

Regular Article

The relation between APOE genotype and cerebral microbleeds in cognitively unimpaired middle- and old-aged individuals



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ABSTRACT

Positive associations between cerebral microbleeds (CMBs) and APOE-ε4 (apolipoprotein E) genotype have been reported in Alzheimer's disease, but show conflicting results. We investigated the effect of APOE genotype on CMBs in a cohort of cognitively unimpaired middle- and old-aged individuals enriched for APOE-ε4 genotype. Participants from ALFA (Alzheimer and Families) cohort were included and their magnetic resonance scans assessed (n = 564, 50% APOE-ε4 carriers). Quantitative magnetic resonance analyses included visual ratings, atrophy measures, and white matter hyperintensity (WMH) segmentations. The prevalence of CMBs was 17%, increased with age ($p < 0.05$), and followed an increasing trend paralleling APOE-ε4 dose. The number of CMBs was significantly higher in APOE-ε4 homozygotes compared to heterozygotes and non-carriers ($p < 0.05$). This association was driven by lobar CMBs ($p < 0.05$). CMBs co-localized with WMH ($p < 0.05$). No associations between CMBs and APOE-ε2, gray matter volumes, and cognitive performance were found. Our results suggest that cerebral vessels of APOE-ε4 homozygous are more fragile, especially in lobar locations. Co-occurrence of CMBs and WMH suggests that such changes localize in areas with increased vascular vulnerability.

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1. Introduction

Cerebral microbleeds (CMBs) are lesions that can be visualized on T2* gradient recall echo (GRE) and susceptibility weighted imaging (SWI) magnetic resonance imaging (MRI) sequences as round foci of hypointensity less than 10 mm in diameter (Haller et al., 2018). They are the result of the deposition in the brain parenchyma of hemosiderin breakdown products due to previous microscopic hemorrhage. It has been suggested that CMBs occur in areas of cerebral small vessels diseases, either due to the presence of cerebral amyloid angiopathy (CAA) or hypertensive vasculopathy (Yates et al., 2014b). The differential or synergistic effects of CAA and arterial hypertension on CMBs are reflected in CMBs' anatomical distribution. Typically, CMBs located in the deep and infratentorial regions are thought to result from hypertensive vasculopathy, while cortico-subcortical CMBs seem to be closely related to CAA (Cordonnier and Van der Flier, 2011).

In the context of Alzheimer's disease (AD), an increased prevalence on CMBs has been reported, together with associations with age and white matter hyperintensities (WMHs) even in asymptomatic individuals (van Rooden et al., 2009). It has also been proposed that CMBs in individuals with concomitant AD might be due to a disruption in the blood-brain barrier integrity caused by high vascular amyloid- β burden (Yates et al., 2014a). The $\epsilon 4$ allele of the apolipoprotein E (APOE) gene is the strongest genetic risk factor for AD. Risk for AD is 2- to 3-fold higher in people with one APOE- $\epsilon 4$ allele and about 12-fold higher in those with 2 (Michaelson, 2014). In addition, a higher number of APOE- $\epsilon 4$ alleles is also associated with higher levels of amyloid- β burden in cognitively normal older people (Reiman et al., 2009) and with an earlier onset of cognitive symptoms (Caselli et al., 2009; Haller et al., 2018; Tzioras et al., 2019). On the other hand, the APOE- $\epsilon 2$ variant is associated with a lower risk of AD (Haller et al., 2018; Reiman et al., 2020). Moreover, APOE genotype also seems to impact the risk of cerebrovascular disease, which is often a comorbidity in AD patients, especially at older ages (Belloy et al., 2019). Even though a relationship between APOE polymorphisms and sporadic CMBs has been proposed, reports show conflicting results. More specifically, the effect of APOE- $\epsilon 4$ and $\epsilon 2$ genotypes on the prevalence and location of CMBs seems to be controversial (Cordonnier et al., 2006; Kim et al., 2005; Martins et al., 2014; Sepehry et al., 2016; Stefaniak et al., 2018).

With this in mind, the aim of this study is to investigate the effect of APOE genotype on CMBs in a cohort of cognitively unimpaired middle- and old-aged subjects with limited cardiovascular risk factors and enriched for APOE- $\epsilon 4$ genotype. We seek to establish whether the prevalence of CMBs differs on the base of APOE genotype, and its association with CMBs localization. Moreover, we aim at elucidating the relationship between CMBs and other known markers of cerebrovascular pathology and AD.

2. Materials and methods

2.1. Study participants

Participants of this study are part of a wider research platform: the ALFA cohort (for Alzheimer and Families; [Clinicaltrials.gov](https://clinicaltrials.gov/ct2/show/study/NCT01835717) Identifier: NCT01835717). With the aim of tracking the evolution of the AD continuum in asymptomatic individuals, ALFA is composed of 2743 cognitively unimpaired participants, aged between 45 and 75, many of them adult children of patients with AD (47.4% with parental history of AD with onset before 75 years). For a full detailed description of the ALFA cohort, see Molinuevo et al. (2016). In brief, participants had a Clinical Dementia Rating (Morris, 1993) equal to 0 and scored within the established cut-offs

for the following neuropsychological battery that included Mini-Mental State Examination ≥ 26 , Memory Impairment Screen ≥ 6 (Böhm et al., 2005; Buschke et al., 1999), Time-Orientation subtest of the Barcelona Test II ≥ 68 (Quiñones-Úbeda, 2009), and semantic fluency (animals) ≥ 12 (Peña-Casanova et al., 2009; Ramier and Hécaen, 1970). All participants were genotyped for the APOE gene (see Section 2.3).

