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Genetic loci for serum lipid fractions and intracerebral hemorrhage

Saloua Akoudad [a, b, c], M. Arfan Ikram [a, b, c], Marileen L.P. Portegies [a, c], Hieab H. Adams [a, b], Daniel Bos [a, b], Albert Hofman [a], Peter J. Koudstaal [c], Andre G. Uitterlinden [a, d], Aad van der Lugt [b], Cornelia M. van Duijn [a], Meike W. Vernooij [a, b, *]

[a] Department of Epidemiology, Erasmus MC, University Medical Center Rotterdam, the Netherlands
[b] Department of Radiology, Erasmus MC, University Medical Center Rotterdam, the Netherlands
[c] Department of Neurology, Erasmus MC, University Medical Center Rotterdam, the Netherlands
[d] Department of Internal Medicine, Erasmus MC, University Medical Center Rotterdam, the Netherlands

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ABSTRACT

Background: Serum total cholesterol and its fractions are inversely associated with intracerebral hemorrhages (ICH) and their potential subclinical precursor, cerebral microbleeds. To ascertain whether there is a genetic basis for this inverse association, we studied established genetic loci for serum total, LDL, and HDL cholesterol, and triglycerides in their association with ICH and microbleeds.

Methods: Data on 161 genetic variants for serum lipids was collected in 9011 stroke-free participants (mean age 65.8, SD 10.2; 57.9% women) of the population-based Rotterdam Study. Participants were followed from baseline (1997–2005) up to 2013 for the occurrence of ICH. A subset of 4179 participants underwent brain MRI for microbleed assessment between 2005 and 2011. We computed genetic risk scores (GRS) for the joint effect of lipid variants. Cox proportional hazards and logistic regression models were used to investigate the association of GRS of lipid fractions with ICH and microbleeds.

Results: After a mean follow-up of 8.7 (SD 4.1) years, 67 (0.7%) participants suffered an ICH. Microbleed prevalence was 19.6%. Higher genetic load for high serum total and LDL cholesterol was associated with an increased risk of ICH. Higher genetic load for high serum LDL cholesterol was borderline associated with a higher prevalence of multiple lobar microbleeds.

Conclusions: Genetic susceptibility for high serum total and LDL cholesterol is positively associated with incident ICH and borderline associated with multiple lobar microbleeds. We did not find a genetic basis for the previously reported inverse association between serum lipid levels and ICH.

1. Introduction

Hypercholesterolemia is an established modifiable risk factor for ischemic cardiovascular disease, including ischemic stroke. Paradoxically, high serum total cholesterol has been associated inversely with clinical intracerebral hemorrhages (ICH) [1–4] and their potential subclinical precursor, cerebral microbleeds [5–8]. Profound insight into this seemingly contradictory association is essential given the growing concern of adverse ICH events in persons vigorously treated with lipid-lowering medication [9–11].

To date, studies have focused solely on serum lipid levels to investigate the inverse association with ICH. Some studies reported that low total cholesterol drove this association [1,2,4], whilst others pointed towards a specific lipid fraction [3,5,6]. Results from these studies are, however, limited by the fact that serum lipid levels were measured only once, and associations may partly be explained by residual confounding due to unmeasured determinants, such as diet. No study reported on a potential genetic basis for the inverse association of serum lipids with ICH. Studying genes that influence serum lipid levels may provide more robust, unconfounded associations, as genes should not be susceptible to changes in lifestyle or environment.

We investigated 161 known genetic loci for serum total, HDL, and LDL cholesterol, and triglycerides and studied their associations with risk of ICH and with presence of their potential subclinical precursor, cerebral microbleeds.

* Corresponding author. Erasmus MC, University Medical Center, Na28-18, Postbus 2040, 3000 CA, Rotterdam, the Netherlands.
E-mail address: m.vernooij@erasmusmc.nl (M.W. Vernooij).

