalae and hippocampi. The significant *APOE*-*CLU* interactions were found in the volumes of the left amygdala and right hippocampus, as well as the shape of the left amygdala; moreover, the combination of *APOE* ε4 allele and *CLU* C allele showed negative additive effects on the volumes and shapes of bilateral hippocampi and amygdalae. Overall, these results may improve our understanding of the complex roles of multiple genes in the structural architecture of the hippocampus and amygdala.

We observed the subjects with 2 *APOE* ε4 allele were younger than those with 0 or 1 *APOE* ε4 allele.

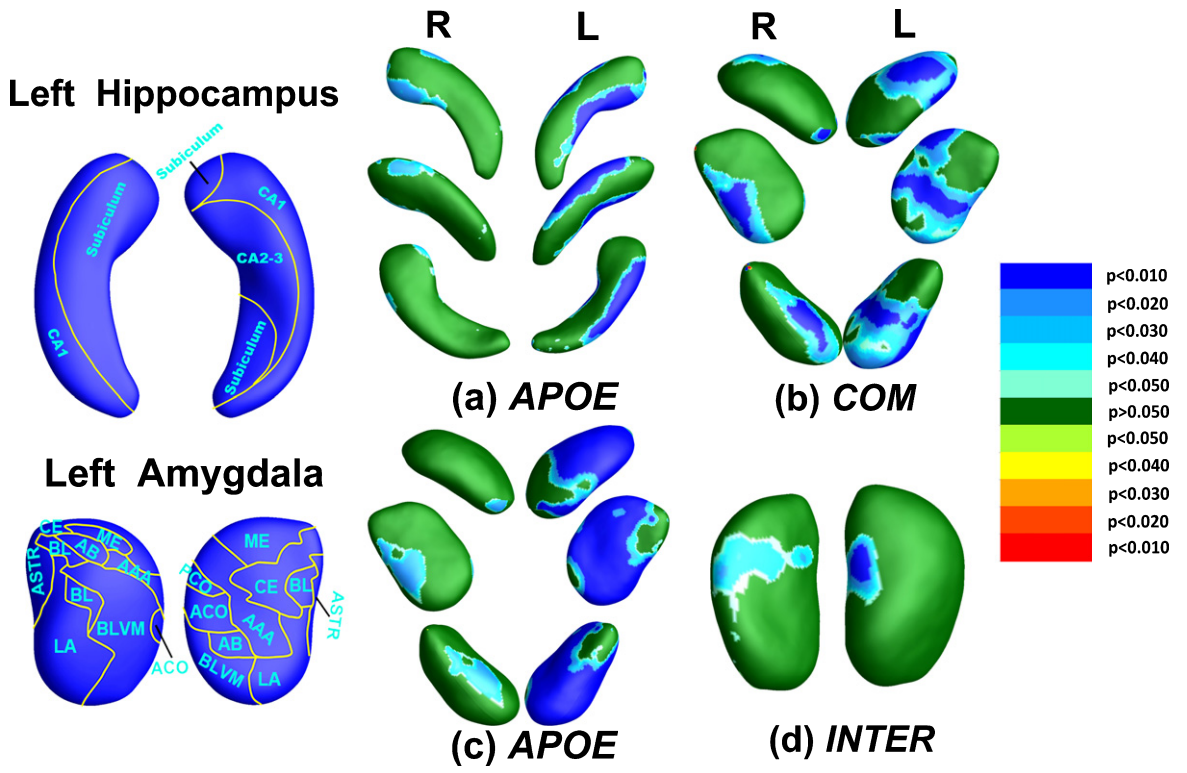

Fig. 4. (a): the relationships between carrying more *APOE* ε4 alleles and regional atrophy of the bilateral hippocampi at 2-year follow-up. (b): the combined effects of *APOE-CLU* on regional deformations of bilateral amygdalae at 2-year follow-up. (c): the effects of *APOE* genotype on regional atrophy of bilateral amygdalae at 2-year follow-up. (d): the interaction effect of *CLU* and *APOE* on regional deformations of the left amygdala at 2-year follow-up. DarkGreen-to-Blue hues indicate subregions in which higher risk-allele loading is associated with atrophy of the surfaces, DarkGreen-to-Red hues indicate regions in which higher risk-allele loading is associated with greater expansion of the surfaces. For all statistical maps, the color bar encodes the FDR-corrected *p*-values for the observed effects.