A subgroup of 608 ALFA participants without MRI contraindications was selected to participate in the present study ([Clinicaltrials.gov](https://clinicaltrials.gov/ct2/show/study/NCT02198586) Identifier: NCT02198586) according to their APOE genotype, preferentially including APOE- $\epsilon 4$ and APOE- $\epsilon 2$ allele carriers (Cacciaglia et al., 2018). The rest of the participants were selected to sex and age match the previous ones. From the 608 participants invited, 595 agreed to undertake MRI scans and 575 provided valid MRIs. Finally, we removed 11 individuals due to the presence of incidental findings or those that did not have a T2* for rating CMBs, leaving the total sample size to 564 participants. This recruitment strategy, maximizing the number of APOE- $\epsilon 4$ and APOE- $\epsilon 2$ alleles, enabled us to reach an unprecedented sample size for these fewer common genotypes in a unicentric study, allowing us to assess their specific effect on CMBs.

Recorded basic sociodemographic and clinical data included familiar history for AD and smoking status (active smokers, former smokers, and non-smokers) as reported in previous studies (Salvadó et al., 2019). The participant underwent an extensive neuropsychological assessment as described in Molinuevo et al. (2016). Neuropsychological test results were transformed into z-scores and then grouped into the relevant cognitive domains as already reported (Brugulat-Serrat et al., 2019). As the study population was cognitively unimpaired, we investigated the domains mostly related to AD, that is, memory and executive functions (Weintraub et al., 2018).

2.2. Vascular risk factors

Vascular risk factors were extensively reported elsewhere (Salvadó et al., 2019). Criteria for the assessment of such risk factors are reported in the Appendix. REGICOR (Registre Gironí del Cor [Girona Heart Registry]) score predicts the 10-year cardiovascular mortality risk and it is developed specifically for the Catalan population. It is calculated based on sex, age, smoking status, systolic blood pressure, and total blood cholesterol levels; it has a range of 1–33 with higher score values indicating an increased risk (Marrugat et al., 2003; Rojas et al., 2018).

Cardiovascular risk factors were also used to derive the probability of dementia in 20 years using the CAIDE (cardiovascular risk factors, aging, and incidence of dementia) dementia risk score (Kivipelto et al., 2006). CAIDE risk score was calculated taking into account the age, education, sex, systolic blood pressure > 140 mm Hg, body mass index, total cholesterol, and physical activity in the first model. CAIDE score (range 0–15, with higher score meaning higher risk) is known to be associated to WMH load, APOE- $\epsilon 4$ status, and CSF biomarkers (Ecay-Torres et al., 2018; Enache et al., 2016; Kaffashian et al., 2013).

2.3. APOE genotyping

Total DNA was obtained through proteinase-K digestion and alcohol precipitation of the blood cellular fraction. Samples were genotyped, using the APOE-F 5'-TTGAAGGCCTA CAAATCGGAAC TG-3' and APOE-R 5'-CCGGCTGCCCAT CTCCTCCATCCG-3' primers, for 2 single nucleotide polymorphisms, rs429358 and rs7412. Thus, the possible APOE alleles were determined as follows: $\epsilon 1$, rs429358 (C) + rs7412 (T); $\epsilon 2$, rs429358 (T) + rs7412 (T); $\epsilon 3$, rs429358 (T) + rs7412 (C); and $\epsilon 4$, rs429358 (C) + rs7412 (C).

2.4. Magnetic resonance imaging

MRI scans were acquired in a 3T scanner (GE Discovery MR750W 3T). The protocol included an isotropic 3D T1-weighted sequence (matrix size of $256 \times 256 \times 160$; repetition time (TR)/echo time (TE)/inversion time (TI) = 8.0/3.7/450 ms, number of signals averaged = 1, flip angle = 8°) and 3 clinical T2-weighted sequences with a voxel size of $1 \times 1 \times 3 \text{ mm}^3$, that is, T2W fast spin echo (TR/TE = 5000/85 ms, flip angle = 110°), fluid attenuated inversion recovery (FLAIR: TR/TE/TI = 11,000/90/2600 ms, flip angle = 160°), and T2* gradient echo (GRE: TR/TE = 1300/23 ms, flip angle = 15°). All scans were visually assessed for quality and incidental findings by a neuroradiologist, who also rated them with medial temporal atrophy, posterior cortical atrophy, and global cortical atrophy scales (Koedam et al., 2011; Pasquier et al., 1996; Scheltens et al., 1992). Total intracranial volume (TIV) and cortical volumes were calculated using the Geodesic information flows label fusion framework, using T1-weighted images (Cardoso et al., 2015).

2.5. WMH visual assessment and volume quantification

All FLAIR images were assessed for WMHs of probable vascular origin and rated using Fazekas scale ranging from 0 to 3 (0: none or a single punctate WMH lesion; 1: multiple punctate lesions; 2: beginning confluent lesions [bridging]; 3: large confluent lesions) (Fazekas et al., 1987). WMH segmentation and quantification was performed with a validated method as described by Sudre et al. (2015). This automated segmentation is based on a hierarchical Gaussian mixture model that takes as input co-registered T1-weighted and FLAIR images and dynamically chooses the number of Gaussian components necessary to model each tissue, separating healthy tissue from unexpected observations (outliers). It incorporates spatial information derived from subject-specific statistical atlases and applies consistency rules across neighbors via a Markov Random Field. After convergence of the model, candidate lesion voxels are selected based on intensity and location heuristics. The formed connected components are then automatically classified based on anatomical rules to eliminate potential artifacts.

Finally, the resulting probability map is integrated to provide the final volume of WMH in mm^3 .

2.6. CMB visual assessment and quantification

CMBs were defined as foci of hypointensity $<10 \text{ mm}$ in diameter in the T2* GRE sequences (Fig. 1). The visual assessment of CMBs was performed by consensus of 2 experienced raters blinded to all clinical data and APOE genotype according to the Brain Observer MicroBleeds Scale criteria (Cordonnier et al., 2009). Moreover, the localization of CMBs was marked using ITK-snap (www.itksnap.org) (Yushkevich et al., 2006).

2.7. Statistical analyses

Comparisons of demographic, clinical, and radiological characteristics among the different APOE groups were performed using parametric tests. For descriptive purposes, the general prevalence of CMBs in our study sample was calculated and stratified by the APOE genotype and the collation of the CMBs (lobar and deep).