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2. Methods

2.1. Study population

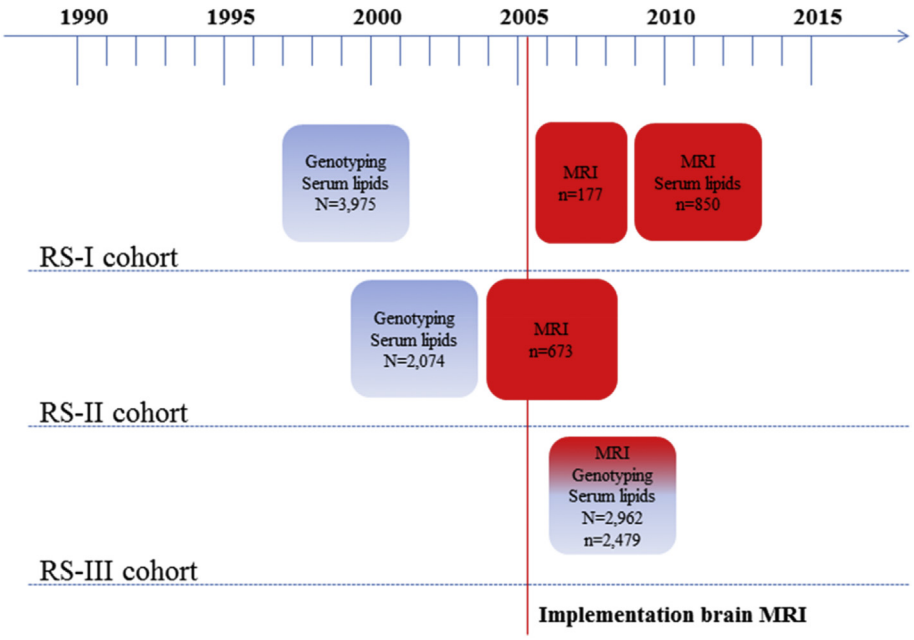
This study was conducted within the Rotterdam Study, a prospective population-based cohort study aimed at investigating determinants and consequences of chronic diseases in an aging population [12]. In 1990, 7983 persons (78% of invitees) were included in the initial study wave (RS-I). In 1999, the cohort was expanded by 3011 participants (67% of invitees) (RS-II). The cohort expanded a second time in 2006 with 3932 participants (65% of invitees) (RS-III). The total of 14,926 participants enrolled, were invited to undergo home interviews and various physical and laboratory examination at the research center every 4 years. Genotyping was done in 1997 (RS-I), 1999 (RS-II), and 2006 (RS-III). Of the 14,926 participants, 9011 were genotyped and stroke-free at baseline (Fig. 1). From 2005 onwards, brain MRI, including microbleeds assessment, was performed in those without MRI contraindications (pacemakers, claustrophobia) [13,14]. Of the 9011 stroke-free participants who were included at baseline, 4179 underwent brain MRI scanning between 2005 and 2011.

2.2. Genotyping

Participants were genotyped using Illumina HumanHap 550 Duo BeadChip or the Illumina Infinium II HumanHap 610 Quad Arrays. Genotyping was done at the Human Genotyping Facility Genetic Laboratory department of Internal Medicine, Erasmus MC, Rotterdam, the Netherlands [14]. Variant-specific quality controls included filters for call rate (>98%), minor allele frequency (>0.1%), Hardy–Weinberg equilibrium (p-values>10−6), and differential missingness by outcome or genotype (mishap test in PLINK, http://pngu.mgh.harvard.edu/purcell/plink/). Markov Chain Haplotyping (MaCH) package (http://www.sph.umich.edu/csg/abecasis/MACH, version 1.0.15 or 1.0.16 software) was used for imputation to the 1000 Genomes Phase I Version 3 reference panel (all populations). For each imputed variant, quality of imputation was estimated as the ratio of the empirically observed dosage variance to the expected binomial dosage variance. For this study, we extracted data on 161 variants, 73 which have been related to serum concentrations of total cholesterol, 56 to LDL, 71 to HDL, and 39 to triglycerides (Supplementary Table 1) [15]. Imputation quality (R^2) for the serum lipid variants was >0.60 (mean = 0.96).

2.3. Assessment of stroke

Stroke was defined as a syndrome of rapidly developing clinical signs of focal or global disturbance of cerebral function, with symptoms lasting 24 h or leading to death, with no apparent cause other than of vascular origin [16]. A history of stroke was assessed in all participants upon study entry using home interviews and was confirmed by reviewing medical records. Participants were subsequently followed for stroke occurrence through automated linkage of general practitioners' medical records with the study database. Medical records from nursing homes and from general practitioners of participants who moved out of the study area were checked on a regular basis. Research physicians reviewed all potential strokes using hospital discharge letters and information from general practitioners. An experienced vascular neurologist verified the stroke diagnoses [17]. Computed tomography reports



Blue boxes: population at risk of intracerebral hemorrhages (ICH) (baseline). Red boxes: subgroup of the population at risk of ICH that underwent MRI for the first time. Note that in some cases serum lipid levels were assessed at visits preceding MRI.

