

A Multi-Cohort Study of ApoE ϵ 4 and Amyloid- β Effects on the Hippocampus in Alzheimer's Disease

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Abstract. The apolipoprotein E (APOE) gene has been consistently shown to modulate the risk of Alzheimer's disease (AD). Here, using an AD and normal aging dataset primarily consisting of three AD multi-center studies ($n = 1,781$), we compared the effect of APOE and amyloid- β (A β) on baseline hippocampal volumes in AD patients, mild cognitive impairment (MCI) subjects, and healthy controls. A large sample of healthy adolescents ($n = 1,387$) was also used to compare hippocampal volumes between APOE groups. Subjects had undergone a magnetic resonance imaging (MRI) scan and APOE genotyping. Hippocampal volumes were processed using FreeSurfer. In the AD and normal aging dataset, hippocampal comparisons were performed in each APOE group and in $\epsilon 4$ carriers with positron emission tomography (PET) A β who were dichotomized (A β +/A β -) using previous cut-offs. We found a linear reduction in hippocampal volumes with $\epsilon 4$ carriers possessing the smallest volumes, $\epsilon 3$ carriers possessing intermediate volumes, and $\epsilon 2$ carriers possessing the largest volumes. Moreover, AD and MCI $\epsilon 4$ carriers possessed the smallest hippocampal volumes and control $\epsilon 2$ carriers possessed the largest hippocampal volumes. Subjects with both APOE $\epsilon 4$ and A β positivity had the lowest hippocampal volumes when compared to A β - $\epsilon 4$ carriers, suggesting a synergistic relationship between APOE $\epsilon 4$ and A β . However, we found no hippocampal volume differences between APOE groups in healthy 14-year-old adolescents. Our findings suggest that the strongest neuroanatomic effect of APOE $\epsilon 4$ on the hippocampus is observed in AD and groups most at risk of developing the disease, whereas hippocampi of old and young healthy individuals remain unaffected.

Keywords: Alzheimer's disease, amyloid, APOE $\epsilon 4$, hippocampus, magnetic resonance imaging, mild cognitive impairment

INTRODUCTION

The apolipoprotein E (APOE) gene is a well-established genetic risk factor for the development of late-onset Alzheimer's disease (AD) and since its discovery, a large body of research has been conducted to explain its role in AD pathophysiology [1–3]. The APOE $\epsilon 4$ allele, a genetic risk factor known to substantially increase the risk of AD in a dose-dependent fashion, is associated with higher amyloid- β (A β) deposition [4, 5]. By contrast, the APOE $\epsilon 2$ allele has been suggested to confer a protective effect against AD [6].

Hippocampal volumetry has been shown to be a sensitive, albeit non-specific marker of neurodegeneration in AD. Previously, it has been used to

demonstrate accelerated rates of hippocampal atrophy in $\epsilon 4$ carriers with amnesic mild cognitive impairment (MCI) [7, 8]. Although the exact temporospatial relationship between A β and tau in the pathological cascade of AD is unclear, it has been suggested that both proteinopathies may have a synergistic effect on neuronal toxicity [9]. Emerging data also suggests that these pathological processes that influence cognitive decline in AD are moderated by APOE $\epsilon 4$ through both A β -dependent and A β -independent mechanisms [10]. However, evidence for the direct mechanistic actions of APOE $\epsilon 4$ is mixed.

Despite the numerous studies, our limited understanding of APOE $\epsilon 4$ risk in asymptomatic individuals and individuals with varying stages of AD pathophysiology warrants further study. Evidence

has suggested that equivocal findings in neuroimaging studies of APOE $\epsilon 4$ may be attributed to the lack of specificity between changes that are due to normal aging and those that manifest as a result of pathological neurodegeneration [11]. Furthermore, some APOE neuroimaging findings originate from studies with limitations in sample size and hence study power [12]. This is further exacerbated by the varying distribution of APOE $\epsilon 4$ carrier status across different samples. However, recent neuroimaging APOE studies with well-characterized samples (between 400–700 subjects) are beginning to address this methodological issue [13, 14]. Also, recent neuroimaging evidence from young APOE $\epsilon 4$ carriers has shown that structural and functional alterations in the brain may precede A β pathology [15, 16]. This had led researchers to postulate that APOE $\epsilon 4$ may exert neurodevelopmental changes that provide a foothold for the pathological cascade of AD later in life.

In order to capture the heterogeneity of varying AD risk, we combined several well-characterized cohort studies to evaluate the neuroanatomic effect of APOE genotype on hippocampal volumes. The first dataset in our study, known as the AD and normal aging dataset ($n = 1,781$) consisted of imaging data from three large multi-center AD consortiums, (i) The Alzheimer's Disease Neuroimaging Initiative (ADNI), (ii) the AddNeuroMed study (ANM), and (iii) the Australian Imaging, Biomarkers and Lifestyles (AIBL) study, as well as a sample of non-demented individuals from the Swedish National Study on Aging and Care in Kungsholmen (SNAC-K) and a dementia study from King's College London, UK (BRC-AD). The sample of non-demented individuals from the SNAC-K study were considered separate from healthy controls as the recruitment criteria for these subjects was epidemiological in nature and we could not exclude the possibility of memory impairment in some subjects. In our second dataset, we used a large sample of healthy 14-year-old adolescents ($n = 1,387$) in order to determine the neurodevelopmental effect of APOE $\epsilon 4$ genotype on hippocampal volume. This is particularly important because adolescence is a time of substantial dynamic neurobiological and behavioral changes. These changes are often beneficial and can optimize the brain for adult maturation, but can also confer neural vulnerabilities for certain types of psychiatric or neurological illness.

The aim of this study was to firstly evaluate the neuroanatomic effect of APOE genotype on the

hippocampus in the AD and normal aging dataset. Previous studies have demonstrated a linear effect of APOE genotype on disease risk, with $\epsilon 2$ carriers possessing a low risk of developing AD, and $\epsilon 4$ carriers possessing the greatest risk [17]. We aimed to test if this linear stepwise effect of APOE genotype also imparted a similar neuroanatomic effect on the hippocampus across the different stages of AD pathophysiology. We then tested the neuroanatomic effect of APOE genotype on hippocampal volumes of healthy 14-year-old adolescents to determine the role of APOE in adolescent brain development.

On the other hand, converging evidence suggests that APOE $\epsilon 4$ modifies A β accumulation and may have downstream effects on tau neurodegeneration [18]. To further elucidate the mechanisms of this proposed synergistic relationship, we aimed to examine whether high levels of A β deposition would lead to greater hippocampal loss compared to low levels of A β deposition in APOE $\epsilon 4$ carriers.

MATERIALS AND METHOD

Datasets

Cohort specific inclusion criteria and details of the study design can be found in previous publications [19–23]. Table 1 provides details of the number of subjects from each cohort included in the AD and normal aging dataset.

AD and Normal Aging Dataset ($n = 1,781$)

Alzheimer's Disease Neuroimaging Initiative ($n = 779$)

A detailed description of the study design can be found on the ADNI webpage (<http://www.adni-info.org>). Data was obtained for subjects from the ADNI online database (<http://adni.loni.usc.edu>). Subjects were between 55 and 90 years of age. ADNI was approved by the institutional review board and ethics committees of participating institutions, and written informed consent was obtained from all participants or their next of kin.