We defined 2 variables for CMBs, namely CMB presence (with CMBs dichotomized as either present or absent) and CMB number (continuous) to study the prevalence and severity of CMB, respectively. The presence of CMBs was studied using logistic regression models and the number of CMBs with linear regression models, in both cases after accounting for the confounding effects of age and sex. Since APOE- $\epsilon 4$ homozygotes had a higher prevalence of hypercholesterolemia, this factor was also included as a confounder, but resulted to be insignificant and was removed in the final models. APOE genotype was included in the models using 3 different coding schemes. On the one hand, as a categorical variable for non-carriers, $\epsilon 4$ heterozygotes and $\epsilon 4$ homozygotes. On the other, as a continuous variable coding the number of $\epsilon 4$ alleles. Finally, as a dichotomous variable for $\epsilon 4$ non-carriers and carriers.

Combined with the 2 CMB-related variables, this coding scheme resulted in 6 different statistical models that allowed us to study the different APOE- $\epsilon 4$ inheritance modes: additive (proportional to the number of $\epsilon 4$ alleles), recessive ($\epsilon 4$ homozygotes vs. the rest), and

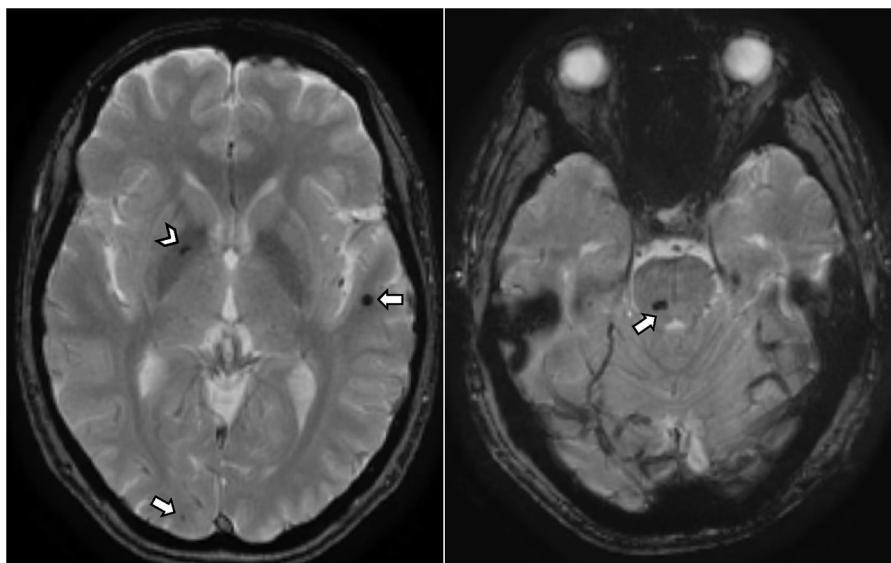


Fig. 1. Representative image of CMBs. CMBs were defined as small round foci of hypointensity at T2*-weighted MR imaging (arrows) and they were assessed according to BOMBS criteria. Based on their location, they were stratified as lobar (cortex gray-white junction, subcortical white matter) or deep (basal ganglia, internal and external capsule, thalamus, brainstem, cerebellum). CMBs had to be distinguished from cerebral calcifications especially in the basal ganglia. Calcifications demonstrated varying degrees of signal intensity and a more ill-defined shape (arrow-head).

dominant ($\epsilon 4$ carriers vs. non-carriers) for both the presence and number of CMBs. Afterward, to analyze the effect of APOE genotype on CMB location, we proceeded to repeating these analyses stratifying for the location of CMBs, lobar or deep. We considered the additive model as the principal one, while the recessive model was more exploratory and the dominant model was mostly performed to compare our results with other literature findings.

Moreover, we studied the relationship between the presence and number of CMBs respectively with APOE- $\epsilon 2$ genotype. To do so, we categorized APOE genotype into the following categories: APOE- $\epsilon 2$ carriers, APOE $\epsilon 2/\epsilon 4$, APOE- $\epsilon 3$ homozygous, or APOE- $\epsilon 4$ carriers.

Afterward, we sought to establish the potential of CMBs as a marker of cerebrovascular disease. To do that, we tested whether the presence of CMBs was associated with WMH volume with a linear regression model, after correcting for age and sex. In order to assess the co-localization of CMBs and WMHs, Euclidean distance maps from the WMHs were derived. For each subject, 1000 random samples across the white matter + deep gray matter volumes were used to estimate a theoretical random distance distribution between each CMB and WMH. For each detected CMB, the empirical distance between the closest WMH and CMB was then compared to the theoretical random distance using a *t*-test and further categorized as <5, 5–10, 10–15, or >15 mm. The distribution of CMBs and their respective division into distance categories was then investigated per lobe.

Then, we proceeded to investigate the relationship between CMBs and REGICOR and CAIDE scores respectively to test whether the location of CMBs was predictive of increased risk of higher cardiovascular risk of dementia due to vascular burden. To do so, we built a linear regression model having the number of deep CMBs as predictor and REGICOR score as outcome (as both REGICOR and deep CMBs are supposed to be associated with systemic cardiovascular risk factors), and another linear regression model having the number of lobar CMBs as predictor and the CAIDE score as outcome (as CAIDE and lobar CMBs should indicate higher risk of AD). Both models were corrected for age, sex, and APOE- $\epsilon 4$ genotype.

Finally, we investigated the relationship of CMBs with known markers of AD, such as atrophy markers and cognitive performance. Atrophy markers were defined as cortical volume, hippocampal volume, and precuneus volume. Three independent logistic regression models were built to establish whether each of these volumes was predictive of the presence of CMBs. Covariates of these models were age, sex, and TIV. Cognitive performance was defined as memory and executive function composite scores. Two logistic

regression analyses were performed with presence of CMBs as a predictor, memory and executive performance respectively as outcomes, and age, sex, and years of education as covariates.