N= total population for ICH analyses; n= total population for cerebral microbleed analyses.

Fig. 1. Schematic overview of the study population.

were used to distinguish intracerebral hemorrhages from ischemic strokes. Strokes were classified as unspecified if neuroimaging was absent. Follow-up was complete until January 1st 2013 accounting for 77,991 (98.2% of potential) person-years. In total, 7.5% of the person-years follow-up was collected via records of nursing homes and records of general practitioners of patients who moved out of the study area.

2.4. Brain MRI and microbleed assessment

A multi-sequence MRI protocol was used on a 1.5-Tesla MRI scanner (GE Healthcare, Milwaukee, WI) [13]. Cortical infarcts were defined as focal lesions affecting the cortical gray matter on FLAIR, T1-weighted, and T2-weighted sequences. Microbleeds were detected using a custom-made accelerated 3-dimensional T2*-weighted gradient-recalled echo sequence (repetition time = 45 ms, echo time = 31, matrix size = 320 × 244, flip angle = 13, field-of-view = 25 × 17.5 cm^2, parallel imaging acceleration factor = 2, 3D acquisition with 96 slices encoded with a slice thickness of 1.6 mm zero padded to 192 slices of 0.8 mm, acquisition time 5 min 55 sec) [18]. Microbleeds were defined as small, round areas of signal loss on T2*-weighted images and their presence, number, and location were rated by trained research-physicians [18].

2.5. Covariates

Serum total cholesterol, HDL cholesterol, and triglyceride levels were determined using an automated enzymatic procedure (Hitachi analyzer, Roche Diagnostics, Washington DC). LDL cholesterol was calculated using the Friedwald formula (LDL cholesterol = total cholesterol − HDL cholesterol − triglycerides/2.17), in those with triglyceride levels ≤4.51 mmol/L [19]. If serum lipids levels were not assessed at time of MRI, serum lipid levels of a preceding visit were used (Fig. 1). Records on lipid-lowering medication use (ATC-code C10), blood-pressure lowering medication (ATC-code C02-03, c07-09), and antithrombotic medication (ATC-code B01AA, B01AB, B01AC, B01AX) were retrieved from local pharmacies serving the study area. Blood pressures measurements were averaged over 2 readings measured within a single center visit using a random-zero sphygmomanometer. Smoking habits were defined as ever versus never smoking. Diabetes mellitus was defined as having fasting glucose levels of ≥7.0 mmol/L or the use of glucose-lowering medication. Body-mass-index was calculated as weight (in kilograms) divided by height (in meters) squared.

2.6. Statistical analysis

We computed weighted genetic risk scores (GRS) for total, LDL, and HDL cholesterol, and triglycerides by summing the number of serum lipid fraction effect alleles and weighting them by the reported effect estimate of each lipid variant (73 variants for total cholesterol, 56 variants for LDL cholesterol, 71 variants for HDL cholesterol, and 39 variants for triglycerides using results from the GWAS on serum lipids) [15]. Note that most variants were pleiotropic and thus there was overlap across the risk scores.

Cox proportional hazards models were used to compute the estimated hazard ratios (HR) and 95% confidence intervals (CI) for the association of serum lipid fractions and GRS of lipid fractions with incident ICH. Logistic regression models were used to estimate odds ratios (OR) and 95% CI for the association of serum lipid fractions and GRS of lipid fractions with presence of cerebral microbleeds on MRI. Microbleeds were categorized by their location and count (single and multiple strictly lobar and deep or mixed microbleeds versus no microbleeds) [18]. We fitted 3 models for the main analyses, adjusting for age and sex in the first model, additionally for serum lipid levels and lipid-lowering medication in the second model, and cardiovascular risk factors (blood pressures, smoking habits, diabetes mellitus, body-mass-index, blood pressure-lowering and antithrombotic medication use) in the third model. Variance inflation factor was calculated to detect possible multicollinearity in the models described above. For microbleeds, analyses were repeated after excluding participants with cortical infarcts on MRI (N = 151). Also, we repeated the analysis for ICH and cerebral microbleeds after excluding *APOE* alleles from the GRS.

Missing cardiovascular covariate data (≤7%) were imputed based on sex, age, and cardiovascular risk factors using logistic regression models. Analyses were done using IBM SPSS statistic for Windows, Version 21.0 (IBM Corp., Armonk, NY), using an alpha-value of 0.05.