- i. *AD ($n = 177$)*: General inclusion/exclusion criteria: 1) MMSE scores between 20 and 26; 2) CDR of 0.5 or 1.0; 3) met NINCDS/ADRDA criteria for probable AD.
- ii. *MCI ($n = 383$)*: General inclusion/exclusion criteria: 1) subjects had MMSE scores between 24 and 30 (inclusive); 2) memory complaint

Table 1
Number of subjects obtained from each cohort study in the AD and normal aging dataset ($n = 1,781$)

Cohort Study	Clinical Diagnosis	Number of subjects (%)
Alzheimer's Disease Neuroimaging Initiative ($n = 779$)	Alzheimer's disease	177 (22.7%)
	Mild Cognitive Impairment	383 (49.2%)
	Healthy Controls	219 (28.1%)
AddNeuroMed study ($n = 303$)	Alzheimer's disease	109 (36.0%)
	Mild Cognitive Impairment	97 (32.0%)
	Healthy Controls	97 (32.0%)
Australian, Imaging, Biomarkers and Lifestyles (AIBL) study ($n = 228$)	Alzheimer's disease	46 (20.2%)
	Mild Cognitive Impairment	42 (18.4%)
	Healthy Controls	140 (61.4%)
Biomedical Research Centre for Dementia, King's College London (BRC-AD) study ($n = 89$)	Alzheimer's disease	33 (37.1%)
	Healthy Controls	56 (62.9%)
Swedish National study on aging and care in Kungsholmen (SN-ACK) ($n = 382$)	Healthy Controls	382 (100%)

had objective memory loss measured by education adjusted scores on Wechsler Memory Scale Logical Memory II; 3) CDR of 0.5; 4) absence of significant levels of impairment in other cognitive domains, essentially preserved activities of daily living, and an absence of dementia.

- iii. *Cognitively normal (CN) (n = 219)*: General inclusion/exclusion criteria: 1) MMSE scores between 24 and 30 (inclusive); 2) CDR of zero; 3) they were non-depressed, non-MCI, and non-demented.

AddNeuroMed (ANM) study (n = 303)

Information regarding the study design and enrolment criteria has been previously described elsewhere [21, 24]. All AD and MCI subjects were recruited from the local memory clinics of one of the six participating sites while the control subjects were recruited from non-related members of the patient's families, caregiver's relatives, and social centers for the elderly or GP surgeries. ANM was approved by the South London and Maudsley NHS Foundation Trust ethics committee and other ethical review boards of each participating country. Ethics committee approval was obtained at each of the participating centers in accordance with the Alzheimer's Associations published recommendations.

- i. *AD (n = 109)*: Inclusion criteria: 1) ADRDA/NINCDS and DSM-IV criteria for probable AD. 2) Mini-Mental State Examination (MMSE) score ranged from 12 to 28; 3) age 65 years or above. Exclusion criteria: 1) Significant neurological or psychiatric illness other than AD; 2) Significant unstable systematic illness or organ failure.

- ii. *MCI (n = 97) and CN (n = 97)*: Inclusion criteria: 1) MMSE score range between 24 and 30; 2) Geriatric Depression Scale score less than or equal to 5; 3) age 65 years or above; 4) medication stable; 5) good general health. Exclusion criteria: 1) met the DSM-IV criteria for dementia; 2) significant neurological or psychiatric illness other than AD; 3) significant unstable systematic illness or organ failure.

- iii. *The distinction between MCI and CN individuals was based on two criteria*: 1) subject scores 0 on Clinical Dementia Rating Scale (CDR) = CN; 2) Subject scores 0.5 on CDR = MCI. For the MCI subjects, it was preferable that the subject and informant reported occurrence of memory problems. All AD subjects had a CDR score of 0.5 or above.

The Australian, Imaging, Biomarkers, and Lifestyles (AIBL) study (n = 228)

The AIBL study is a prospective longitudinal study of aging, integrating data from neuroimaging, biomarkers, lifestyle, clinical, and neuropsychological analysis. Detailed information about the study design has been described in previous publications [20, 25]. CN individuals were recruited by advertisement in the community while MCI and AD patients were recruited from tertiary memory disorders clinics or private geriatricians, psychiatrists, and neurologists that subspecialize in dementia. All participants were at least 60 years of age, in good general health with no history of stroke or other neurological disease. The institutional ethics committees of Austin Health, St Vincent's Health, Hollywood Private Hospital and Edith Cowan University approved the AIBL study, and

all volunteers gave written informed consent before participating.

- i. *AD* ($n = 46$): Inclusion criteria: 1) all met NINCDS-ADRDA criteria for probable AD; 2) had a CDR of 1 or more.
- ii. *MCI* ($n = 42$): Inclusion criteria: 1) met criteria of subjective and objective cognitive difficulties in the absence of significant functional loss; 2) had a CDR of less than 1. 52 MCI participants fulfilled criteria for “amnesic” MCI, and 5 were non-amnesic cases (4 were non-amnesic multi-domain and 1 was non-amnesic single domain).
- iii. *CN* ($n = 140$): Inclusion criteria: participants were separated in those who reported subjective memory complaints ($n = 95$) and those who did not ($n = 82$), according to their response to the question: “Do you have any difficulty with your memory?”

Biomedical Research Centre for Mental Health and Dementia Cohort, King’s College London (BRC-AD) ($n = 89$)

BRC-AD is a neuroimaging study which was designed to establish imaging markers for the earlier detection and diagnosis of AD. Data was collected at the Institute of Psychiatry, Psychology, and Neuroscience, King’s College London, UK. A total of 89 subjects (AD: 33, CN:56) were obtained with APOE data for this study. Diagnostic inclusion and exclusion criteria for this study were exactly the same as for the ANM study.

Swedish National study on Aging and Care in Kungsholmen (SNAC-K) study ($n = 382$)

Participants were recruited from a larger population-based epidemiological study, the SNAC-K. In this study, participants were randomly selected to take part from the island of Kungsholmen in central Stockholm to examine aging in late adulthood [26]. During the first data collection, a subsample of non-institutionalized and non-disabled participants were randomly selected to undergo MRI. Participants with dementia diagnoses, schizophrenia diagnosis, bipolar disorder diagnosis, self-reported stroke, stroke observed on MRI, self-reported Parkinson’s disease, or epilepsy, were excluded. The study design has been described in detail elsewhere [22]. We used a sample of 459 individuals from SNAC-K, who underwent MRI imaging and APOE genotyping. 77 subjects with suboptimal MRI images and

neurological and/or psychiatric conditions were excluded. The SNAC-K study was population-based, therefore subjects in this sample were considered a heterogeneous sample of elderly participants and treated separate from our sample CN individuals.

The SNAC-K study complies with the declaration of Helsinki, and has been approved by the ethical committee at Karolinska Institutet. All subjects gave informed consent, and in the case of severe cognitive impairment consent was collected from next-of-kin.