Statistical significance level was set at a *p*-value <0.05. Analyses were conducted in R version 3.6.0 (R Foundation for Statistical Computing, Vienna, Austria).

3. Results

3.1. Demographics and clinical data

The distribution of APOE alleles in the population was the following: 7 (1.2%) individuals were APOE- $\epsilon 2$ homozygous, 111 (19.7%) APOE- $\epsilon 2/\epsilon 3$, 43 (7.6%) APOE- $\epsilon 2/\epsilon 4$, 162 (28.7%) APOE- $\epsilon 3$ homozygous, 168 (29.8%) APOE- $\epsilon 3/\epsilon 4$, and 73 (12.9%) APOE- $\epsilon 4/\epsilon 4$ (Fig. 2A). The sociodemographic data, clinical information, cognitive tests performance, and the MRI visual rating scales of the study participants (*n* = 564) are described in Table 1. Overall, APOE- $\epsilon 4$ homozygotes were significantly younger (*p* = 0.001), probably because with increasing age they are more likely to convert to mild cognitive impairment or dementia and to the fact that these subjects are expected to experience onset of cognitive dysfunction earlier (Vemuri et al., 2010). Moreover, they reported the presence of hypercholesterolemia in a significantly higher number of cases with respect to the rest of the study population (*p* < 0.05; Table S1). CAIDE dementia risk score was generally low in the whole sample due to the low prevalence of vascular risk factors such as hypertension, body mass index >30 kg/m², or hypercholesterolemia, and the high prevalence of physical activity and high education levels in the study participants. The variance of the Mini-Mental State Examination scores was very limited as the population was cognitively unimpaired due to the known ceiling effect of this test (range = 26–30, median = 29). MRI visual rating scales showed no statistical differences among the 3 APOE- $\epsilon 4$ genotype groups with the exception of a weak difference in posterior cortical atrophy (*p* < 0.05). Volumetric outcomes were available for a subset of 554 subjects.

3.2. The effect of APOE- $\epsilon 4$ on the prevalence and number of CMBs

The crude prevalence of CMBs was 14.3% in APOE- $\epsilon 4$ non-carriers, 19.0% in APOE- $\epsilon 4$ heterozygotes, and 20.5% in APOE- $\epsilon 4$ homozygotes, and the presence of CMBs was significantly associated with age (β = 0.20, 95% confidence interval [CI]: 0.06–0.51, *p* < 0.05) (Fig. 2B).

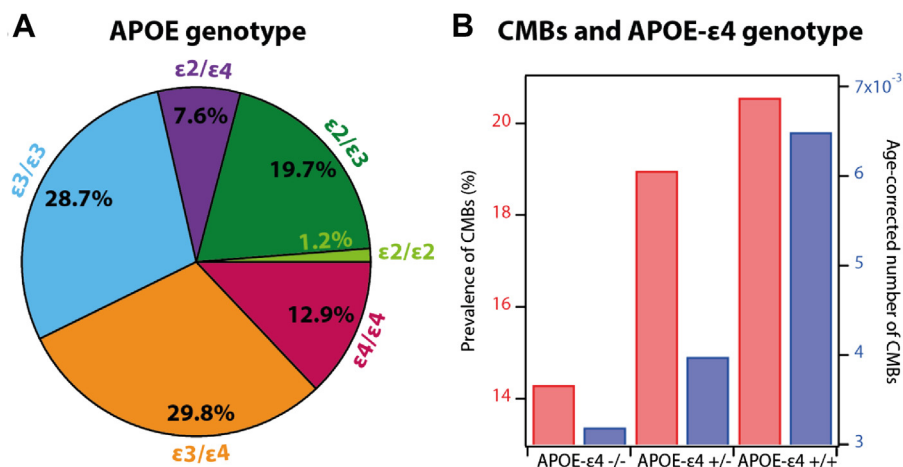


Fig. 2. Prevalence of CMBs (red) and age-corrected number of CMBs (blue) according to APOE- $\epsilon 4$ genotype.

Table 1
Descriptive characteristics of the study population by APOE-ε4 genotype

Parameter	APOE-ε4 non-carriers	APOE-ε4 heterozygotes	APOE-ε4 homozygotes	p-value
Demographics				
Sample size	280	211	73	
Age, mean (SD)	58.3 (7.5)	58.4 (7.2)	54.9 (6.1)	0.001 ^a
Sex: male (%)	102 (36.4)	95 (45.0)	26 (35.6)	0.119
Years of education, mean (SD)	13.6 (3.6)	13.8 (3.5)	13.4 (3.5)	0.604
Familiar history for AD, present (%)	251 (89.6)	196 (92.9)	68 (93.2)	0.376
Cardiovascular risk factors				
CAIDE, mean (SD)	5.9 (2.5)	6.1 (2.6)	5.7 (2.1)	0.432
REGICOR score, mean (SD), NA = 22	4.2 (3.0)	4.6 (3.1)	4.1 (2.1)	0.372
Neuropsychological tests				
MMSE, mean (SD), NA = 1	29.0 (1.1)	29.0 (1.1)	29.2 (0.8)	0.457
Executive function composite z score, mean (SD), NA = 12	-0.02 (0.67)	-0.01 (0.63)	0.15 (0.62)	0.148
Memory composite z score, mean (SD), NA = 13	-0.04 (0.94)	0.05 (0.83)	0.21 (0.85)	0.103
Radiological Visual Rating Scales				
GCA, mean (SD)	1.8 (0.5)	1.9 (0.5)	1.7 (0.5)	0.087 ^b
PCA, mean (SD)	1.9 (0.6)	2.0 (0.6)	1.8 (0.5)	0.026 ^a
MTA, mean (SD)	1.8 (0.7)	1.8 (0.6)	1.8 (0.7)	0.711
Fazekas score, mean (SD)	1.6 (0.6)	1.6 (0.6)	1.6 (0.7)	0.596
CMBs, present (%)	40 (14.3)	40 (19.0)	15 (20.5)	0.26
Lobar CMBs	31 (11.1)	29 (13.7)	11 (15.1)	0.535
Deep CMBs	10 (3.6)	15 (7.1)	5 (6.8)	0.185
Quantitative MR outcomes				
Cortical volume (cm ³), mean (SD), NA = 10	489.9 (45.2)	492.1 (42.7)	497.3 (42.1)	0.429
WMH volume (cm ³), mean (SD), NA = 10	3.2 (4.0)	3.1 (3.0)	3.8 (4.4)	0.364
TIV (cm ³), mean (SD), NA = 10	1386.7 (130.5)	1394.2 (125.3)	1399.4 (116.9)	0.685

Significance value was set at $p < 0.05$. Variables indicated with NA (not available) had missing data.

