# Neurovascular imaging markers of brain aging

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# Risk Factors Associated With Incident Cerebral Microbleeds According to Location in Older People

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## ABSTRACT

### Objectives

The spatial distribution of cerebral microbleeds (CMBs), which are asymptomatic precursors of intracerebral hemorrhage, reflects specific underlying microvascular pathologies of cerebral amyloid angiopathy (lobar) and hypertensive vasculopathy (deep brain structures). Relatively little is known about occurrence of and modifiable risk factors for developing CMBs, especially in a lobar location, in the general population of older people. The objective of this study was to investigate whether lifestyle and lipid factors predict new CMBs in relation to their location.

### Methods

Population-based sample of the Age, Gene / Environment Susceptibility (AGES)-Reykjavik Study of 2,635 individuals aged 66-93 years. Participants underwent a baseline brain MRI examination in 2002-2006, and returned for a repeat brain MRI in 2007-2011. Lifestyle and lipids factors assessed at baseline, i.e. smoking, alcohol drinking, body mass index, serum levels of total cholesterol, high-density lipoprotein, low-density lipoprotein and triglycerides. Brain MRI-detected incident CMBs, which were further categorized into one of two locations: strictly lobar or deep.

### Results

During a mean follow-up of 5.2 years, 486 people (18.4%) developed new CMBs, of whom 308 had strictly lobar and 178 had deep CMBs. In the multivariate log-binomial regression model adjusting for baseline cardiovascular risk factors including blood pressure and antihypertensive use, prevalent CMBs and markers of cerebral ischemic small vessel disease, heavy alcohol consumption (versus light to moderate, relative risk [RR] 2.94; 95%CI, 1.23 to 7.01) predicted incident CMBs in a deep location. Baseline being underweight (versus normal weight, 2.41; 1.21 to 4.80), current smoking (1.47; 1.11 to 1.94), higher serum high-density lipoprotein cholesterol (per SD increase 1.13; 1.02 to 1.25) and lower triglycerides (per SD decrease in natural log-transformed triglycerides 1.17; 1.03 to 1.33) were each significantly associated with an increased risk for strictly lobar CMBs but not with deep CMBs.

### Conclusions

Lifestyle and lipids risk profiles for CMBs were similar to those for symptomatic intracerebral hemorrhage and differed for lobar and deep CMBs. Modification of these factors could have the potential to prevent new onset CMBs, particularly those occurring in a lobar location.

## INTRODUCTION

Cerebral microbleeds (CMBs), visualized as hypointense lesions on T2*-weighted gradient echo MRI, frequently occur in healthy older people.[1,2] CMBs are an asymptomatic precursor of intracerebral hemorrhage (ICH)[3,4] and their presence is associated with an increased risk of (recurrent) ischemic stroke,[5] cognitive impairment[6] and mortality.[7] Histopathologically, CMBs represent hemosiderin deposits from microvascular leakage.[8] Similar to ICH, the pathophysiology of CMBs may differ according to their location, with lobar (cortical-subcortical) CMBs attributable to cerebral amyloid angiopathy and deep (basal ganglia, thalamus and brainstem) CMBs to hypertensive vasculopathy.[3]

Apart from high blood pressure, little is known about other potentially modifiable risk factors for the occurrence in the general population of new CMBs, especially in lobar locations.[1,9-11] On the other hand, the modifiable risk factors for ICH have been extensively investigated; establishing an overlap in the risk profiles for CMBs and ICH may pave the way for early detection of people at an increased risk of ICH, which is a devastating condition with no curative treatment options. For example, the adverse effects of lifestyle variables, such as low or high extremes of body mass index (BMI) and excessive alcohol intake, have been reported to be associated with the development of ICH. Whether these factors also predispose to CMBs at a particular location has not yet been well explored.[1,12] Furthermore, low serum lipid levels have long been recognized as an important risk factor for ICH[13-15] and also relate to the presence of CMBs in previous studies.[9,11,15] However, results are inconsistent with respect to CMBs locations and it remains unknown which serum lipid fractions are most closely associated with CMBs. To date, longitudinal data are scarce[11] and have been limited by relatively small sample sizes. Therefore we further examined the incidence and location of CMBs, and whether a spectrum of modifiable lifestyle and lipid factors predict new CMBs in relation to their location in the large population-based Age, Gene/ Environment Susceptibility (AGES)-Reykjavik Study.

## METHODS

### Participants

For the present study, we used longitudinal data from the AGES-Reykjavik Study, which originates from the Reykjavik Study, as described fully elsewhere.[16] Briefly, from 2002 to 2006, 5,764 surviving men and women born 1907–1935 of the Reykjavik Study cohort underwent an extensive physical and brain examination. From 2007 to 2011, there was a follow-up examination including repeated brain MRI scans. The study was approved by

the Icelandic National Bioethics Committee (VSN 00-063), and by the National Institute on Aging Intramural Institutional Review Board.

Of the 4,497 participants who had brain MRI scans and no dementia at baseline (Supplementary Figure 1), 547 had died, 154 were lost to follow-up (could not be contacted by any means), and 808 declined further participation between baseline and follow-up. Of the 2,988 participants in the follow-up examination, brain MRI imaging data were missing on 353 individuals due to contraindications (n=127), refusal/nonattendance (n=197), or technical reasons (i.e. no qualitatively acceptable MRI data available for all necessary sequences, n=29). Therefore 2,635 people who had complete and reliable baseline and follow-up MRI scans provided data in the analyses. Compared with people who participated in the first examination only, those in both MRI examinations were younger, had higher education, were less often underweight or treated with anticoagulants, and had more favorable profiles of cardiovascular risk factors and disease (Supplementary Table 1).