Neuroimaging-Genetics IMAGEN Study ($n = 1,387$)

This is the first European multi-center study combining genetics with behavioral and neuropsychological measures, functional and structural neuroimaging, and genome-wide association analyses in 2,000 healthy 14-year-old adolescents. A description of the study design are provided in Schumann et al. [23]. We selected 1,387 healthy adolescents with available MRI data and ApoE status information.

Genome-wide genotyping was performed using Illumina Quad 610 and 660 arrays (San Diego, CA, USA). Quality control of the genome-wide data was performed and samples with the following criteria were excluded: genotype call rate < 95%, and those with discordance between clinical and genotypic gender. Single nucleotide polymorphisms (SNP) quality control filters were used as described in the ENIGMA consortium imputation protocol (<http://enigma.ini.usc.edu/protocols/genetics-protocols/>). Further details on the imputation of unobserved SNP’s to determine APOE status are described in detail elsewhere [27].

Image acquisition

High resolution 3D T1-weighted MRI were acquired for each subject and a comprehensive quality control procedure was applied to all MR images according to the AddNeuroMed study quality control framework [24, 28].

ADNI: The protocol included a high resolution T1 weighted sagittal 3D MP-RAGE volume (voxel size $1.1 \times 1.1 \times 1.2$ mm³), and axial proton density with T2 weighted fast spin echo images. MRI scanner protocols from models of General Electric (GE) Healthcare, Philips Medical Systems, and Siemens Medical Solutions were supported.

ANM: Data acquisition took place using six different 1.5T MR systems (4 General Electric, 1 Siemens, and 1 Picker). At each site a quadrature birdcage coil was used for RF transmission and reception. Data acquisition was designed to be compatible with the ADNI. The imaging protocol included a high resolution sagittal 3D T1-weighted MPRAGE volume (voxel size $1.1 \times 1.1 \times 1.2 \text{ mm}^3$) and axial proton density / T2-weighted fast spin echo images. The BRC-AD study protocol was designed to be the same as the ANM protocol.

AIBL: T1-weighted MRI was obtained using the ADNI 3D Magnetization Prepared Rapid Gradient Echo (MPRAGE) sequence, with $1 \times 1 \text{ mm}$ in-plane resolution and 1.2 mm slice thickness, TR/TE/T1 = 2300/2.98/900, flip angle 9° and field of view 240×256 and 160 slices. T2 FSE and FLAIR sequences were also obtained. The AIBL protocol has been described in extensive detail previously [20].

SNAC-K: MRI scanning was undertaken on a 1.5T scanner (Philips Intera, Netherlands) on which 3D FFE (fast field echo) T1, Axial SE (spin echo) Proton Density/T2, DTI (Diffusion Tensor Imaging), and Axial FLAIR (fluid-attenuated inversion recovery) were acquired. In this study, the 3D FFE T1 images (TR = 15 ms, TE = 7 ms, Flip angle = 15° , number of slices = 128, thickness = 1.5 mm, in-plane resolution = $0.94 \times 0.94 \text{ mm}$, no gap, Field of view = 240, matrix = 256×256) were used.

IMAGEN: MRI images were acquired using 3T MRI systems from major manufacturers (Siemens, Philips, Bruker, and General Electric). The protocol included a high-resolution 3D T1-weighted ultra-fast gradient echo volume (voxel size $1.1 \times 1.1 \times 1.1 \text{ mm}^3$) and axial proton density T2-weighted fast spin echo images based on the ADNI study protocol.

Aβ PET methods

Data on Aβ imaging with positron emission tomography (PET) was accessed for a total of 95 ADNI and 57 AIBL $\epsilon 4$ carriers. Aβ imaging for these subjects was conducted using either ^{11}C -Pittsburgh Compound B (PiB), or ^{18}F -florbetapir. PET scans that were acquired as close as possible to the structural MRI scans in the ADNI and AIBL study were chosen. The PET imaging methodology of the ADNI and AIBL studies has been extensively described elsewhere [29, 30]. For PiB PET, the measure of amyloid burden was calculated by averaging the ratio of cortical to cerebellar signal (SUVR) measurements

from frontal, parietal, anterior cingulate, and parietal regions of interest [29]. For ^{18}F -florbetapir, the SUVR was calculated for 6 pre-defined regions of interest (frontal, temporal, parietal, anterior cingulate, posterior cingulate, and precuneus). The whole cerebellum was used as a reference region for both PiB and ^{18}F -florbetapir PET.

Of the 95 subjects that underwent PET neuroimaging in the ADNI study, 66 were scanned using ^{18}F -florbetapir and 29 using PiB. For the AIBL study, all subjects underwent PET neuroimaging using PiB. The global measure of amyloid burden was used to define participants as Aβ positive (Aβ+) and Aβ negative (Aβ-). PiB participants were classified as Aβ+ when the measure of amyloid burden was ≥ 1.5 [30] and ^{18}F -florbetapir participants were classified as Aβ+ if the measure of amyloid burden was ≥ 1.11 [31].

Image analysis

Volumetric segmentation of the hippocampus was performed using FreeSurfer (5.1.0). FreeSurfer utilizes an affine rigid linear transformation and combines spatial information about voxel intensity relative to a probability distribution for tissue classes [32]. The FreeSurfer segmentation process includes motion correction of volumetric T1-weighted images, removal of non-brain tissue using a hybrid watershed/surface deformation procedure [33], automated Talairach transformation, segmentation of the subcortical white matter and deep grey matter volumetric structures (including hippocampus, amygdala, caudate, putamen, ventricles) [34], intensity normalization [33], tessellation of the grey matter white matter boundary, automated topology correction [35, 36], and surface deformation following intensity gradients to optimally place the grey/white and grey/cerebrospinal fluid borders at the location where the greatest shift in intensity defines the transition to the other tissue class [37–39]. Further details of the segmentation approach have been described previously [34]. Quality control of hippocampal images were performed by visualizing the subcortical segmentation borders of the hippocampus for every subject. Images reflecting a poor segmentation of the hippocampal structures were excluded from the study. Hippocampal volumes were normalized by subject intracranial volume ($\text{volume}_{\text{normalized}} = \text{volume}_{\text{raw}} * 1,000 / \text{intracranial volume}$) to correct for individual differences in head size.

Statistical analysis

To compare demographic statistics, Fisher's exact tests and ANOVA with Tukey-Kramer HSD *post-hoc* tests were used. A linear mixed model regression was used to compare hippocampal volumes by APOE genotype ($\epsilon 2/\epsilon 3/\epsilon 4$). In accordance with previous work, 44 $\epsilon 2/\epsilon 4$ individuals were excluded from the analysis due to the opposing protective effect of the $\epsilon 2$ allele and detrimental effect of $\epsilon 4$ allele [40]. The model treated subject age, gender, and baseline diagnosis as fixed terms in the final model. Interaction terms for APOE genotype and diagnosis were also included and image acquisition site was included as a random effect term.