Key: AD, Alzheimer's disease; APOE, apolipoprotein E; CAIDE, cardiovascular risk factors, aging, and incidence of dementia; CMB, cerebral microbleed; GCA, global cortical atrophy; MMSE, mini-mental state examination; MTA, medial temporal atrophy; PCA, posterior cortical atrophy; REGICOR, Registre Gironi del Cor (Girona Heart Registry); SD, standard deviation; TIV, total intracranial volume; WMH, white matter hyperintensity.

^a $p < 0.05$.

^b $p < 0.1$.

In the dominant model, we showed a trend of higher prevalence and higher number of CMBs in APOE-ε4 carriers with respect to non-carriers ($p < 0.1$; dominant model, Table 2). On the other hand, the number of CMBs was significantly associated with the number APOE-ε4 alleles ($p < 0.05$; additive model, Table 2). Going even more in depth, using the exploratory recessive model, the number of CMBs was significantly higher in APOE-ε4 homozygotes with respect to non-carriers ($\beta = 0.21$, 95% CI: 0.06–0.36,

$p < 0.01$) and heterozygotes ($\beta = 0.17$, 95% CI: 0.02–0.32, $p < 0.05$) (Table 2).

When stratifying the results on the basis of lobar or deep location of CMBs, we showed that the number of lobar CMBs tends to increase with higher number of APOE-ε4 alleles ($\beta = 0.07$, 95% CI: 0.002–0.13, $p = 0.056$; additive model, Table 3). More specifically, the number of lobar CMBs was significantly higher in APOE-ε4 homozygotes compared to APOE-ε4 non-carriers ($\beta = 0.19$, 95% CI:

Table 2
The association between APOE-ε4 alleles and the presence or number of CMBs

Additive model				
	Presence of CMBs		Number of CMBs	
	β (95% CI)	p-value	β (95% CI)	p-value
Number of APOE-ε4 alleles	0.31 (-0.01 to 0.62)	0.055	0.09 (0.02–0.16)	0.012 ^a
Recessive model				
Reference group: APOE-ε4 -/-	Presence of CMBs		Number of CMBs	
	β (95% CI)	p-value	β (95% CI)	p-value
APOE-ε4 +/+	0.606 (-0.09 to 1.27)	0.079	0.21 (0.06–0.36)	0.006 ^b
APOE-ε4 +/-	0.328 (-0.16 to 0.82)	0.185	0.04 (-0.06 to 0.15)	0.429
Reference group: APOE-ε4 +/-	Presence of CMBs		Number of CMBs	
	β (95% CI)	p-value	β (95% CI)	p-value
APOE-ε4 +/+	0.28 (-0.42 to 0.94)	0.424	0.17 (0.02–0.33)	0.031 ^a
Dominant model				
Reference group: APOE-ε4 non-carriers	Presence of CMBs		Number of CMBs	
	β (95% CI)	p-value	β (95% CI)	p-value
APOE-ε4 carriers	0.40 (-0.05 to 0.85)	0.085	0.09 (-0.01 to 0.18)	0.082

The presence of CMBs was studied using logistic regression models and the number of CMBs with linear regression models. All models were corrected for age and sex. Significance value was set at $p < 0.05$.

Key: APOE, apolipoprotein E; CI, confidence interval; CMB, cerebral microbleed.

^a $p < 0.05$.

^b $p < 0.01$.

Table 3
The difference between lobar and deep CMBs

Location of CMB	Additive model	Presence of CMBs		Number of CMBs	
		β (95% CI)	<i>p</i> -value	β (95% CI)	<i>p</i> -value
	Number of APOE- ϵ 4 alleles	0.28 (–0.08 to 0.64)	0.127	0.07 (–0.002 to 0.13)	0.056
	Recessive model				
	Reference group: APOE- ϵ 4 –/–	Presence of CMBs		Number of CMBs	
		β (95% CI)	<i>p</i> -value	β (95% CI)	<i>p</i> -value
Lobar CMBs	APOE- ϵ 4 +/+	0.58 (–0.22 to 1.33)	0.135	0.19 (0.04–0.33)	0.012 ^a
	APOE- ϵ 4 +/-	0.25 (–0.30 to 0.80)	0.377	–0.01 (–0.11 to 0.09)	0.909
	APOE- ϵ 4 +/+	0.34 (–0.47 to 1.09)	0.394	0.19 (0.04–0.34)	0.013 ^a
	Dominant model				
	Reference group: APOE- ϵ 4 non-carriers	Presence of CMBs		Number of CMBs	
		β (95% CI)	<i>p</i> -value	β (95% CI)	<i>p</i> -value
	APOE- ϵ 4 carriers	0.33 (–0.18 to 0.84)	0.206	0.04 (–0.05 to 0.14)	0.365
	Additive model				
		Presence of CMBs		Number of CMBs	
		β (95% CI)	<i>p</i> -value	β (95% CI)	<i>p</i> -value
Deep CMBs	Number of APOE- ϵ 4 alleles	0.48 (–0.04 to 1.00)	0.068	0.04 (0.01–0.07)	0.013 ^a
	Recessive model				
	Reference group: APOE- ϵ 4 –/–	Presence of CMBs		Number of CMBs	
		β (95% CI)	<i>p</i> -value	β (95% CI)	<i>p</i> -value
	APOE- ϵ 4 +/+	0.86 (–0.36 to 1.95)	0.086	0.08 (–0.02 to 0.15)	0.017 ^a
	APOE- ϵ 4 +/-	0.72 (–0.09 to 1.58)	0.138	0.03 (–0.01 to 0.08)	0.155
	APOE- ϵ 4 +/+	0.13 (–1.04 to 1.15)	0.809	0.05 (–0.02 to 0.12)	0.169
	Dominant model				
	Reference group: APOE- ϵ 4 non-carriers	Presence of CMBs		Number of CMBs	
		β (95% CI)	<i>p</i> -value	β (95% CI)	<i>p</i> -value
	APOE- ϵ 4 carriers	0.75 (–0.01 to 1.58)	0.059	0.05 (0.003–0.09)	0.036 ^a