## Brain MRI and CMBs assessment

High-resolution brain MRI scans were all acquired on the same study-dedicated 1.5-T scanner (Signa Twinspeed, General Electric Medical Systems) following a similar MRI protocol, described elsewhere,[2] at both time points. A 2-dimensional T2*-weighted gradient echo-type echo planar sequence (GRE-EPI) was used for CMBs detection.[2] CMBs were defined as a focal area of signal void within the brain parenchyma that is visible on T2*-weighted GRE-EPI and smaller or invisible on T2 weighted fast pin echo scans.[2]

Two trained radiographers, blinded to the baseline CMBs scan, identified CMBs on the follow-up scan. If identified, the baseline CMBs scan was examined to determine whether the CMBs were present in the same slice location. If so, the follow-up CMBs were labeled ‘prevalent’; if not, the CMBs were labeled ‘incident’. Each CMB on the follow-up scan was evaluated in terms of size and anatomical location. A total count of CMBs per person was generated based on individually labeled CMBs as region specific estimates. CMBs were counted in lobar regions (frontal, parietal, temporal, and occipital); and in deep or infratentorium (basal ganglia and thalamus, corpus callosum, and infratentorial including brain stem and cerebellum) regions. People with ≥1 new CMBs restricted to lobar regions were considered to have strictly lobar CMBs and those with CMBs in a deep or infratentorial region, with or without concomitant lobar CMBs were considered to have deep CMBs. Intra-rater reliability (kappa) based on two ratings within a 6-month interval was 0.75 and 0.73 respectively, and the statistics of inter-rater agreement was 0.70, indicating good reliability.

## Lifestyle and lipids risk factors

Information on baseline lifestyle and lipids risk factors was gathered by questionnaire, laboratory and physical examinations.[16] Cigarette smoking was dichotomized as current versus noncurrent (never/former) smokers. Alcohol consumption was categorized into 4 groups based on drinking status and current weekly alcohol consumption (drinks/week): abstainers, former drinkers, light to moderate (women 1-7; men 1-14) and heavy (women >7; men>14).[17] BMI was calculated as weight (kg) divided by height squared ($m^2$) and further categorized into 4 groups according to the WHO guidelines: underweight <18.5, normal weight 18.5-24.9, overweight 25-29.9 and obese >30. Fasting total cholesterol, HDL-cholesterol and triglyceride levels were determined on a Hitachi 912 instrument using comparable enzymatic procedures (Roche Diagnostics, Mannheim, Germany).[18] All measurements fulfilled the criteria of the National Institute of Health/National Cholesterol Education Program for precision and accuracy of lipids measurements. LDL-cholesterol was calculated using the Friedewald equation.[18]

## Statistical analysis

All continuous variables were normally distributed except for the triglyceride levels and white matter hyperintensity volume (WMHV) for which natural logarithmic transformations of both were used. The cumulative incidence of CMBs was estimated in 10-year baseline age strata and separately in strata of presence/absence of CMBs at baseline MRI. To estimate the relative risk, we applied log-binominal regression[19] to examine the association of putative risk factors with CMBs incidence. The log-binominal model produces an unbiased estimate of the adjusted relative risk (RR) when the incidence of the outcome is greater than 10%.[20]All analyses were initially adjusted for age and sex (model 1), followed by additional adjustment for brain MRI examination interval between baseline and follow-up scans, head coil, systolic blood pressure, use of antihypertensive medications, use of anticoagulants/aspirin, prevalent CMBs, subcortical infarcts and WMHV (model 2). We additionally adjusted analyses of lipid levels and CMBs for statin use. These analyses were also performed stratified by incident CMBs location. Interactions between putative risk factors and other covariates were assessed in the fully-adjusted models. To test the robustness of the results, we did several sensitivity analyses, details of which are described in the supplements (Supplementary Methods). Analysis was conducted using Stata version 12.

# RESULTS

## Incidence of CMBs

Table 1 shows the study population characteristics according to incident CMBs categories. The mean age of the study population at baseline was 74.6 years and 59% were women. Overall, 486 of the 2635 participants (18.4%) during a mean period of 5.2 years developed new CMBs on MRI, of whom 145 (5.5%) had multiple new CMBs (eTable 2). Among people with new CMBs, 308 (63%) had incident strictly lobar CMBs and 178 (37%) had deep CMBs (eFigure 1). Of these who had incident CMBs located in a deep brain region, 66 people also had one or more incident lobar CMBs. The 5-year cumulative incidence of any CMBs increased with age at baseline from 16.0% in people aged 65 to 74 years to 28.6% in the oldest participants (>85 years). The similar pattern of incidence by age was also observed for multiple CMBs. CMBs incidence was slightly higher for men than for women in all age groups (overall 21.9% vs. 16.1%; eFigure 2) and higher for participants with CMBs at baseline compared with those without (31.2% vs. 15.8%). Moreover, participants with multiple CMBs at baseline had the highest incidence (48.4%) (eFigure 3).

Among participants without baseline CMBs (n=2,186), 346 (15.8%) had completely new onset CMBs (eTable 2). Of those with both baseline and incident CMBs (n=140), 78 had a single baseline CMB and 62 had multiple CMBs at baseline. Further, there were 66 participants who had baseline and incident CMBs occurring in a strictly lobar location and 33 with both in a deep location.

**Table 1.** Baseline characteristics (2002-2006) of the study population (n=2,635) according to cerebral microbleeds (CMBs) incidence category

| | No incident CMBs (N=2,149) | All incident CMBs (N=486) | Strictly lobar incident CMBs (N=308) | Deep incident CMBs (N=178) |
|---|---|---|---|---|
| Age, years | 74.5 (4.7) | **75.3 (4.9)*** | 75.1 (5.0) | **75.8 (4.8)*** |
| Men, % | 39.4 | **48.8*** | **48.4*** | **49.4*** |
| Primary education level, % | 20.5 | 18.6 | 19.0 | 18.1 |
| Type 2 diabetes,% | 8.8 | **11.8*** | 11.4 | 12.4 |
| APOE ε4 allele carrier, % | 25.6 | 29.0 | 30.2 | 27.0 |
| **Lifestyle risk factors** | | | | |
| Body mass index categories, kg/m$^2$, % | | | | |
| Underweight (<18.5) | 0.6 | **1.7*** | **2.0*** | 1.1 |
| Normal weight (18.5-24.9) | 28.4 | 31.3 | 31.9 | 30.3 |
| Overweight (25.0-29.9) | 47.0 | 45.4 | 45.9 | 44.4 |
| Obesity (>30) | 24.0 | 21.7 | 20.2 | 24.2 |