Hippocampal comparisons were also performed in a sample of subjects from the ADNI and AIBL cohorts who were divided into A β + and A β - participants. Pairwise multiple comparisons were corrected using a stringent Bonferroni correction method. The R statistical software environment, version 3.1.1, was used to perform all statistical analyses in RStudio. The nlme v3.1-117 [41] package was used to create linear-mixed effects models and multcomp v1.3-6 [42] package for *post-hoc* comparisons.

RESULTS

Demographic characteristics

In the AD group, a difference in age between APOE groups was significant ($p = 0.016$) with $\epsilon 2$ carriers being significantly older than $\epsilon 3$ carriers and $\epsilon 4$ carriers. For MCI subjects, $\epsilon 2$ and $\epsilon 3$ carriers had significantly higher MMSE scores than $\epsilon 4$ carriers ($p = 0.016$). No other demographic characteristics differed between the groups (Table 2).

Comparing the effect of APOE genotype on hippocampal volume in the AD, and normal aging dataset ($n = 1,781$) and the IMAGEN study of healthy 14-year-old adolescents ($n = 1,387$)

In AD patients, $\epsilon 4$ carriers had significantly smaller hippocampal volumes than non-carriers. A significant linear stepwise reduction in hippocampal volume was observed with $\epsilon 2$ carriers possessing the largest volumes, $\epsilon 3$ carriers possessing intermediate volumes and $\epsilon 4$ carriers possessing the smallest volumes. This pattern was also observed in the MCI group (Table 3). The effect of the $\epsilon 4$ allele on hippocampal volume was found to be moderately

dose-dependent in AD patients [left region: Cohen's $d = 0.10$, $p \leq 0.0001$; right region: Cohen's $d = 0.22$, $p \leq 0.0001$] and MCI subjects [left region: Cohen's $d = 0.15$, $p \leq 0.0001$; right region: Cohen's $d = 0.19$, $p \leq 1$].

The effect of APOE genotype on hippocampal volumes in CN individuals was not significant (left region: $p = 0.052$; right region: $p = 0.053$) and the magnitude of this non-significant difference in both regions was small (Cohen's $d = 0.16$; effect size $r = 0.08$). This non-significant pattern was also observed in elderly individuals from the SNAC-K study, as well as in healthy adolescents from the IMAGEN study (Table 3). Figure 1 displays hippocampal volumes by APOE genotype in the AD and normal aging dataset and IMAGEN study of adolescents.

Hippocampal volumes of AD $\epsilon 4$ carriers were found to be significantly smaller than MCI, CN, and non-demented $\epsilon 4$ carriers. This pattern was also observed in MCI $\epsilon 4$ carriers who had significantly smaller volumes than CN, and non-demented $\epsilon 4$ carriers (Table 4). In carriers of the $\epsilon 2$ allele, the MCI and CN groups possessed significantly larger hippocampal volumes than $\epsilon 2$ carriers of the AD group (Table 5).

Comparison of hippocampal volumes by ApoE $\epsilon 4$ and A β deposition

In this analysis, $\epsilon 4$ carriers from the ADNI and AIBL cohort studies were selected and divided into A β + and A β - participants to assess if the effect of APOE $\epsilon 4$ on the hippocampus is modified by levels of A β deposition. Descriptive information for this sample is shown in Table 6. Among $\epsilon 4 + CN$ individuals, hippocampal volumes between A β + and A β - participants did not significantly differ (left region: $p = 0.692$; right region: $p = 0.946$). MCI A β + participants were found to have significantly smaller hippocampal volumes than MCI A β - participants for the right hippocampus (left hippocampus: $p = 0.295$; right hippocampus: $p = 0.054$). Hippocampal volumes of MCI A $\beta + \epsilon 4 +$ participants did not differ when compared to AD A $\beta + \epsilon 4 +$ participants. AD A $\beta + \epsilon 4 +$ participants possessed significantly smaller hippocampal volumes than (i) the CN A $\beta - \epsilon 4 +$ group (left region: Cohen's $d = -1.05$; $p \leq 0.001$; right region: Cohen's $d = -1.03$; $p \leq 0.001$) and the (ii) CN A $\beta + \epsilon 4 +$ group (left region: Cohen's $d = -1.09$; $p \leq 0.001$; right region: Cohen's $d = -1.02$; $p \leq 0.001$). The complete results for these comparisons are shown in Table 7. There was no interaction

Table 2

Demographic characteristics of 1) the AD and normal aging dataset by subject group and 2) the IMAGEN study of healthy adolescents

	$\epsilon 2$ carriers (<i>n</i> = 344)	$\epsilon 3$ carriers (<i>n</i> = 1754)	$\epsilon 4$ carriers (<i>n</i> = 1070)	<i>p</i> -value
	$\epsilon 2/\epsilon 3$ (<i>n</i> = 326) $\epsilon 2/\epsilon 2$ (<i>n</i> = 18)	$\epsilon 3$ Homozygotes	$\epsilon 4/\epsilon 3$ (<i>n</i> = 893) $\epsilon 4/\epsilon 4$ (<i>n</i> = 177)	
1) AD and Normal Aging Dataset (<i>n</i> = 1,781)				
i) AD (<i>n</i> = 375)				
Number of subjects (<i>n</i> (%))	17 (4.5)	125 (33.3)	223 (59.5)	—
Age (years)	78.1 ± 6.6	76.9 ± 7.4	74.9 ± 6.7	0.016
Male (<i>n</i> (%))	4 (23.5)	56 (44.8)	112 (50.2)	0.085
Education	12.0 ± 3.6	11.8 ± 5.1	12.2 ± 4.5	0.847
MMSE	21.3 ± 5.8	22.1 ± 4.1	22.1 ± 3.7	0.734
ICV (mL)	1437 ± 165	1520 ± 166	1532 ± 188	0.110
ii) MCI (<i>n</i> = 522)				
Number of subjects (<i>n</i> (%))	29 (5.6)	233 (44.6)	260 (49.8)	—
Age (years)	75.6 ± 7.1	75.3 ± 7.5	74.2 ± 6.9	0.186
Male (<i>n</i> (%))	15 (2.9)	144 (27.6)	152 (29.1)	0.508
MMSE	27.6 ± 1.4	27.1 ± 1.8	26.8 ± 1.8	0.016
ICV (mL)	1491 ± 173	1558 ± 173	1559 ± 169	0.123
iii) CN individuals (<i>n</i> = 512)				
Number of subjects (<i>n</i> (%))	66 (12.9)	275 (53.7)	171 (33.4)	—
Age (years)	75.8 ± 6.5	75.9 ± 6.3	75.6 ± 6.2	0.936
Male (<i>n</i> (%))	28 (5.5)	139 (27.1)	84 (16.4)	0.495
MMSE	28.9 ± 1.1	29.0 ± 1.1	29.1 ± 1.1	0.537
ICV (mL)	1506 ± 174	1521 ± 162	1518 ± 160	0.797
iv) SNAC-K (<i>n</i> = 382)				
Number of subjects (<i>n</i> (%))	45 (11.8)	239 (62.6)	98 (25.7)	—
Age (years)	67.9 ± 7.8	70.1 ± 8.6	69.5 ± 8.6	0.269
Male (<i>n</i> (%))	15 (3.9)	102 (26.7)	37 (9.7)	0.420
MMSE	29.2 ± 0.9	29.2 ± 1.0	29.1 ± 1.1	0.365
ICV (mL)	1478 ± 265	1509 ± 248	1522 ± 275	0.649
2) IMAGEN (<i>n</i> = 1,387)				
Number of subjects (<i>n</i> (%))	187 (13.5)	882 (63.6)	318 (22.9)	—
Age (years)	14.4 ± 0.4	14.5 ± 0.4	14.5 ± 0.4	0.523
Male (<i>n</i> (%))	97 (7.0)	437 (31.5)	152 (11)	0.674
Body Mass Index (BMI)	20.6 ± 3.2	20.7 ± 3.6	20.8 ± 3.3	0.843
CANTAB SWM	31.3 ± 5.3	31.2 ± 5.5	31.0 ± 5.5	0.866
Verbal IQ	109.6 ± 15.7	111.6 ± 15.5	111.0 ± 14.9	0.258
Nonverbal IQ	106.5 ± 14.9	107.8 ± 14.4	107.7 ± 14.1	0.523
ICV (mL)	1472 ± 155	1487 ± 156	1490 ± 162	0.397