The presence of CMBs was studied using logistic regression models and the number of CMBs with linear regression models. All models were corrected for age and sex. Significance value was set at $p < 0.05$

Key: APOE, apolipoprotein E; CI, confidence interval; CMB, cerebral microbleed.

^a $p < 0.05$.

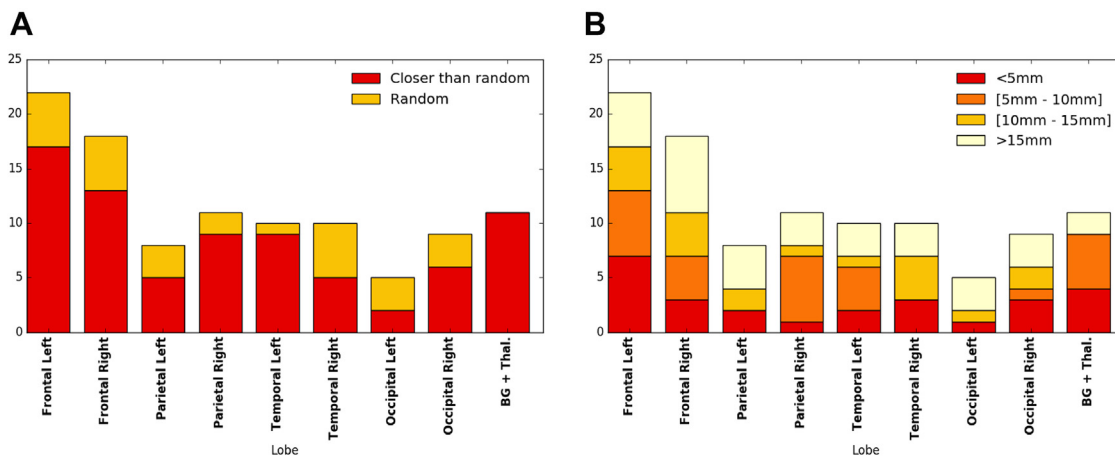


Fig. 3. Distance between CMBs and WMHs. (A) shows the distribution of CMB distance to WMH compared to random sampling per lobe per side (Left/Right). (B) shows WMH distance to CMBs per lobe per side categorized as <5 mm, 5–10 mm, 10–15 mm, >15 mm (Left/Right). The y-axis reports the number of CMBs. Abbreviations: BG, basal ganglia; Thal, thalamus.

0.04–0.33, $p < 0.05$) and APOE- $\epsilon 4$ heterozygotes ($\beta = 0.19$, 95% CI: 0.04–0.34, $p < 0.05$; recessive model, Table 3).

The number of deep CMBs was higher in APOE- $\epsilon 4$ carriers with respect to non-carriers ($\beta = 0.05$, 95% CI: 0.003–0.09, $p < 0.05$; dominant model, Table 3), and increased significantly with the number of APOE- $\epsilon 4$ alleles ($\beta = 0.04$, 95% CI: 0.01–0.07, $p < 0.05$; additive model, Table 3). There were statistically significant differences in the number of deep CMBs between APOE- $\epsilon 4$ homozygotes and non-carriers ($\beta = 0.08$, 95% CI: –0.02 to 0.15, $p < 0.05$), but not between APOE- $\epsilon 4$ heterozygotes and non-carriers, or between APOE- $\epsilon 4$ homozygotes and heterozygotes (recessive model, Table 3).

3.3. APOE-2 genotype does not protect from the formation of CMBs

A description of the APOE- $\epsilon 2$ sample is reported in Table S2. APOE- $\epsilon 2$ carriers were significantly younger ($p < 0.001$) and demonstrated a significantly lower prevalence of hypercholesterolemia ($p < 0.001$) with respect to the other groups. APOE- $\epsilon 2$ carriers did not show a significantly different prevalence or number of CMBs compared to APOE $\epsilon 3/\epsilon 3$ homozygotes, APOE $\epsilon 2/\epsilon 4$, or APOE- $\epsilon 4$ carriers (Table S3).

3.4. The relation of CMBs with known markers of cerebrovascular diseases

There was a weak but not statistically significant association between total WMH volume and the presence of CMBs ($p = 0.07$), but no relation was found between total WMH volume and the number of CMBs (not shown). WMH volume was not associated preferentially with either lobar or deep CMBs (not shown).

Local co-occurrence of CMBs and WMHs was investigated in comparison with random sampling, and it was demonstrated that CMBs are spatially associated with WMHs (Fig. 3A). More specifically, most of the CMBs showed a distance <15 mm than WMHs (Fig. 3B).

There was no association between REGICOR score and the number of deep CMBs ($\beta = 0.08$, $R^2 = 0.02$, 95% CI: –0.05 to 0.18, $p = 0.17$) (Table S4A). CAIDE score had no relation with the number of lobar CMBs ($\beta = 0.17$, $R^2 = 0.07$, 95% CI: –0.02 to 0.41, $p = 0.12$) (Table S4B).