3. Results

Baseline characteristics of the study population are presented in Table 1. During a mean follow-up of 8.7 years (SD 4.1), 67 (0.7%) participants suffered an ICH. The prevalence of lobar microbleeds and the prevalence of deep or infratentorial microbleeds in those who underwent MRI (n = 4179) was respectively 12.9% and 6.7%. GRS of total, LDL, and HDL cholesterol and triglycerides were strongly associated with their corresponding serum lipid fraction (respectively $P = 6.2 \times 10^{-83}$ for total cholesterol, $P = 5.0 \times 10^{-71}$ for LDL cholesterol, $P = 2.4 \times 10^{-102}$ for HDL cholesterol, and $P = 7.0 \times 10^{-96}$ for triglycerides) (Supplementary Table 2). Associations of fasting serum lipid levels with ICH and microbleeds are shown in Supplementary Tables 3 and 4 Although not significant, serum triglycerides associated inversely with ICH risk and microbleed presence. Additionally, serum HDL cholesterol was inversely related to lobar microbleeds, whereas LDL cholesterol was inversely associated with deep or infratentorial microbleeds.

Table 2 shows the association of GRS of serum lipid fractions with incident ICH. Higher GRS of total cholesterol was associated with an increased risk of ICH, even after adjusting for serum total cholesterol, lipid-lowering medication, and cardiovascular risk factors (Model 3 HR 1.32, 95% CI 1.03–1.70, *P* value 0.031). Per standard deviation increase in the GRS of LDL cholesterol the risk of ICH increased significantly (Model 1, age and sex adjusted HR 1.32, 95% CI 1.04–1.68, *P* value 0.024). No associations were found for GRS of HDL cholesterol and triglycerides with ICH (Model 1-3). GRS of LDL was associated with ICH even after excluding *APOE* alleles from the risk score (age and sex adjusted HR: 1.27, 95% CI 1.00–1.62, *P* value 0.053, HR additionally adjusted for serum LDL levels and lipid-lowering medication: 1.32, 95% CI 1.03–1.70, *P* value 0.029, HR additionally adjusted for cardiovascular risk factors: 1.32, 95% CI 1.03–1.70, *P* value 0.028).

The association between GRS of lipid fractions and cerebral microbleeds is presented in Table 3. Higher GRS of LDL cholesterol was borderline associated with a higher prevalence of multiple lobar microbleeds (OR 1.17, 95% CI 1.00–1.38, *P* value 0.054). GRS of lipid fractions were not associated with deep or infratentorial microbleeds. Additional adjustments for serum lipid fractions, lipid-lowering medication, and cardiovascular risk factors did not alter the results (data not shown). Overall, there was no indication of multicollinearity as the variance inflation factor was consistently below 2. The association between GRS of LDL and multiple lobar microbleeds disappeared after excluding *APOE* alleles from the GRS (age and sex adjusted OR 1.03, 95% CI 0.88–1.21, *P* value 0.720).

4. Discussion

In this population-based study, we found that genetic

Table 1
Baseline characteristics of the study population.

	Complete cohort N = 9011	Set with MRI N = 4179	P[a]
Age, years	65.8 (10.2)	64.2 (11.0)	<0.001
Women	5214 (57.9)	2286 (54.7)	0.001
Serum total cholesterol, mmol/L	5.7 (1.0)	5.6 (1.1)	<0.001
Serum LDL cholesterol, mmol/L	3.7 (0.9)	3.5 (0.9)	<0.001
Serum HDL cholesterol, mmol/L	1.4 (0.4)	1.4 (0.4)	<0.001
Serum triglycerides, mmol/L*	1.4 (1.0–1.8)	1.3 (1.0–1.8)	0.836
Use of lipid-lowering medication	1346 (14.9)	1020 (24.4)	<0.001
Systolic blood pressure, mmHg	140.1 (20.8)	139.2 (21.3)	0.053
Diastolic blood pressure, mmHg	78.5 (11.3)	82.3 (10.9)	0.008
Use of blood pressure-lowering medication	5956 (66.1)	1957 (46.8)	<0.001
Use of antithrombotic medication	4350 (48.5)	1229 (29.4)	<0.001
Smoking	6337 (70.3)	2944 (70.4)	0.886
Diabetes mellitus	906 (10.1)	352 (8.4)	0.003
Body mass index, kg/m^2	27.2 (4.1)	27.4 (4.1)	0.928

Values represent mean (standard deviation) or *median [interquartile range] for continuous variables, and number (percentage) for categorical variables.
[a] *P* value for the difference in baseline characteristics in the complete cohort and cohort with MRI.