MMSE, Mini-Mental State Examination; ICV, intracranial volume; CANTAB SWM, Cambridge Neuropsychological Test Automated Battery [CANTAB] Spatial Working Memory; IQ, intelligence quotient. Data are Mean ± SD. Percentages are displayed in parentheses.

observed between gender and $\epsilon 4$ status in hippocampal volumes between groups. Hippocampal differences between the different A β + and A β - participants are shown in Fig. 2.

DISCUSSION

Prior neuroimaging studies of APOE $\epsilon 4$ have helped define our current observation of structural changes in the brain, but the mechanisms associated with the detrimental effect of APOE $\epsilon 4$, particularly across the different stages of AD, still remains poorly understood. With recent studies proposing a neurodevelopmental foothold of APOE $\epsilon 4$ on the brain [43, 44], understanding whether atrophy in AD-susceptible areas, such as the hippocampus, are

attributed to pre-clinical manifestations of the disease, or whether these constitute a part of non-specific normal aging is of great importance for earlier diagnosis.

Here we present the largest cross-sectional multi-cohort study of APOE and hippocampal volume to date (*n* = 3,168) and discuss a number of key findings. Firstly, a linear neuroanatomic effect of the APOE genotype was observed for hippocampal volumes of AD and MCI subjects, whereby $\epsilon 4$ carriers presented with the lowest volumes, $\epsilon 3$ homozygotes possessed intermediate volumes, and $\epsilon 2$ carriers possessed the largest volumes. As expected, $\epsilon 4$ carriers in the AD and the MCI group had significantly lower hippocampal volumes when compared to CN individuals and a population-based sample of elderly

Table 3
Hippocampal volume results by ApoE genotype in each subject group

	$\epsilon 2$ carriers	$\epsilon 3$ Homozygotes	$\epsilon 4$ carriers	p -value	t -value ($\epsilon 4$ versus $\epsilon 2$ carrier)	Pairwise Difference ($\epsilon 2 > \epsilon 3 > \epsilon 4$)
<i>Alzheimer's disease</i> ($n = 365$)						
Left Hippocampus	2.00 (0.11; 1.77–2.22)	1.91 (0.04; 1.83–1.97)	1.80 (0.02; 1.75–1.84)	0.0001	–2.2	$\epsilon 4 < \epsilon 3 = 0.0006$; $\epsilon 4 < \epsilon 2 = 0.07$; $\epsilon 3 < \epsilon 2 = 0.86$
Right Hippocampus	2.04 (0.12; 1.80–2.29)	1.98 (0.04; 1.91–2.06)	1.81 (0.02; 1.77–1.86)	<0.0001	–2.3	$\epsilon 4 < \epsilon 3 = <0.0001$; $\epsilon 4 < \epsilon 2 = 0.042$; $\epsilon 3 < \epsilon 2 = 0.99$
<i>Mild Cognitive Impairment</i> ($n = 522$)						
Left Hippocampus	2.22 (0.06; 2.09–2.35)	2.09 (0.03; 2.03–2.14)	1.97 (0.02; 1.93–2.02)	<0.0001	–3.4	$\epsilon 4 < \epsilon 3 = 0.0004$; $\epsilon 4 < \epsilon 2 = 0.0015$; $\epsilon 3 < \epsilon 2 = 0.19$
Right Hippocampus	2.27 (0.06; 2.14–2.39)	2.13 (0.03; 2.07–2.18)	2.01 (0.02; 1.97–2.05)	<0.0001	–3.3	$\epsilon 4 < \epsilon 3 = <0.0001$; $\epsilon 4 < \epsilon 2 = 0.0019$; $\epsilon 3 < \epsilon 2 = 0.22$
<i>Healthy Controls</i> ($n = 512$)						
Left Hippocampus	2.40 (0.04; 2.32–2.47)	2.36 (0.02; 2.32–2.40)	2.30 (0.03; 2.25–2.36)	0.052	–1.9	$\epsilon 4 < \epsilon 3 = 0.076$; $\epsilon 4 < \epsilon 2 = 0.367$; $\epsilon 3 < \epsilon 2 = 0.865$
Right Hippocampus	2.43 (0.04; 2.35–2.51)	2.39 (0.02; 2.34–2.51)	2.33 (0.03; 2.27–2.39)	0.053	–2.0	$\epsilon 4 < \epsilon 3 = 0.085$; $\epsilon 4 < \epsilon 2 = 0.115$; $\epsilon 3 < \epsilon 2 = 0.827$
<i>Population-based sample of non-demented elders: SNAC-K study</i> ($n = 382$)						
Left Hippocampus	2.72 (0.08; 2.56–2.87)	2.56 (0.03; 2.49–2.62)	2.56 (0.06; 2.45–2.67)	0.395	–1.9	$\epsilon 4 < \epsilon 3 = 0.660$; $\epsilon 4 < \epsilon 2 = 0.367$; $\epsilon 3 < \epsilon 2 = 0.669$
Right Hippocampus	2.66 (0.08; 2.50–2.82)	2.53 (0.03; 2.46–2.59)	2.53 (0.06; 2.42–2.64)	0.509	–2.0	$\epsilon 4 < \epsilon 3 = 0.625$; $\epsilon 4 < \epsilon 2 = 0.530$; $\epsilon 3 < \epsilon 2 = 0.868$
<i>Healthy 14-year-old adolescents: IMAGEN study</i> ($n = 1,387$)						
Left Hippocampus	2.88 (0.02; 2.84–2.93)	2.86 (0.01; 2.84–2.88)	2.88 (0.02; 2.84–2.92)	0.972	–0.3	$\epsilon 4 < \epsilon 3 = 0.714$; $\epsilon 4 < \epsilon 2 = 0.960$; $\epsilon 3 < \epsilon 2 = 0.611$
Right Hippocampus	2.95 (0.02; 2.91–3.00)	2.91 (0.01; 2.89–2.93)	2.94 (0.02; 2.91–2.97)	0.751	–0.8	$\epsilon 4 < \epsilon 3 = 0.399$; $\epsilon 4 < \epsilon 2 = 0.731$; $\epsilon 3 < \epsilon 2 = 0.136$

Data are mean (SE; min-max). Mean values of normalized hippocampal volumes are reported.

non-demented individuals from the SNAC-K study. These findings are in agreement with previous studies demonstrating a distinct neuroanatomic effect of APOE genotype on brain structure, as well as studies reporting smaller hippocampal volumes in MCI subjects with prodromal stages of AD. Furthermore, our finding of larger hippocampal volumes in $\epsilon 2$ carriers in older healthy groups supports the $\epsilon 2$ allele's suggested effect of protection against neurodegeneration. Previous cellular models have advocated its role in a disease staving protective effect, in particular its ability to modify the neuropathological effects of A β accumulation [45].