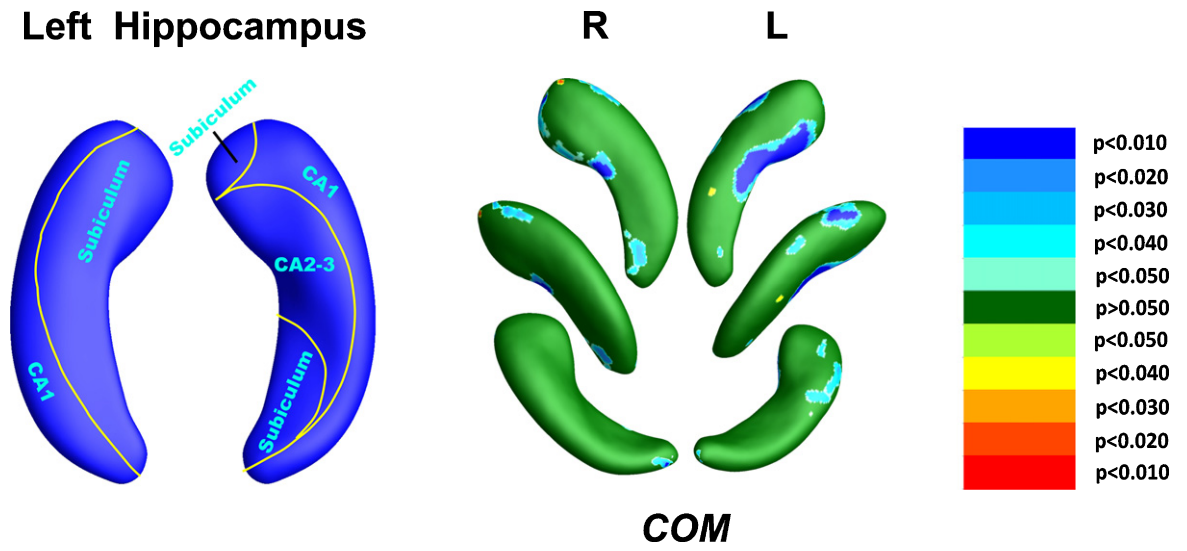

Fig. 5. The uncorrected maps depicting the combined effects of *APOE* and *CLU* genotypes on the regional deformations of the bilateral hippocampi at 2-year follow-up. DarkGreen-to-Blue hues indicate subregions in which higher risk-allele loading is associated with atrophy of the surfaces, DarkGreen-to-Red hues indicate regions in which higher risk-allele loading is associated with greater expansion of the surfaces.

Age was significantly different among the groups and the association between the *APOE* and hippocampal (or amygdalar) morphometrics may be related to an age effect [48, 81]. To overcome the potential confounder of age, sex, and brain size, all analyses were corrected for baseline age, sex, and ICV. In agreement with previous studies [82, 83], we found that *APOE* ε4 carriers had inferior cognitive performances as compared to non-ε4 carriers in non-demented elders. Previous research demonstrated that the MMSE scores were negatively correlated with the volumes of the hippocampus and amygdala [84, 85], suggesting that MMSE score decline was probably a concrete manifestation of morphological atrophy in the hippocampus and amygdala.

Hippocampal and amygdalar degeneration are the most typical characteristic of AD. The volume loss of the hippocampus and amygdala in non-demented individuals has been reported to increase the risk of developing AD [38, 39] and to be associated with higher cerebrospinal fluid tau level (a hallmark of AD pathology) [86–88]. Recent studies have reported gene dose effects of *APOE* ε4 alleles on the volumes of hippocampus and amygdala in patients with MCI and AD [89, 90], our results indicate that the *APOE* ε4 alleles may affect the hippocampal and amygdala atrophy in a dose-dependent manner in non-demented individuals. In addition, most previous reports [5, 91] have found that the main effects of *APOE* ε4 allele on hippocampal morphology were mainly located in the most vulnerable regions, e.g., CA1 and subiculum [92, 93], our results support these findings and show that this effect is cumulative and spread to the CA 2-3 regions over time. Only one prior research [48] revealed the effect of the *APOE* ε4 genotype on morphological changes of the amygdala. However, they only focused on cross-sectional studies. Our results mapped the morphological deformations in the subregions of the amygdala over time and found the atrophy of surfaces primarily started in the ASTR, LA, and AAA and extended toward the whole surfaces over time. The main effects of *CLU* were not found on volumes and shapes of the hippocampus, which is in line with previous studies [41, 79]. In contrast, our findings showed that the *CLU* genotype can exert a significant effect on the volumes of the left amygdala at baseline and bilateral amygdala at 2-year follow-up. Although the mechanisms of the volume loss of the bilateral amygdalae are unclear, we speculate that it may be due to the ventricular expansion affecting the gray and white matter degeneration in nearby subcortical regions [30], for example, the amygdala. However, the effects of *CLU* variant on regional atrophy of bilateral amygdalae did not pass FDR correction at baseline and 2-year follow-up. This may be due to the heavy burden of multiple tests (15,000 vertexes for each side of the amygdala), which was underpowered to detect small genetic effects [30]. While at least 20 genes have been identified as being associated with AD, *APOE* is the strongest genetic risk factor [94]. This mechanism also may be consistent with our current observations of volume and shape which show that *APOE* has a greater effect on hippocampal and amygdala atrophy than *CLU*.