**Table 1.** Baseline characteristics (2002-2006) of the study population (n=2,635) according to cerebral microbleeds (CMBs) incidence category (continued)

| | No incident CMBs (N=2,149) | All incident CMBs (N=486) | Strictly lobar incident CMBs (N=308) | Deep incident CMBs (N=178) |
|---|---|---|---|---|
| Current smoker, % | 9.9 | **14.2*** | **15.6*** | 11.8 |
| Alcohol drinking, % | | | | |
| Abstainers | 20.9 | 18.7 | 17.1 | 21.5 |
| Former drinkers | 10.0 | 10.0 | 9.2 | 11.3 |
| Current light to moderate drinkers | 68.2 | 69.5 | 72.1 | 65.0 |
| Current heavy drinkers | 0.8 | **1.9*** | 1.6 | 2.3 |
| **Blood pressure measures** | | | | |
| Systolic blood pressure, mmHg | 140.7 (19.7) | **143.0 (19.9)*** | 142.5 (19.1) | **143.8 (21.2)*** |
| Diastolic blood pressure, mmHg | 74.0 (9.1) | **75.2 (9.9)*** | 74.9 (9.1) | **75.8 (11.2)*** |
| Pulse pressure | 66.7 (17.4) | 67.7 (17.5) | 67.5 (17.6) | 68.0 (17.5) |
| Hypertension, % | | | | |
| Mild[a] | 61.9 | 60.3 | 60.4 | 60.1 |
| Severe[a] | 14.3 | **17.9*** | 16.9 | 19.7 |
| **Lipid levels** | | | | |
| Serum total Cholesterol, mmol/L | 5.67 (1.13) | 5.61 (1.13) | 5.59 (1.10) | 5.63 (1.18) |
| HDL cholesterol, mmol/L | 1.59 (0.43) | 1.62 (0.46) | 1.62 (0.45) | 1.61 (0.48) |
| LDL cholesterol, mmol/L | 3.53 (1.03) | 3.47 (0.99) | 3.46 (0.99) | 3.48 (1.00) |
| Triglycerides, mmol/L, median (quartile range) | 1.06 (0.80-1.43) | **1.00 (0.74-1.39)*** | **0.98 (0.74-1.35)*** | 1.04 (0.76-1.48) |
| **Medication use** | | | | |
| Use of antihypertensive medications, % | 60.3 | 60.0 | 57.5 | 63.5 |
| Use of anticoagulants/aspirin, % | 25.5 | 28.1 | 28.3 | 27.9 |
| Statin, % | 24.2 | 22.0 | 22.4 | 21.4 |
| **Cardiovascular disease** | | | | |
| Coronary artery disease, % | 17.8 | 20.0 | 20.1 | 18.5 |
| Stroke, % | 4.8 | 5.6 | 5.2 | 6.2 |
| **Brain MRI measures** | | | | |
| Subcortical infarct, % | 6.5 | **11.1*** | 8.8 | **15.2*** |
| White matter hyperintensities, ml median (quartile range) | 10.9 (6.3-19.7) | **16.6(8.7-29.6)*** | **15.8(8.7-28.5)*** | **18.3(9.4-31.7)*** |
| Presence of CMBs on baseline MRI, % | 14.4 | **28.8*** | **26.0*** | **33.7*** |

*p<0.05 (compared with the no-incident CMBs group); baseline characteristics were compared among incident CMBs categories using two-sample t-test or the Wilcoxon-Mann-Whitney test for continuous variables and chi-squared tests for categorical variables. [a]Mild hypertension was defined as 140 ≤ systolic blood pressure (SBP) <160 mmHg, 90 ≤ diastolic blood pressure (DBP) <100 mmHg, or the use of antihypertensive medications and severe hypertension was defined as SBP/DBP ≥160/100 mmHg regardless of medication use.

## Incidence of CMBs and lifestyle factors

In the fully adjusted multivariate binomial regression model, baseline being underweight, current smoking and heavy current alcohol consumption were all significantly associated with a higher incidence of CMBs (Table 2). When stratified according to CMBs location, heavy alcohol drinking (versus light to moderate) predicted incident CMBs in deep (RR 2.94; 1.23 to 7.01) but not lobar regions. Both being underweight (versus normal weight RR 2.41; 1.12 to 4.80) and a current smoker (RR 1.47; 1.11 to 1.94) were associated with incident strictly lobar CMBs but not with deep CMBs. No association was observed for other alcohol drinking or BMI categories.