The absence of APOE-dependent hippocampal volume loss in CN individuals and in a population-based sample of elderly individuals suggested that APOE $\epsilon 4$ may not be independently associated with hippocampal atrophy in normal aging. Although the findings from previous APOE studies in non-demented individuals are mixed, our findings replicate a number of earlier neuroimaging studies showing no effect of APOE $\epsilon 4$ on regions such as the hippocampus in normal aging [46–48]. One explanation for the discrepant APOE findings in CN individuals may be related to differences in defining those that fulfil the criteria for pre-clinical AD from subjects showing typical normal aging [11]. Recent

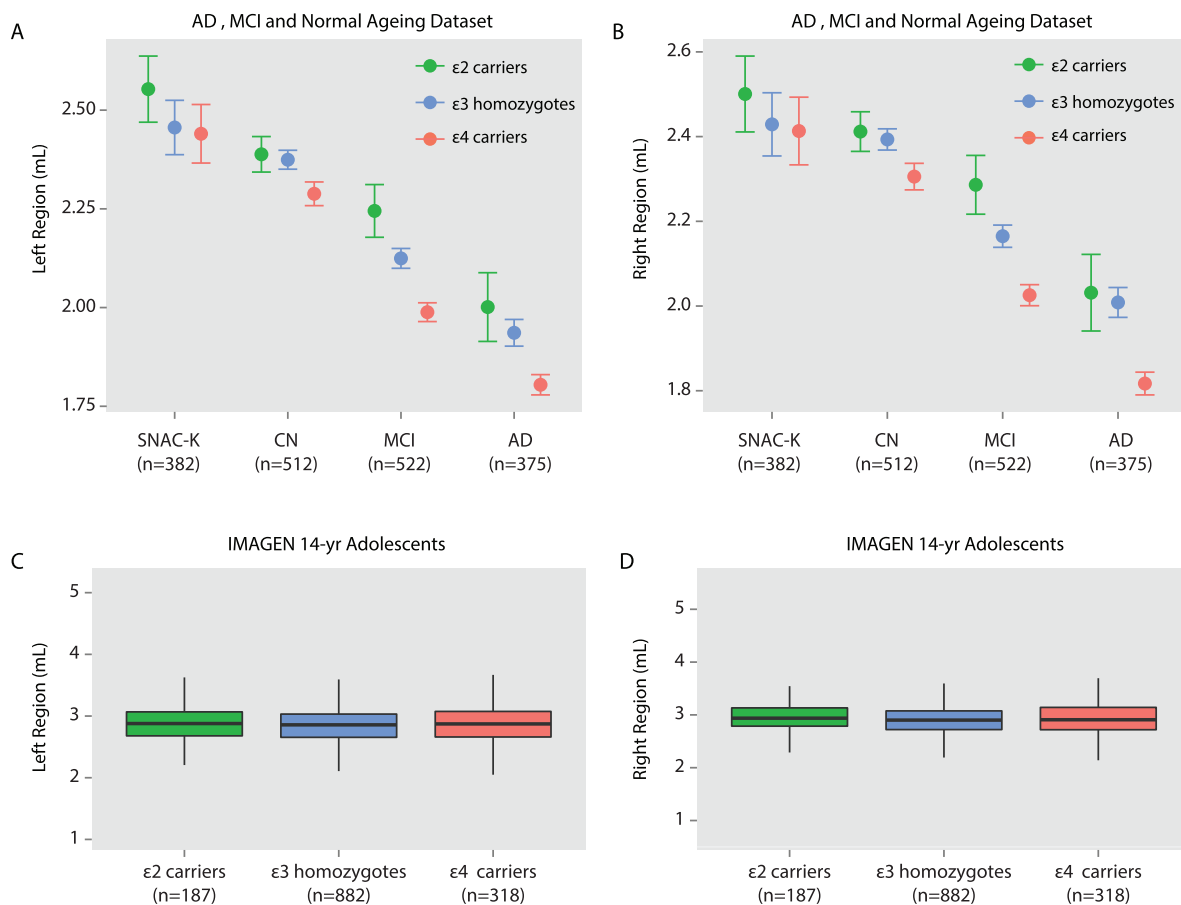


Fig. 1. Hippocampal volumes and ApoE genotype in the AD and normal aging dataset and IMAGEN study. Shown are hippocampal volumes from (a) the left and (b) the right region in the AD and normal aging dataset by baseline diagnosis ApoE genotype ($\epsilon 2$ carriers, $\epsilon 3$ homozygotes, and $\epsilon 4$ carriers). Hippocampal volumes from (c) the left and (d) right region of healthy 14-year-old adolescents in the IMAGEN study. SNAC-K indicates Swedish National study on Aging and Care in Kungsholmen.

Table 4
Hippocampal volume comparisons of ApoE $\epsilon 4$ carriers ($\epsilon 4+$) from different subject groups

	AD $\epsilon 4+$ group (<i>n</i> = 223)	MCI $\epsilon 4+$ group (<i>n</i> = 260)	healthy controls $\epsilon 4+$ group (<i>n</i> = 171)	SNAC-K elderly $\epsilon 4+$ group (<i>n</i> = 98)	<i>p</i> -value	Pairwise Difference (<i>t</i> -value; <i>p</i> -value)
	$\epsilon 3/\epsilon 4$ (<i>n</i> = 159) $\epsilon 4/\epsilon 4$ (<i>n</i> = 64)	$\epsilon 3/\epsilon 4$ (<i>n</i> = 203) $\epsilon 4/\epsilon 4$ (<i>n</i> = 57)	$\epsilon 3/\epsilon 4$ (<i>n</i> = 151) $\epsilon 4/\epsilon 4$ (<i>n</i> = 20)	$\epsilon 3/\epsilon 4$ (<i>n</i> = 84) $\epsilon 4/\epsilon 4$ (<i>n</i> = 14)		
Left Hippocampus	1.79 (0.02; 1.75–1.84)	1.97 (0.02; 1.93–2.02)	2.30 (0.03; 2.25–2.36)	2.56 (0.06; 2.45–2.67)	<0.0001	AD versus MCI = –5.7; <0.001 AD versus CTL = –14.0; <0.001 AD versus SNAC-K = –8.7; <0.001 MCI versus CTL = –9.0; <0.001 MCI versus SNAC-K = –6.3; <0.001 SNAC-K versus CTL = 1.9; 0.208
Right Hippocampus	1.81 (0.02; 1.77–1.86)	2.01 (0.02; 1.97–2.05)	2.33 (0.03; 2.27–2.39)	2.53 (0.06; 2.42–2.64)	<0.0001	AD versus MCI = –6.0; <0.001 AD versus CTL = –13.4; <0.001 AD versus SNAC-K = –7.9; <0.001 MCI versus CTL = –8.1; <0.001 MCI versus SNAC-K = –5.3; <0.001 SNAC-K versus CTL = 1.2; 0.579

Data are presented as the mean of normalized hippocampal volumes ($\text{Volume}/\text{ICV} \times 1000$). SE with min-max is shown in parentheses.