In the main-effect analysis, we often observed that the hippocampal asymmetry in non-demented elders ($R > L$), which was primarily due to the greater degree of atrophy of right hippocampal volume with the increasing number of *APOE* risk-allele. This finding is in line with the results of several other cohort studies [95–97]. However, for the shape measurements, the atrophic areas of the left hippocampus were more enlarged, after FDR correction. We can only speculate the reason for the inconsistent manifestation of the shape and volume in the asymmetric fashion, which is mainly because of the heavy multiple test burden: the small genetic effects failed to pass the FDR correction. Additionally, among all the analyses, the amygdalar shape was mostly consistent with the amygdalar volume in the asymmetry pattern ($L > R$). Our results are in agreement with those of a previous study (in terms of *APOE* genotype's effects) [98]. These inconsistent findings may reflect the complexity in the effects of genetic factors on brain structures. The same SNP may show a different degree of influence on the different brain structures on the left and right sides.

We also found complex epistatic interaction effects ($APOE \times CLU$) of *APOE* and *CLU* on the volumes of the left amygdala and right hippocampus, as well as the shape of left amygdala. In the subgroup of individuals with the *CLU* CC subgroup, carrying more *APOE* ε4 allele was associated with faster atrophy in bilateral hippocampi and amygdalae at 2-year follow-up. The subjects with both *APOE* ε4 allele and *CLU* CC show the strongest volume loss of hippocampus and amygdala before the onset of any cognitive symptom, which indicates the synergistic effects of *APOE* and *CLU* on the subcortical structures. Numerous studies have reported the *APOE* ε4 allele increases Aβ deposition and reduces Aβ efflux from the brain [99, 100]. Moreover, *APOE* or *CLU* knockout in an AD mouse model leads to similar effects on the

accumulation of Aβ, a biomarker of AD [31]. Thus, the *APOE* and *CLU* risk genotypes might affect Aβ deposition through similar pathways, ultimately resulting in the development of AD [101]. The potential synergistic effects on Aβ deposition or clearance may have led to the observed *APOE*-*CLU* interaction on the vulnerable subcortical structure. Within the subgroup of *CLU* TT, carrying more *APOE* ε4 allele was not associated with volumes of bilateral hippocampi and amygdalae, whereas higher ε4 allele load was significantly correlated with the volume loss in the bilateral hippocampi and amygdalae in the *CLU* C-allele subgroup. These findings show that *CLU* might modulate the expression of *APOE* ε4, which may be supported by the findings that *CLU* and *APOE* can influence each other's expression in physiologic and pathologic states [102, 103]. According to the aforementioned findings, we can infer that synergistic effects of *APOE* and *CLU* may increase the risk of developing AD by affecting the volume loss in the hippocampus and amygdala at 2-year follow-up.

Interestingly, the combinations of *CLU* and *APOE* genotypes have additively modified the risk of AD [28]. Previous research has identified that they have additive effects on medial temporal lobe activity [29] and ventricular expansion [30]. We also examined the combined effects of the two SNPs on the volumes and shapes of the hippocampus and amygdala. Although the *CLU* risk genetic factor may not individually show different effects on volumes and shapes of the hippocampus, the combination of the *APOE* and *CLU* may exhibit meaningful influences on the hippocampus and amygdala. The most obvious observation was that the combination of the two SNPs produced greater reduction in the volumes and shapes of bilateral amygdalae than that of any of the individual SNP at baseline and 2-year follow-up. However, this finding was not replicated in the hippocampal volume. A possible explanation is that the *CLU* variant may regulate the *APOE* influences in volumes and shapes of bilateral hippocampi, leading to an insufficient detection power in combined effects. Moreover, the regions of atrophy and expansion regions both presented in the hippocampal surfaces, which may explain why no significant difference between the combined effects and hippocampal volumes at the baseline. The reason for this phenomenon is not yet clear, but we speculate that it may be caused by the artificial grouping of a combination of the two SNPs. Nonetheless, the combination of the two SNPs had stronger effects in the shapes and volumes of the hippocampus and amygdala over time and they were stronger than either of the individual effects in the atrophy progress of the amygdala. Thus, it seems plausible that a combination of *APOE* ε4 allele and *CLU* C allele may accelerate the course of the disease.

The current study has several potential limitations. First, the number of carriers with two ε4 alleles is small. Although the epistatic interactions of *APOE* and *CLU* are found in the morphometry of the amygdala and hippocampus at the 2-year follow-up, the sample size of *APOE* ε4 homozygotes carriers may influence statistical ability to detect more subtle effects on brain atrophy. Second, these exploratory observations need to be confirmed in a larger sample size and longer follow-up. Finally, we only focus on the effects of *APOE* and *CLU*, which are only two of multiple AD-related genes. Further work is needed to explore gene-gene interactions among other AD-related genes.

In summary, the present study is the first attempt to explore the epistatic interactions and combined effects between *APOE* and *CLU* on the volumes and shape morphometrics of the hippocampus and amygdala in non-demented elders. We found that the *APOE* ε4/ε4 and *CLU* CC have strong synergistic adverse effects on the hippocampal and amygdalar volumes, which indicates that gene-to-gene interaction is a crucial factor for the pathogenesis of AD. The synergistic effects also suggest that subjects with both *CLU* CC and *APOE* ε4/ε4 genotypes are the population who may need more attention in early preventive interventions.

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SUPPLEMENTARY MATERIAL

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