**Table 2.** Lifestyle and lipid factors and incident cerebral microbleeds according to location

| | **Any incident CMBs (n=486)** | | **Strictly lobar incident CMBs (n=308)** | | **Deep incident CMBs (n=178)** | |
|---|---|---|---|---|---|---|
| | Model 1* | Model 2† | Model 1* | Model 2 † | Model 1* | Model 2 † |
| **Lifestyle factors** | | | | | | |
| Body mass index categories | | | | | | |
| Underweight, vs. Normal weight | **1.75(1.00-3.05)** | **1.89(1.10-3.27)** | **2.16(1.09-4.28)** | **2.41(1.21-4.80)** | 1.40(0.38-5.18) | 1.52(0.41-5.56) |
| Overweight, vs. Normal weight | 0.90(0.75-1.09) | 0.91(0.76-1.10) | 0.89(0.70-1.13) | 0.88(0.70-1.12) | 0.91(0.65-1.26) | 0.93(0.67-1.29) |
| Obesity, vs. Normal weight | 0.89(0.71-1.12) | 0.88(0.71-1.10) | 0.81(0.60-1.10) | 0.80(0.60-1.09) | 1.03(0.70-1.51) | 0.99(0.67-1.45) |
| Smoking | | | | | | |
| Current smoker, vs. former or never | **1.49(1.19-1.86)** | **1.32(1.06-1.64)** | **1.65(1.24-2.19)** | **1.47(1.11-1.94)** | 1.36(0.88-2.10) | 1.21(0.77-1.88) |
| Alcohol drinking | | | | | | |
| Abstainers, vs. light to moderate drinkers | 0.94(0.76-1.17) | 0.96(0.78-1.19) | 0.84(0.63-1.13) | 0.88(0.66-1.17) | 1.12(0.78-1.61) | 1.13(0.79-1.62) |
| Former, vs. light to moderate drinkers | 0.93(0.71-1.22) | 0.83(0.64-1.09) | 0.84(0.58-1.22) | 0.77(0.53-1.11) | 1.07(0.68-1.69) | 0.91(0.58-1.42) |
| Heavy, vs. light to moderate drinker | **1.90(1.11-3.24)** | **1.99(1.18-3.36)** | 1.76(0.80-3.85) | 1.85(0.85-4.03) | **2.73(1.16-6.44)** | **2.94(1.23-7.01)** |

**Table 2.** Lifestyle and lipid factors and incident cerebral microbleeds according to location (continued)

| | Any incident CMBs (n=486) | | Strictly lobar incident CMBs (n=308) | | Deep incident CMBs (n=178) | |
|---|---|---|---|---|---|---|
| | Model 1* | Model 2† | Model 1* | Model 2 † | Model 1* | Model 2 † |
| **Serum lipid measures (mmol/l)‡** | | | | | | |
| Total cholesterol, per SD (1.13) decrease | 0.99(0.91-1.08) | 1.02(0.93-1.13) | 1.01(0.90-1.13) | 1.03(0.91-1.17) | 0.96(0.83-1.11) | 1.02(0.86-1.20) |
| HDL-cholesterol, per SD (0.44) increase | **1.12(1.03-1.22)** | **1.12(1.03-1.21)** | **1.14(1.03-1.27)** | **1.13(1.02-1.25)** | 1.12(0.97-1.30) | 1.14(0.99-1.32) |
| LDL-cholesterol, per SD (1.02) decrease | 1.02(0.94-1.11) | 1.06(0.96-1.16) | 1.03(0.93-1.15) | 1.06(0.93-1.20) | 1.01(0.87-1.16) | 1.08(0.91-1.28) |
| Triglycerides§, per SD (0.44) decrease | 1.07(0.99-1.16) | **1.11(1.01-1.21)** | **1.13(1.02-1.26)** | **1.17(1.03-1.33)** | 0.99(0.86-1.14) | 1.04(0.89-1.21) |

* Model 1 was adjusted for age & sex; † Model 2 was adjusted for age, sex, brain MRI examination interval, head coil, systolic blood pressure, use of antihypertensive medications, use of anticoagulants/aspirin, baseline presence of CMBs, subcortical infarcts and white matter hyperintensities (% intrcranial volume). ‡Model 2 was additionally adjusted for statin use. §Triglycerides levels were natural log-transformed.

## Incidence of CMBs and lipids level

Increasing levels of HDL-cholesterol were significantly associated with an increasing risk of any incident CMBs. Triglyceride levels showed an inverse association with risk of CMBs. These associations were also independent of statin use in the fully-adjusted model and were especially strong for incident strictly lobar CMBs (per SD increase in HDL RR 1.13; 1.02 to 1.25; per SD decrease in natural log-transformed triglycerides RR 1.17; 1.03 to 1.33), whereas there was no significant association with deep CMBs. Neither total nor LDL-cholesterol was associated with CMBs.

## Sensitivity analyses

When we analyzed people with 'strictly' deep CMBs (n=112) excluding those with CMBs in both the lobes and deep structures, results were similar to those reported above for any deep CMBs. Additional adjustment for APOE ε4 genotype or prevalent stroke generated similar results. In stratified analyses, the associations persisted in participants without baseline CMBs and were found to be in the same direction, though not significant, in the smaller sample of people with baseline CMBs. We also repeated the analyses for subgroups stratified by APOE ε4 carriership or statin use. As there was no a priori hypothesis, we considered interactions significant only if $p \leq 0.01$ and none met this level of significance. There were no significant interactions of putative risk factors with other covariates. In location-specific analyses, exclusion of those with discordant

location between baseline and incident CMBs did not essentially change the findings for incident lobar or deep CMBs. In multinomial logistic regression analyses by categorizing the dependent variables of CMBs into no incident CMB, a single CMB, and multiple CMBs, lifestyle factors and increasing levels of HDL-cholesterol were significantly associated with an increased risk of developing a single CMB, but not multiple CMBs (Supplementary Table 3). Analyses on the imputed datasets yielded results similar to those reported in the main analysis.

## DISCUSSION

Five-year cumulative incidence of any CMBs in this population-based cohort of older people was 18.4%. Lifestyle and lipid risk profiles for CMBs were similar to those for ICH and differed according to CMBs location. Heavy current alcohol consumption relative to light to moderate drinking predicted CMBs in a deep region. Baseline underweight (BMI <18.4 kg/m$^2$), current smoking, high serum HDL cholesterol and low triglycerides were all significantly associated with an increased risk of incident strictly lobar CMBs but not with deep CMBs. These associations were independent of major cardiovascular risk factors and ischemic cerebral small vessel disease.