Table 2
Genetic risk scores of lipid fractions and the risk of intracerebral hemorrhage.

Intracerebral hemorrhages							
Hazard ratios (95% confidence interval)							
Genetic risk scores	Events/number at risk	Model 1	*P*	Model 2	*P*	Model 3	*P*
Total cholesterol	67/9011	1.24 (0.98–1.58)	0.073	1.32 (1.03–1.69)	0.029	1.32 (1.03–1.70)	0.031
LDL cholesterol	67/9011	1.32 (1.04–1.68)	0.024	1.38 (1.08–1.77)	0.011	1.40 (1.09–1.79)	0.009
HDL cholesterol	67/9011	1.04 (0.82–1.32)	0.771	1.01 (0.79–1.30)	0.909	1.03 (0.80–1.32)	0.807
Triglycerides	67/9011	0.89 (0.70–1.13)	0.340	0.94 (0.73–1.20)	0.623	0.94 (0.73–1.20)	0.602

Values represent adjusted hazard ratios for intracerebral hemorrhages in relation to genetic risk scores of lipid fractions (increase per Z-scores).
Model 1: adjusted for age and sex.
Model 2: adjusted for age, sex, corresponding lipid fraction (serum total cholesterol for total cholesterol risk score, LDL for LDL risk score, serum HDL for HDL risk score, serum triglyceride for triglyceride risk score), and lipid-lowering medication.
Model 3: as model 2, additionally adjusted for blood pressures, ever smoking, diabetes mellitus, body-mass-index, blood-pressure lowering medication, and antithrombotic medication use.
Abbreviations: LDL = low-density lipoprotein, HDL = high-density lipoprotein.

Table 3
Genetic risk scores of lipid fractions and microbleeds by location and count.

Microbleeds									
Odds ratios (95% confidence interval)									
	Events/total population	Yes vs No	*P*	Events/total population	Single vs No	*P*	Events/total population	Multiple vs No	*P*
Genetic risk scores	**Strictly lobar microbleeds**								
Total cholesterol	539/3900	1.01 (0.92–1.11)	0.832	379/3740	0.96 (0.86–1.07)	0.463	160/3521	1.14 (0.97–1.34)	0.109
LDL cholesterol	539/3900	1.07 (0.97–1.17)	0.179	379/3740	1.02 (0.92–1.14)	0.667	160/3521	1.17 (1.00–1.38)	0.054
HDL cholesterol	539/3900	1.01 (0.92–1.11)	0.771	379/3740	1.00 (0.98–1.11)	0.927	160/3521	1.06 (0.90–1.25)	0.465
Triglycerides	539/3900	0.93 (0.85–1.02)	0.133	379/3740	0.91 (0.82–1.02)	0.098	160/3521	0.98 (0.84–1.15)	0.796
	Deep or infratentorial microbleeds								
Total cholesterol	279/3640	1.01 (0.89–1.15)	0.869	107/3468	0.92 (0.75–1.12)	0.387	172/3533	1.08 (0.92–1.27)	0.371
LDL cholesterol	279/3640	1.00 (0.88–1.14)	0.969	107/3468	0.87 (0.71–1.06)	0.869	172/3533	1.10 (0.94–1.30)	0.238
HDL cholesterol	279/3640	1.04 (0.92–1.18)	0.549	107/3468	1.15 (0.95–1.39)	0.153	172/3533	0.97 (0.83–1.14)	0.694
Triglycerides	279/3640	0.93 (0.82–1.06)	0.272	107/3468	0.88 (0.72–1.06)	0.179	172/3533	0.97 (0.83–1.14)	0.726

Values represent age and sex adjusted odd ratios for categories of single and multiple microbleeds in relation to genetic risk scores for lipid fractions (increase per Z-scores).
Abbreviations: LDL = low-density lipoprotein, HDL = high-density lipoprotein.

susceptibility for high total and LDL cholesterol associated with an increased risk of ICH. In addition, higher GRS of LDL cholesterol associated with a higher prevalence of multiple lobar microbleeds. Associations remained unchanged after adjusting for serum lipid concentrations, lipid-lowering medication, and cardiovascular risk.