3.5. The relation between CMBs and other markers of AD

No significant associations were found between cortical volume, hippocampal volume, or precuneus volume (independent variables) and the presence of CMBs after correction for age, sex, and TIV (Table S5).

Similarly, there was no statistically significant association between the presence of CMBs and performance in the memory and executive function domains (Table S6).

4. Discussion

4.1. APOE- $\epsilon 4$ allele dose-dependent effect on the number of CMBs

In this study, we investigated the relation between CMBs with the APOE genotype, as well as other established biomarkers of AD and vascular pathology in a cohort of late- and middle-aged cognitively unimpaired subjects. As expected, our results showed that the presence and number of CMBs increase with age, as described by other literature reports (Cordonnier et al., 2006; Graff-Radford et al., 2019; Yubi et al., 2018). The main contribution of this work is studying CMB burden in a group of APOE- $\epsilon 4$ homozygotes which allowed us to assess recessive (i.e., APOE- $\epsilon 4$ homozygotes vs. the rest of groups), additive (i.e., proportional to the number of $\epsilon 4$ alleles), and dominant effects (APOE- $\epsilon 4$ carriers vs. non-carriers) of the APOE- $\epsilon 4$ genotype. Our main results are that (1) the number of CMBs parallels the number of APOE- $\epsilon 4$ alleles, with (2) APOE- $\epsilon 4$ homozygotes having a higher number of CMBs with respect to both APOE- $\epsilon 4$ heterozygotes and non-carriers; more specifically (3) APOE- $\epsilon 4$ homozygotes have a higher number of lobar CMBs compared to both APOE- $\epsilon 4$ heterozygotes and non-carriers, and increased number of deep CMBs with respect to non-carriers. All these APOE- $\epsilon 4$ effects showed a trend level with regards the presence of CMBs. Finally, we demonstrated that (4) CMBs co-occur with WMHs. These results confirm the influence of the APOE- $\epsilon 4$ genotype on the CMB burden even in cognitively unimpaired subjects with very low cardiovascular risk levels.

The effect of APOE- $\epsilon 4$ allele dose on CMBs was found to be higher with respect the number of CMBs—a marker of severity—than their mere presence. This result might be indicative that, rather than increasing the prevalence of CMBs, harboring 2 copies of the APOE- $\epsilon 4$ allele may render the brain more fragile to cerebrovascular insults once they appear. The higher number of CMBs in

APOE- ϵ 4 homozygotes with respect to heterozygotes suggests that this effect may be mediated by the deposition of amyloid- β plaques. By the average age of our APOE- ϵ 4 groups, it is expected that about 50% of homozygotes show abnormal levels of amyloid- β compared to only 15% and 10% in the heterozygote and non-carrier group, respectively (Jansen et al., 2018). In addition to plaques in the brain, amyloid- β is deposited in the walls of cerebral capillaries and arteries, causing a disruption in the perivascular drainage of soluble amyloid- β from the brain interstitium (Hawkes et al., 2013, 2011). This is also suggested by the study of Yates et al. (2011) demonstrating that amyloid- β mediates the association between APOE- ϵ 4 status and lobar CMBs. Taken together, these findings suggest that APOE- ϵ 4 homozygotes may not necessarily be more susceptible to the presence of CMBs but their brain may be more fragile to cerebrovascular insults once they appear. We hypothesize that once the amyloid burden reaches a critical threshold, then the erosive action of amyloid- β plaques deposited in the media and adventitia of small vessels of the leptomeninges and cerebral cortex becomes evident and might in time lead to CMB formation and eventually CAA (Viswanathan and Greenberg, 2011). However, we could not directly assess this hypothesis given the absence of amyloid biomarkers in this sample. In addition, other amyloid-independent mechanisms cannot be discarded. An alternative explanation might be that APOE- ϵ 4 makes the brain more vulnerable to CMBs through the disruption of the blood-brain barrier mediated by brain capillary pericytes degeneration, independently of amyloid plaques accumulation (Montagne et al., 2020).

4.2. The role of APOE- ϵ 2 in the formation of CMBs

Literature reports on the role of APOE- ϵ 2 in AD show conflicting results. Although some reports conclude that APOE- ϵ 2 is protective against AD development due to a reduced accumulation of amyloid deposition (Benjamin et al., 1994; Corder et al., 1994; Groot et al., 2018; Grothe et al., 2017; Rebeck et al., 2002), others indicated an increased prevalence of CAA-related cerebral hemorrhages in APOE- ϵ 2 carriers (McCarron et al., 2000; McCarron and Nicoll, 1998; Nicoll et al., 1997; Tai et al., 2016). Our results showed no significant association between APOE- ϵ 2 and CMBs. It must be noted that we decided to exclude subjects with APOE- ϵ 2/ ϵ 4 genotype as it was difficult to disentangle the proportional impact of the 2 alleles on the presence or absence of CMBs. The only other literature report that investigated this relation showed an increased prevalence of CMBs in APOE- ϵ 2 carriers compared to APOE- ϵ 3 homozygous in a small sample of subjects with mild cognitive impairment or Alzheimer's dementia (Groot et al., 2018). Our results might also be driven by the young age of study participants.