CMBs represent the remnants of small asymptomatic ICHs and are associated with an increased risk for symptomatic ICH.[21] The associations with lifestyle factors are all consistent with findings for ICH.[22,23] Furthermore, the region-specific associations suggest these factors have different etiologic roles or are markers of a vulnerable cerebral microvascular system susceptible to specific vascular pathologies such as hypertensive arteriopathy or cerebral amyloid angiopathy. For example, heavy alcohol intake increases the risk of arterial hypertension; in our cohort, 26% of heavy drinkers had severe hypertension, which was higher than those reporting a lower consumption of alcohol. In particular, a transient increase in blood pressure together with cerebral arteriolar vasoconstriction during alcohol exposure might cause rupture of small cerebral arteries.[24] Although the observations with underweight may point to low lipid levels as a potential mechanism, further adjustment for total cholesterol or triglycerides in additional analyses did not eliminate the associations. However, there were few underweight subjects (n=21) and it has been shown in other studies that low BMI may be a prodromal symptom of dementia.[25]

Consistent with previous reports,[13,15,22,26] we observed an inverse association between triglycerides levels and CMBs. HDL-cholesterol was also positively and independently associated with CMBs, which is in accordance with a previous cross-sectional study of HDL and the presence of CMBs in patients with neurological diseases,[27] but it contrasts

with the lack of association in other studies.[1,10,15] Cholesterol and triglycerides are essential structural elements of cell membranes. There is increased permeability of erythrocyte membranes *in vitro* and *in vivo* studies with reduced lipid levels.[28] It has been proposed that lower levels of total cholesterol or triglycerides result in smooth muscle degeneration and endothelial weakness that more readily lead to arterial fragility and microaneurysms which are prone to leakage and rupture.[29,30] It is also possible that low triglyceride levels favor a prohemorrhagic state due to negative correlations with the vitamin K-dependent coagulation factors and with the plasminogen activator inhibitor.[31]

Increased HDL has been speculated to have a 'dual and opposite effect' on cerebral blood vessels with vascular protection from ischemia on one hand and increased vulnerability to vascular rupture on the other hand.[31] Although the underlying mechanisms remain unclear, there are some possible explanations. Cholesteryl ester transfer protein (CETP) mediates the transfer of cholesteryl esters from HDL to triglyceride-rich lipoproteins.[32] This stimulates reverse cholesterol transport from peripheral cells to the liver for excretion. It is possible that CETP deficiency or dysfunction secondary to genetic or environmental variation (e.g. alcohol consumption)[33], causes reduced reverse cholesterol transport; this process is reflected as an increase in HDL cholesterol levels, and contributes to the loss of the anti-atherogenic properties of HDL resulting from its increased cholesterol content and particle size.[34] Furthermore, HDL is involved in the regulation of reverse cholesterol transport at the blood-brain barrier and in the processing of β-amyloid in the brain. Fagan et al.[35] found a positive association between plasma HDL and HDL in central nervous system and the increased HDL levels found in the periphery may reflect increased efflux from the brain.[36] Thus it is possible that alterations in the metabolism and the actions of HDL in the cerebral microvascular subendothelial space may contribute to the vascular deposition of amyloid.[37] Serum 24S-hydroxycholesterol have been proposed as a more specific indicator of brain cholesterol than HDL, and increased levels are observed in patients with Alzheimer's disease.[38]

Our further finding that the association with triglycerides was most robust for strictly lobar CMBs may provide specific etiologic clues and suggest a role through development of amyloid microangiopathy. The association with HDL was also significant for strictly lobar CMBs. However, the risk estimates were very similar for CMBs in both locations and we cannot rule out the possibility that the non-significant result for deep CMBs was due to a lack of statistical power. In the Rotterdam Scan Study[11] an inverse association between serum total cholesterol and incidence of CMBs was found to be strongest for CMBs located in the deep regions. Although not significant, the directions of our findings on LDL-cholesterol and total cholesterol are consistent with the associations observed for triglycerides. Total cholesterol reflects both HDL and LDL subfractions in varying pro-

portions, which may explain why we could not find associations with total cholesterol. It could also be that a smaller sample size, younger age of the cohort, a higher load of baseline CMBs and a higher percentage of participants with severe hypertension (19.2% vs. 15.0%) in their study compared to our study has influenced the findings. Given the detection of CMBs depends on various MRI parameters, different MRI methods may have affected the reported CMBs incidence and thus limited comparisons between studies.

Major strengths of the present study include the large population-based sample of older individuals, the use of standard MRI protocol at both time points, as well as the extensive characterization of participants which enabled us to examine a spectrum of modifiable risk factors as well as adjust for a series of potential confounders. A possible limitation of the study is that selection bias may have influenced the results. People who were included in the analysis were younger and healthier at baseline than those who were excluded. In particular, people with worse vascular risk profile or more severe cerebral small vessel disease (those more likely to develop new CMBs) died or were lost to follow-up before they could be recruited into the follow-up examination. This may have led us to underestimate the true incidence of CMBs and as such the findings in relation to the predictors of CMBs may be affected if selection occurred differentially according to the predictor variables (e.g. the prevalence of being a current smoker at baseline was higher in those who were excluded; bias would occur if the association between current smoking and CMBs in the excluded people differed from what we found in the included sample). On the outcome side, if prevalent and incident CMBs were located in different locations (deep vs. lobar), then it may be more difficult to identify location-specific risk factors. In our sensitivity analyses, we excluded people whose baseline and follow-up CMBs differed in location; results were unaltered for incident strictly lobar or deep CMBs, suggesting this location difference is unlikely to affect our findings. The clinical and prognostic significance of these CMBs is another area of great interest and we are currently investigating the cognitive consequences of CMBs.

## CONCLUSIONS

Our study provides essential and new information on the importance of lifestyle and lipids factors for the development of CMBs. Risk profiles for asymptomatic CMBs are similar to those for symptomatic ICH and differ for lobar and deep CMBs. Reducing the prevalence of lifestyle-based risk factors, including current smoking and heavy alcohol drinking, and monitoring lipid levels during intensive lipid-lowering therapy (e.g. extremely low triglycerides) could have the potential to prevent new onset CMBs, particularly occurring in lobar locations.

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# SUPPLEMENTARY MATERIAL

## Supplementary methods

### *Ischemic cerebral small vessel disease on MRI*

Subcortical infarcts were graded on fluid attenuated inversion recovery sequence, T2-weighted and proton density-weighted sequences at baseline according to criteria described previously.[1] White matter hyperintensity volume was quantified automatically with an image analysis pipeline[2] and calculated as the percentage of total intracranial volume.