Table 5
Hippocampal volume comparisons of ApoE ε2 carriers (ε2+) from different subject groups

	AD ε2+ group (n = 17)	MCI ε2+ group (n = 29)	healthy controls ε2+ group (n = 66)	SNAC-K elderly ε2+ group (n = 45)	p-value	Pairwise Difference (t-value; p-value)
	ε3/ε2 (n = 17) ε2/ε2 (n = 0)	ε3/ε2 (n = 29) ε2/ε2 (n = 0)	ε3/ε2 (n = 64) ε2/ε2 (n = 2)	ε3/ε2 (n = 42) ε2/ε2 (n = 3)		
Left Hippocampus	2.00 (0.11; 1.77–2.22)	2.22 (0.06; 2.09–2.35)	2.40 (0.04; 2.32–2.47)	2.72 (0.08; 2.56–2.87)	0.0003	AD versus MCI = -2.1; 0.141 AD versus CTL = -4.0; <0.001 AD versus SNAC-K = -3.6; 0.002 MCI versus CTL = -1.9; 0.211 MCI versus SNAC-K = -2.1; 0.137 SNAC-K versus CTL = 1.0; 0.753
Right Hippocampus	2.04 (0.12; 1.80–2.29)	2.27 (0.06; 2.14–2.39)	2.43 (0.04; 2.35–2.51)	2.66 (0.08; 2.50–2.82)	0.0035	AD versus MCI = -2.1; 0.144 AD versus CTL = -3.8; <0.001 AD versus SNAC-K = -2.1; 0.129 MCI versus CTL = -1.5; 0.410 MCI versus SNAC-K = -0.9; 0.796 SNAC-K versus CTL = 0.2; 0.998

Table 6
Demographic characteristics of ε4 carriers from the ADNI and AIBL study divided into Aβ+ and Aβ- participants

	AD Aβ+	MCI Aβ+	MCI Aβ-	CN Aβ+	CN Aβ-	p-value
<i>ADNI study (n = 95)</i>						
Number of subjects (n (%))	8 (8.4)	58 (61.0)	7 (7.4)	14 (14.7)	8 (8.4)	—
Age (years)	70.2 ± 7.3	72.5 ± 7.3	77.3 ± 4.9	77.1 ± 3.4	75.1 ± 4.0	0.041
Male (%)	4 (7.1)	37 (66.1)	5 (8.9)	4 (7.1)	6 (10.7)	0.113
MMSE	23 ± 1.7	27.2 ± 1.7	27.1 ± 1.9	29.4 ± 0.8	29.4 ± 0.9	<0.001
ICV (mL)	1556 ± 210	1583 ± 198	1494 ± 176	1429 ± 98	1591 ± 209	0.08
<i>AIBL study (n = 57)</i>						
Number of subjects (n (%))	7 (12.2)	1 (1.8)	3 (5.3)	20 (35.0)	26 (45.6)	—
Age (years)	77.7 ± 7.8	58 ± —	79.7 ± 12.0	78.1 ± 6.5	71.5 ± 5.8	0.001
Male (%)	1 (3.7)	1 (3.7)	1 (3.7)	10 (37.0)	14 (51.9)	0.296
MMSE	19.4 ± 3.8	27.0 ± —	27.7 ± 2.5	27.4 ± 3.1	27.2 ± 4.3	<0.001
ICV (mL)	1466 ± 87	1515 ± —	1592 ± 301	1535 ± 159	1545 ± 165	0.781

MMSE, Mini-Mental State Examination; ICV, intracranial volume. Bolded values are significant at $p < 0.05$ or the $p < 0.001$ level. In the ADNI study, of the 95 subjects who underwent PET neuroimaging, 66 were scanned using ¹⁸F-florbetapir and 29 using ¹¹C-Pittsburgh Compound B (PiB). In the AIBL study, all subjects underwent PET neuroimaging using PiB.

Table 7
Hippocampal volume results in CN individuals, MCI subjects, and AD patient ε4 carriers by levels of Aβ deposition

	CN Aβ- (n = 34)	CN Aβ+ (n = 34)	MCI Aβ- (n = 10) [‡]	MCI Aβ+ (n = 27) [‡]	AD Aβ+ (n = 15)	p-value	Pairwise Difference (Cohen's d; p-value) (versus CN Aβ- ε4+)
Left Hippocampus	2.42 (0.052)	2.39 (0.053)	2.18 (0.095)	1.97 (0.04)	1.88 (0.078)	<0.0001	CN Aβ+ = -0.08; 0.692 MCI Aβ- = -0.42; <0.0001 MCI Aβ+ = -1.26; <0.0001 AD Aβ+ = -1.09; <0.0001
Right Hippocampus	2.45 (0.056)	2.44 (0.058)	2.27 (0.01)	1.94 (0.043)	1.90 (0.084)	<0.0001	CN Aβ+ = -0.01; 0.946 MCI Aβ- = -0.29; 0.147 MCI Aβ+ = -1.26; <0.0001 AD Aβ+ = -1.02; <0.0001

Data are presented as the estimated marginal mean of normalized hippocampal volumes and SE in parentheses. [‡]MCI follow diagnosis was available for ADNI (0–36 months) and AIBL (0–54 months).

studies have since shown that APOE ε4 is linked to Aβ deposition and may exert a synergistic effect to promote cognitive decline [49, 50].