Strengths of this study include the prospective population-based design aiding generalizability, the large number of participants, which enhances statistical power, and the virtually complete follow-up for ICH. Our findings have to be interpreted in light of some limitations. First, strokes were classified as unspecified in the absence of neuroimaging, thus some ICH cases may have been misclassified. In addition, the number of ICH cases in our study was small. Second, our study population comprised mostly persons of European ancestry. Third, the RS-I and RS-II subcohorts used in this study were part of the GWAS that identified the serum lipid genes, and accounted for 7.3% of the entire discovery cohort. Fourth, for 850 participants we adjusted for serum lipid levels that were measured in visits preceding brain MRI (Fig. 1). Yet, it should be noted that as we cannot date microbleed occurrence on baseline MRI scans, measuring lipid concentrations at the same time as

microbleed assessment does not necessarily lead to better adjustments of potential confounding.

In our study, a higher genetic load for high serum LDL was associated with an increased risk of ICH. Although our findings are in line with another observational study that investigated serum lipid levels and incident ICH [20], we were unable to corroborate findings from the majority of studies reporting inverse associations of total cholesterol [1–3,5,21,22], LDL cholesterol [2,3,5,21,22], and HDL cholesterol [3,5,22] with ICH. This includes findings from a meta-analysis of 23 prospective cohort and nested prospective case-control studies accounting for 7960 ICH cases (5.6% of the total meta-analysis study population) [3], which reported that lower serum total and LDL cholesterol related to an increased risk of ICH. No associations were found for HDL cholesterol or triglycerides. The mechanism by which low serum lipids would influence the pathogenesis of ICH is unclear but it has been suggested that low lipid levels negatively affect the preservation of vessel wall integrity [23–25]. Low total cholesterol levels may cause smooth muscle cells to degenerate, which weakens the endothelial layer of intracerebral vessel walls. This causes vascular hyperpermeability and precipitates the extravasation of erythrocytes [23–25]. On the other hand, low total cholesterol may also be a secondary phenomenon in ICH patients with hypertension and excessive alcohol usage [4].

Our study provides no evidence for a genetic basis for the inverse association of lipid fractions and incident ICH. The disparity between our findings and that of previous studies may partly be explained by the fact that serum lipid fractions were typically measured only once, whereas lipid genes provide a more reliable lifetime exposure risk to elevated serum lipids levels. Also, individuals at highest risk of ICH may have had more aggressive risk factor management (i.e., lipid-lowering medication use, low-fat diet, increased physical activity). Insufficient adjustment for these factors in data analysis may have led to confounding in studies investigating serum lipid levels and ICH. Additionally, residual confounding due to unmeasured factors may also have influenced their findings to some extent. Our results provide more robust associations as genes are not susceptible to changes in lifestyle or environment. Finally, most studies investigating serum lipid levels did not control for competing risk of ischemic stroke, coronary heart disease or mortality. Thus, in these studies, less people with elevated serum lipid levels may have been at risk for ICH due to prior occurrence of other cardiovascular events or death.

The associations between GRS of lipid fraction and multiple strictly lobar microbleeds followed the same trend as that of intracerebral hemorrhages, suggesting that these types of intracranial bleedings may share common pathophysiological pathways.

If we presume multiple lobar microbleeds to be precursors of ICH [26,27], our findings suggest that high genetic load for high serum LDL cholesterol facilitates the progression of cerebral vasculopathy. This progression may particularly be mediated by the presence of *APOE* risk alleles, as we only found an association between GRS of LDL and multiple lobar microbleeds once we included *APOE* alleles in the GRS. After excluding *APOE* alleles, we found no reason to assume that genetic susceptibility for high serum lipids differs across cerebral vasculopathies, as results were similar for microbleeds in regions typically affected by cerebral amyloid angiopathy (lobar regions) and for microbleeds in regions characteristically affected by hypertensive arteriopathy (deep or infratentorial regions).

In conclusion, in a large population-based cohort we found that higher genetic load for high serum total and LDL cholesterol increases the risk of ICH and was borderline significantly associated with a higher prevalence of multiple lobar cerebral microbleeds. We found no genetic support for the paradoxical inverse association of serum lipid fractions with ICH and microbleeds as reported previously by our and other observational studies. Our current findings suggest that high LDL cholesterol is amongst the modifiable risk factors for ICH. However, since these findings are in contrast with the majority of the previous published studies, these observations could be considered hypothetical and need confirmation in studies with larger sample size and repeated LDL cholesterol measurements.

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Disclosures

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.atherosclerosis.2016.01.024.

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