### *Other measurements*

Systolic (SBP) and diastolic (DBP) blood pressures were measured in recumbent position with a standard mercury sphygmomanometer, and the mean of two consecutive measurements was used. Hypertension was defined as SBP ≥140 mmHg, DBP≥90 mmHg or by the use of antihypertensive medications. Severity of hypertension was further categorized into mild and severe hypertension according to 2003 WHO criteria.[3] Type 2 diabetes was ascertained through self-reported history of diabetes, use of blood glucose-lowering medication or a fasting blood glucose level≥7.0 mmol/l. Baseline use of medications (e.g. statin and anticoagulants) was assessed from vials presented at the clinic. Prevalent stroke was identified from the registry or self-report of a doctor's diagnosis of stroke. Apolipoprotein E (APOE) genotyping was carried out using standard DNA amplification and restriction isotyping. Participants were categorized as APOE ε4 carriers (allele combinations ε3/ε4 or ε4/ε4) or noncarriers (allele ε2/ε2 or ε2/ε3 or ε3/ε3). APOE ε2ε4 carriers were grouped separately.

### *Statistical analysis*

We analyzed the deep CMBs group excluding participants with concomitant incident lobar CMBs and reran analyses for incident 'strictly' deep CMBs. We repeated analyses additionally adjusting for APOE ε4 carriership or prevalent stroke at baseline. We performed stratified analyses according to baseline presence of CMBs. In location-specific analyses, we excluded participants who had baseline CMBs in a different location from their incident CMBs (e.g. people with baseline deep CMBs and incident lobar CMBs). We also constructed multinomial logistic regression models to relate CMBs by number (a single or multiple [≥2]) to putative risk factors. Finally, we used multiple multivariate imputation of variables with ten imputed datasets to replace missing values on potential confounders, amounting to 2.4% of total values.

## SUPPLEMENTARY REFERENCES

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**Supplementary Table 1.** Baseline characteristics (2002-2006) of participants (n=4497) who had a second MRI assessment and for those who refused or were dead/lost to follow up.

| | Baseline partici-pants with a second MRI (N=2,635) | Baseline Participants without a second MRI (N=1,161) † | Participants dead or lost to follow-up (N=701) |
|---|---|---|---|
| Age, years | 74.6 (4.8) | **77.8(5.3)*** | **79.0 (5.8)*** |
| Men, % | 41.1 | **37.4*** | **50.5*** |
| Primary education level, % | 20.1 | **27.2*** | **25.1*** |
| **Lifestyle risk factors** | | | |
| Body mass index categories, kg/m$^2$, % | | | |
| Underweight (<18.5) | 0.8 | **1.8*** | **2.9*** |
| Normal weight (18.5-24.9) | 29.0 | **33.6*** | **35.6*** |
| Overweight (25.0-29.9) | 46.7 | 43.4 | **41.6*** |
| Obesity (>30) | 23.5 | 21.2 | **20.0*** |
| Current smoker, % | 10.7 | 12.7 | **14.9*** |
| Alcohol drinking, % | | | |
| Abstinence | 20.5 | **26.5*** | 21.8 |
| Former drinkers | 10.0 | **13.7*** | **15.0*** |
| Current light to moderate drinkers | 68.5 | **59.2*** | **62.3*** |
| Current heavy drinkers | 1.0 | 0.7 | 0.3 |
| **Blood pressure measures** | | | |
| Systolic blood pressure, mmHg | 141.1(19.7) | **144.1(20.2)*** | **143.9(21.9)*** |
| Diastolic blood pressure, mmHg | 74.2(9.3) | 73.5(9.8) | 73.5(10.3) |
| Pulse pressure, mmHg | 66.9(17.4) | **70.5(18.4)*** | **70.4(19.4)*** |
| Hypertension, % | 77.6 | **82.6*** | **85.9*** |
| **Type 2 diabetes**,% | 9.3 | **13.9*** | **14.1*** |
| **Lipid levels** | | | |
| Serum total Cholesterol, mg/dL | 219(44) | 220(46) | **211(48)*** |
| HDL cholesterol, mg/dL | 62(17) | 63(18) | 61(17) |
| LDL cholesterol, mg/dL | 136(39) | 137(41) | **130(42)*** |
| Triglycerides, mg/dL, median (quartile range) | 92(70-127) | 93(68-128) | 89(68-124) |
| **Medication use** | | | |
| Use of anticoagulants, % | 5.5 | **9.1*** | **13.2*** |
| Aspirin use, % | 21.9 | **25.7*** | 22.7 |
| Statin, % | 23.8 | **20.4*** | 21.5 |
| **Cardiovascular disease** | | | |
| Coronary artery disease, % | 18.1 | **21.2*** | **25.1*** |
| Stroke, % | 4.9 | **8.6*** | **9.1*** |
| **Ischemic cerebral small vessel disease** | | | |

**Supplementary Table 1.** Baseline characteristics (2002-2006) of participants (n=4497) who had a second MRI assessment and for those who refused or were dead/lost to follow up. (continued)

| | Baseline participants with a second MRI (N=2,635) | Baseline Participants without a second MRI (N=1,161) † | Participants dead or lost to follow-up (N=701) |
|---|---|---|---|
| Subcortical infarct on baseline MRI, (%) | 8.6 | **12.7*** | **17.1*** |
| White matter hyperintensities on baseline MRI, ml median (quartile range) | 11.6(6.6-21.5) | **14.8(7.9-28.5)*** | **18.4(10.2-34.9)*** |
| **APOE carrier status** | | | |
| APOE ε4 allele carrier | 24.3 | **28.2*** | 25.6 |

* Mean (SD), median (quartile range), or percentage is significantly different (P<0.05) from participants with a second MRI; † Participants in the follow-up examination refusing a second MRI (n=808) and participants with no (complete) MRI examinations (N=353); ‡ Participants who were dead (n=547) or lost to follow up (n=154); Data are missing among participants with a second MRI for BMI (n=1), education (n=14), use of anticoagulants/aspirin (n=277), serum LDL (n=2), Type 2 diabetes (n=2), smoking (n=8), alcohol drinking (n=22) , white matter hyperintensities (n=62), and APOE genotype (n=6).