To test whether an Aβ-dependent effect of APOE ε4 would be associated with greater hippocampal

volume loss, we examined the combined effect of APOE ε4 and Aβ on the hippocampus. We found no significant differences in hippocampal volume between CN Aβ+ ε4+ individuals and CN Aβ- ε4+ individuals, suggesting that in healthy individuals

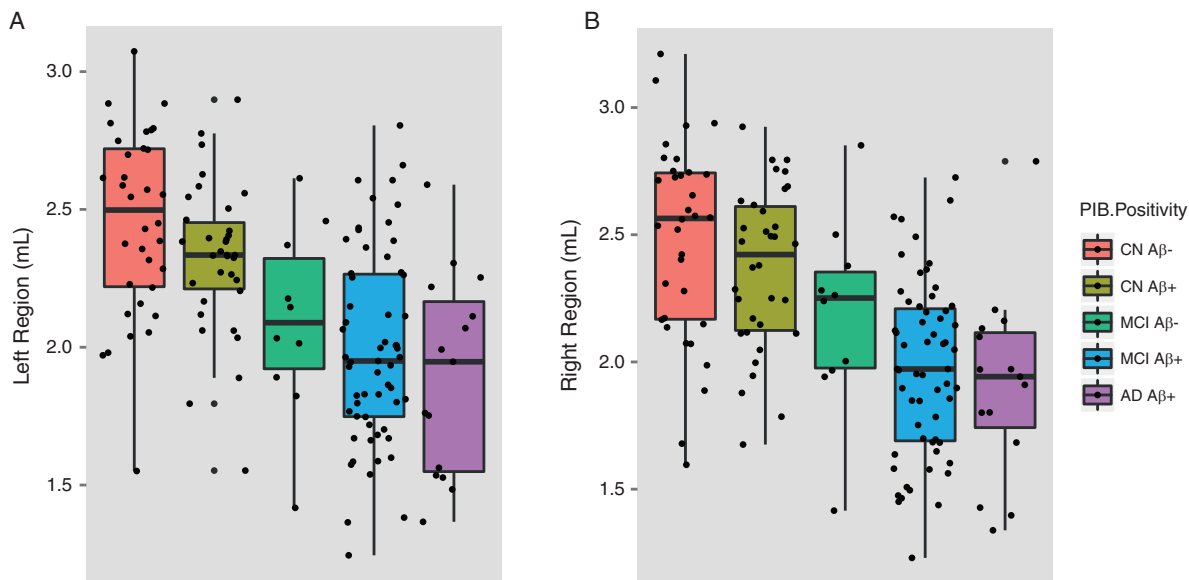


Fig. 2. Hippocampal volumes of $\epsilon 4$ carriers by diagnosis and A β . Data was used from the ADNI and AIBL cohorts for subjects that had available PET A β . MCI subjects were further divided by follow-up diagnosis in ADNI (0–36 months) and AIBL (0–54 months). Shown are baseline hippocampal volumes from (a) the left and (b) the right region. Note: There were no MCI-nc A β - $\epsilon 4$ carriers that converted to AD during the current follow-up period of the ADNI and AIBL studies. A β , amyloid- β ; CN, cognitively normal; CN A β -, A β - negative CN group; CN A β +, A β positive CN group; MCI, mild cognitive impairment; MCI A β -, A β negative MCI group; MCI-c, MCI non-converter; MCI-nc A β +, A β positive MCI-nc group; MCI-c, MCI-converter; MCI-c A β +, A β positive MCI-c group; AD, Alzheimer's disease; AD A β +, A β positive AD group.

there is no effect of APOE $\epsilon 4$ and A β on the hippocampus. This is consistent with previous studies that have shown that the relationship between APOE $\epsilon 4$, A β , and brain atrophy is mediated by CSF p-tau181 levels [51, 52] and, in the absence of abnormal p-tau181 levels, there is no synergistic relationship between APOE $\epsilon 4$ and A β deposition. Hippocampal volumes of MCI A β + $\epsilon 4$ + individuals did not differ when compared to AD A β + $\epsilon 4$ + participants, suggesting that a similar degree of hippocampal loss that is expected in AD has already manifested in MCI subjects. Our findings are supported by empirical evidence which has shown that APOE induces intracellular degradation of A β peptides facilitating synaptotoxicity, neuroinflammation, and tau hyperphosphorylation [5, 53]. It is therefore possible that A β and the $\epsilon 4$ in conjunction impart greater levels of neuronal toxicity and injury in the presence of hippocampal neurodegeneration.

Recent studies have suggested an A β independent effect of the APOE $\epsilon 4$ on neuronal integrity as another explanation for the gene's effect on brain structure [47, 54]. A study by Dean and colleagues [44] argued that an early neurodevelopmental foothold of APOE on the brain may render individuals more susceptible to the toxic and downstream neurodegenerative

effects of A β later in life. However, findings from our large sample of 14-year-old adolescents showed that there were no hippocampal volume differences present between the APOE groups, suggesting that $\epsilon 4$ carriers are unlikely to be at risk in adolescence, but may perhaps develop a greater risk later in life. Similar studies, such as that of O'Dwyer and colleagues [15] reported lower hippocampal volumes in $\epsilon 4$ carriers aged in their mid-twenties. Although differences may be attributed to the methodological approach adopted for the automated segmentation of the hippocampus, we cannot exclude the possibility of a low APOE penetrance in our young sample. Nevertheless, previous studies in younger $\epsilon 4$ carriers have shown that APOE plays a fundamental role in modulating brain function in the absence of any differences in brain volume [47].

An important caveat when interpreting the results of this study is that the multi-cohort data was cross-sectional and a more complete understanding of how hippocampal trajectories vary with age would require longitudinal data. In particular, more neuroimaging studies need to be conducted into typical cognitive aging across the lifespan [18] in order to differentiate between brain changes that are associated with typical normal aging from those that arise from

APOE ε4 dependent mechanisms and β-amyloidosis. Additionally, when combining data across cohort studies, it is important to consider study design differences that may complicate the interpretation of our results. For instance, the use of different AD diagnostic criteria across the different cohort studies may contribute to a level of diagnostic variability between groups. Participants from the ADNI and AIBL study were also highly educated, had few comorbidities, and were of Caucasian background. As such, future prospective studies with more representative samples should be conducted to address how these comorbidities, namely the presence of vascular disease, could potentially influence the size of the hippocampus. We demonstrated that systematic bias was not present in our dataset when comparing hippocampal volumes between APOE groups within each cohort study separately (Supplementary Material). Additional factors such as the use of two different PET radioligands meant that SUVR values of tracer uptake could not be compared as a single continuous measure. However, ongoing working groups such as the Centiloid project will further enable a more standardized approach for the direct comparability of results across different labs when different tracers and methods of analysis are employed [55].

Despite these limitations, this is first multi-cohort neuroimaging study of APOE genotype that attempts to characterize the differential risk of APOE on hippocampal volumes of subjects with varying stages of AD. Using metadata and clinical phenotyping pooled across several cohort studies, we were systematically able to demonstrate differential effects of the different APOE gene polymorphisms on hippocampal volume. An independent sample of healthy 14-year-old adolescents also provides an understanding into the role of APOE in adolescent brain neurodevelopment.

In conclusion, our findings in the largest APOE neuroimaging dataset show that hippocampal volume loss is present in patients with AD and in subjects with an increased risk of developing AD, particularly subjects with memory impairment. However, healthy older individuals did not show APOE ε4 dependent changes in the hippocampus, suggesting that the relationship between APOE ε4 and Aβ may be mediated by the presence of neurodegeneration. The same pattern was also observed in healthy young adolescents who possessed no hippocampal differences between different APOE groups. Our study thus shows hippocampal volume loss is moderated by APOE ε4 and Aβ in AD and the MCI stages of the AD pathological

process. The influence of these three markers could be considered as prognostic tools in clinical trials and therapeutic interventions of AD.

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SUPPLEMENTARY MATERIAL

The supplementary material is available in the electronic version of this article: <http://dx.doi.org/10.3233/JAD-161097>.

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