4.3. CMBs occur in areas vulnerable to cerebrovascular damage together with WMH

To our knowledge, this is the first study showing a spatial co-occurrence of CMBs and WMH. This finding suggests that CMBs occur in areas affected by vascular damage. In a recent study, APOE- ϵ 4 genotype was demonstrated to be the major driver of accumulation of WMH volume independently of AD diagnosis (Sudre et al., 2017). Even though not differing in overall WMH volume (Salvadó et al., 2019), APOE- ϵ 4 homozygotes in the sample here studied show a higher prevalence of pathologic levels of WMHs compared to heterozygotes and non-carriers (Rojas et al., 2018). One potential explanation to these apparently contradictory findings is that the brain of APOE- ϵ 4 homozygotes is more vulnerable to neurovascular insults and, once vascular pathology appears, it progresses faster than in the other genotypic groups. WMHs are thought to be a marker of cerebral small vessel disease, therefore a possible

pathophysiological explanation linking WMHs with CMBs is that they both might be due to partial ischemia of the cerebral tissue and degradation of the blood-brain barrier (Wardlaw et al., 2015). In line with the proposed vascular etiology, WMHs are associated with cardiovascular risk factors such as hypertension (Abraham et al., 2016), diabetes (Brundel et al., 2014), and smoking (Power et al., 2015), and occur with greater frequency in AD patients compared with normal controls (Holland et al., 2008; Provenzano et al., 2013). Interestingly, parenchymal WMHs are also related to CAA (Esiri et al., 2015; Holland et al., 2008; Zipfel et al., 2009). We believe that WMHs and CMBs are two different ways of expression of both cerebrovascular and neurodegenerative processes, due to the synergistic effect of cardiovascular risk factors and amyloid deposition.

The association between WMH volume and the presence of CMBs was weak probably because of the low vascular burden in this cohort, as reflected also by the CAIDE and REGICOR risk scores. As the study participants have a positive family history of AD and might be aware of their relatively high genetic predisposition, they might follow a healthy lifestyle in order to keep a low cardiovascular burden and hence lower their risk of developing dementia later in life. We suspect that our lack of significant associations between CAIDE and lobar CMBs on one side and REGICOR and deep CMBs on the other side is due to the same reason.

4.4. No association between CMBs and known AD atrophy markers or cognition

Previous reports suggest that in AD the APOE- ϵ 4 allele is associated with greater vulnerability to gray matter loss (Chen et al., 2015; Haller et al., 2018; Liu et al., 2010; Lupton et al., 2017; Pievani et al., 2009; Spampinato et al., 2011). A recent study demonstrated a weak association between reduced gray matter volume in some brain regions and APOE- ϵ 4 genotype or familiar history of AD, but no overall differences in cortical volume between APOE- ϵ 4 carriers and non-carriers in cognitively healthy middle-aged subjects (Ten Kate et al., 2016). However, in the same sample studied here and as compared to heterozygotes and non-carriers, cognitively unimpaired APOE- ϵ 4 homozygotes showed reduced gray matter volume in AD-relevant regions, such as the hippocampus, which became apparent after the fifth decade of life (Cacciaglia et al., 2018). We found no significant associations between the presence of CMBs and typical cortical areas of degeneration in AD, namely hippocampal volume, precuneus volume, and the whole cortical volume. Moreover, clinico-pathological studies have shown independent contributions of small vessel disease (Snowdon, 1997) and CAA (Launer et al., 2012) to cognitive impairment in AD. Also, we found no statistically significant association between the presence of CMBs and performance in the memory and executive function domains. Probably, gray matter volume loss and drop in cognitive performance are later-stage processes of neurodegeneration driven by APOE- ϵ 4, and our study participants were generally too young to yet show strong effects.

4.5. Strengths and limitations of this study

This study has some strengths and limitations that should be noted. One of the strengths is the unprecedented composition of our cohort, formed by old- and middle-aged cognitively unimpaired participants enriched for APOE status with limited cardiovascular burden. This composition allowed us to study the presence of CMBs without the confounding effect of other comorbidities and to study the differences in CMB load as a function of APOE genotype. Nevertheless, the characteristics of our cohort also caused some difficulties that resulted in some limitations. The high percentage of relatively young and CMB-free individuals and the low

cardiovascular burden resulted in weaker associations. Another limitation was the technique used to detect the presence of CMBs, which were assessed on T2*-weighted MR images. CMBs can be visualized as small round foci of hypointensity less than 10 mm in diameter at both T2*-weighted MRI and SWI (Haller et al., 2018). However, studies applying SWI showed twice the prevalence rates for CMBs compared to studies using T2*-weighted MRI (Haller et al., 2018). Although SWI allows for a higher sensitivity in the detection of CMBs, T2* imaging has been more frequently used in AD studies and thus this made our results more comparable with those found in literature (ten Kate et al., 2018). Moreover, different statistical models were built to scrutinize the relationship between APOE genotype and CMBs from different perspectives and make our results more comparable with other literature findings, but they were not corrected for multiple comparisons. This was not done as the different models were not independent, hence correction for false discovery rate could increase the number of false negatives and render over-conservative results, especially given the composition of our cohort. Nevertheless, in light of this, results should be taken with a note of caution. Another obvious limitation is the cross-sectional nature of our study, which prevents us from assessing the impact of CMB load and its longitudinal change on the clinical progression of the studied individuals. Finally, the lack of participants' amyloid status is also a limitation of the study and further research is needed to investigate the role of CSF biomarkers in the formation of CMBs.

5. Conclusions

The number of CMBs parallels the number of APOE- ϵ 4 alleles, suggesting that APOE- ϵ 4 genotype more vulnerable brain to cerebrovascular insults. APOE- ϵ 4 homozygotes show a higher number of lobar CMBs with respect to APOE- ϵ 4 heterozygotes and non-carriers, probably indicating a dose-dependent mechanism which may partially explain their increased risk of AD. Finally, co-occurrence of CMBs and WMH suggest that these lesions might occur in areas of the brain with higher vascular fragility, possibly sharing a common pathophysiology.

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Silvia Ingala: Methodology, Formal analysis, Data curation, Writing - review & editing. **Linda Mazzai:** Writing - review & editing. **Carole H. Sudre:** Formal analysis. **Gemma Salvadó:** Data curation. **Anna Brugulat-Serrat:** Data curation. **Viktor Wottschel:** Data curation. **Carles Falcon:** Data curation. **Grégory Operto:** Data curation. **Betty Tijms:** Data curation. **Juan Domingo Gispert:** Project administration, Investigation, Supervision. **José Luis**

Molinuevo: Investigation, Data curation, Supervision. **Frederik Barkhof:** Methodology, Data curation, Supervision.

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Appendix A. Supplementary data

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