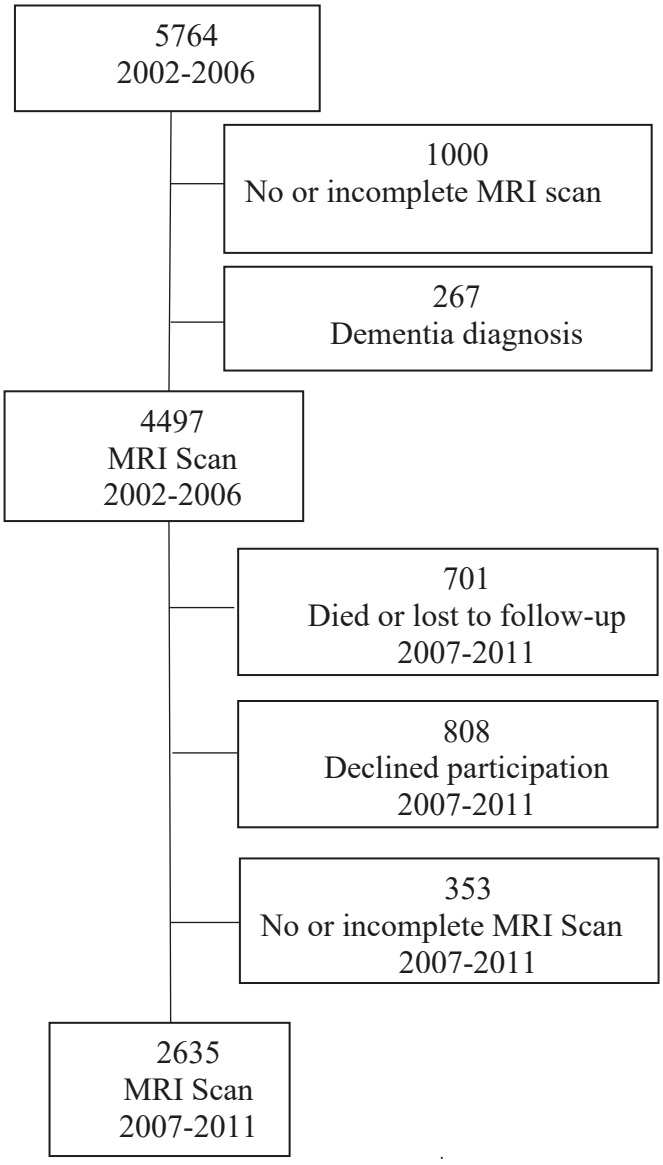
**Supplementary Table 2.** Incidence of CMBs by 10-year age group in strata of the presence of cerebral microbleeds (CMBs) on baseline MRI

| | Overall | | | | No Prevalent CMBs on baseline MRI | | | | Prevalent CMBs on baseline MRI | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **Age range, yrs** | No.of persons | Incident CMBs No.(%) | Single incident CMB No.(%) | Multiple incident CMBs No.(%) | No.of persons | Incident CMBs No.(%) | Single incident CMB No.(%) | Multiple incident CMBs No.(%) | No.of persons | Incident CMBs No.(%) | Single incident CMB No.(%) | Multiple Incident CMBs No.(%) |
| 65-74 | 1404 | 225 (16.0) | 171 (12.2) | 54 (3.9) | 1203 | 166(13.8) | 138(11.5) | 28(2.3) | 201 | 59 (29.4) | 33(16.4) | 26(12.9) |
| 75-84 | 1161 | 241(20.8) | 160 (13.8) | 81 (7.0) | 924 | 167(18.1) | 122(13.2) | 45(4.9) | 237 | 74(31.2) | 38(16.0) | 36(15.2) |
| 85-97 | 70 | 20(28.6) | 10 (14.3) | 10 (14.3) | 59 | 13(22.0) | 5(8.5) | 8(13.6) | 11 | 7(63.6) | 5(45.5) | 2(18.2) |
| Total | 2635 | 486 (18.4) | 341(12.9) | 145(5.5) | 2186 | 346(15.8) | 265(12.1) | 81(3.7) | 449 | 140(31) | 76(16.9) | 64(14.3) |

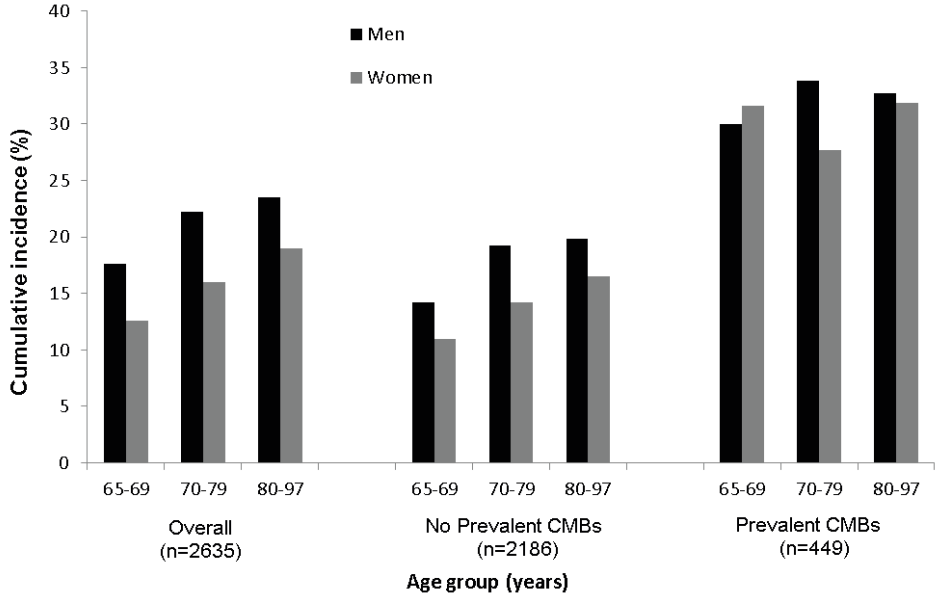
**Supplementary Table 3.** Association of lifestyle and lipid factors with incident single and multiple cerebral microbleeds (CMBs)

| | Single CMB (n=341) | | Multiple (≥2) CMBs (n=145) | |
|---|---|---|---|---|
| | Model 1* | Model 2† | Model 1* | Model 2† |
| **Lifestyle factors** | | | | |
| Body mass index categories | | | | |
| Underweight | **3.65 (1.47-9.08)** | **3.31 (1.31-8.37)** | No cases | No cases |
| Normal weight | 1.00 (reference) | 1.00 (reference) | 1.00 (reference) | 1.00 (reference) |
| Overweight | 0.90 (0.69-1.18) | 0.89 (0.68-1.18) | 0.84 (0.57-1.23) | 0.80 (0.53-1.19) |
| Obesity | 0.95 (0.69-1.31) | 0.93 (0.67-1.29) | 0.72(0.44-1.17) | 0.69(0.41-1.16) |
| Smoking | | | | |
| Former or never | 1.00 (reference) | 1.00 (reference) | 1.00 (reference) | 1.00 (reference) |
| Current smoker | **1.69(1.21-2.35)** | **1.57(1.11-2.21)** | 1.48 (0.88-2.49) | 1.13 (0.65-1.96) |
| Alcohol drinking | | | | |
| Abstinence | 0.87(0.64-1.19) | 0.89(0.65-1.23) | 1.01(0.65-1.58) | 1.07(0.67-1.70) |
| Former | 0.88(0.59-1.31) | 0.80(0.53-1.21) | 1.04(0.60-1.80) | 0.94(0.53-1.67) |
| Light to moderate | 1.00 (reference) | 1.00 (reference) | 1.00 (reference) | 1.00 (reference) |
| Heavy | **2.51 (1.03-6.11)** | **2.57 (1.05-6.28)** | 1.84 (0.41-8.15) | 2.44 (0.53-11.24) |
| **Serum lipid measures (mg/dL)ǂ** | | | | |
| Total cholesterol, per 1-SD (44) decrease | 0.94(0.83-1.06) | 0.97(0.84-1.12) | 1.13(0.95-1.36) | 1.13(0.90-1.42) |
| HDL-cholesterol, per 1-SD (17) increase | **1.18(1.04-1.32)** | **1.17(1.03-1.32)** | 1.08(0.90-1.29) | 1.12(0.93-1.35) |
| LDL-cholesterol, per 1-SD (39) decrease | 0.97(0.86-1.09) | 1.01(0.88-1.16) | 1.17(0.98-1.39) | 1.17(0.94-1.47) |
| Triglycerides§, per 1-SD (39) decrease | 1.13(0.99-1.28) | 1.14(0.99-1.30) | 1.02(0.86-1.22) | 1.09(0.90-1.33) |

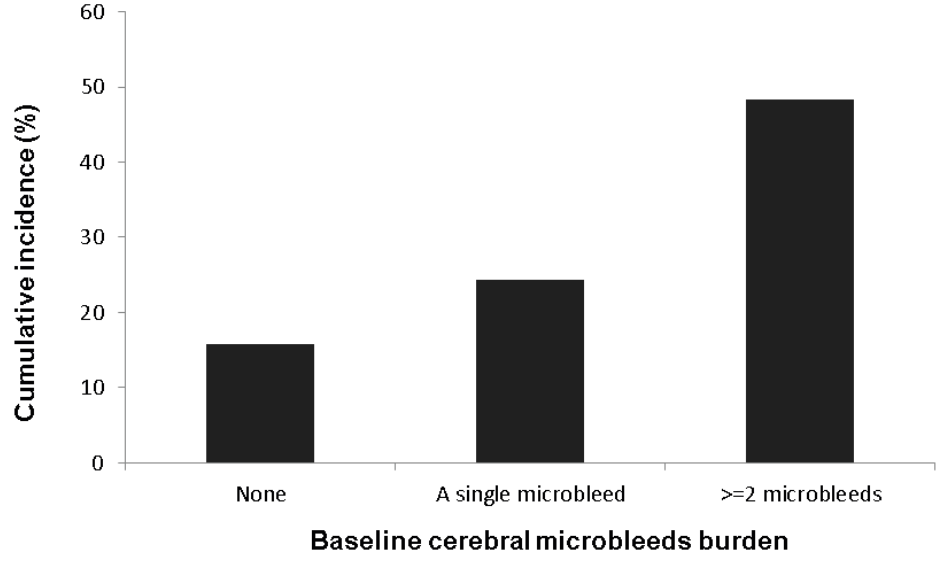
Participants with no incident CMBs (n=2,149) were held as a referent group for multinominal logistic modeling and results were presented as relative risk ratio (95%CI). * Model 1 was adjusted for age & sex;† Model 2 was adjusted for age, sex, brain MRI examination interval, head coil, systolic blood pressure, use of blood pressure lowering drugs, use of anticoagulants/aspirin, baseline presence of CMBs, subcortical infarcts and white matter hyperintensities (% intrcranial volume); §Natural logarithmic-transformation was used.




**Supplementary Figure 1.** Study population




**Supplementary Figure 2.** Cumulative incidence (%) of cerebral microbleeds (CMBs) over a 5-year period for men and women in 10-year age groups according to the presence of CMBs on baseline MRI




**Supplementary Figure 3.** Cumulative incidence (%) of cerebral microbleeds (CMBs) over a 5-year period according to the baseline CMBs burden