# Longitudinal Serum Lipid Trends and APOE in Primary Intracerebral Hemorrhage

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</tr>
</tbody>
</table>
# TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>Name</th>
<th>Page Number</th>
</tr>
</thead>
<tbody>
<tr>
<td>Project Summary</td>
<td>3 - 5</td>
</tr>
<tr>
<td>Manuscript 1</td>
<td>6 - 37</td>
</tr>
<tr>
<td>Manuscript 2</td>
<td>38 - 64</td>
</tr>
</tbody>
</table>
PROJECT SUMMARY

Primary intracerebral hemorrhage (ICH) comprises 10-15% of all strokes in the Western population, with annual incidence of 20-30 per 100,000 persons in the US. However, ICH accounts for nearly 50% of all stroke-related mortality and 30% of stroke-related costs due to its clinical severity. The economic repercussions are significant, with estimates of over $20 billion in lifetime care costs for 70,000 annual cases in the US alone. This burden is only expected to grow in step with increases in life expectancy as incidence rates of ICH increase dramatically with age. Although several genetic and environmental risk factors have been implicated in the occurrence of ICH, including ε2 and ε4 alleles of the APOE gene, hypertension and dyslipidemia, the underlying ICH pathophysiology and biological mechanisms linking the roles of APOE and associated environmental risk factors remain unclear.

While hypercholesterolemia is known to increase risk of cardiac disease and ischemic stroke, there has increasingly been a preponderance of evidence suggesting an inverse relationship with risk of ICH. Multiple observational and randomized-controlled studies have reported an association between hypercholesterolemia and reduced ICH risk, and improvements in ICH-related outcomes. However, it remains indeterminate whether the relationship is causal or associative, and whether individual lipid fractions have independent contributions towards ICH risk. Prior studies examining association between serum lipid levels and ICH risk have mostly relied on single-point measurements pre- or post-ICH, with the majority utilizing measurements obtained at time of ICH. These measurements are unlikely representative of long-term serum lipid levels due to large measurement inconsistencies from variable sampling time, and confounding by medical, environmental, and dietary exposures. In particular, serum cholesterol levels have been reported to decline precipitously within days after both acute ischemic or hemorrhagic strokes, with levels subsequently rising above baseline (drawn at time of stroke) by 90 days post-event. This observation is not unique to cerebrovascular disease. The trend of declining serum lipid levels around time of acute illnesses was similarly seen in post-surgical patients, those hospitalized for non-cerebral diseases including sepsis and acute myocardial infarction, and patients in severe pain.

In order to address the limitations of inter- and intra-individual serum lipid variability, we proposed to study the relationship between cholesterol and risk of ICH using serial serum lipid measurements and focusing on patterns of change in serum lipid levels over time in a case-control cohort. The central hypothesis of the study is that ICH patients will show distinct patterns of serum lipid changes, with decline in serum levels preceding the occurrence of ICH if lipids were in the causal pathway for ICH risk. We tested this hypothesis by (1) comparing temporal serum lipid changes in ICH cases versus controls comprising of acutely ill patients hospitalized for non-cerebral illnesses using mixed-effects modeling to allow evaluation of the impact of random ‘critical events’ on time intervals, enabling dissection of changes in serum lipid trends over distinct, biologically relevant time periods. We also attempted to clarify the contributions of different circulating lipid fractions towards ICH risk. In addition, given known APOE pleiotropy in lipoprotein metabolism and ICH risk, we attempted to determine whether APOE allelic status might influence temporal serum lipid trends in ICH. Notably, the presence of a subset of ICH patients with known APOE genotype status represented a unique opportunity to disentangle lipid-dependent and lipid-independent mechanisms of APOE towards development of ICH. We hypothesized that APOE genotype may influence temporal serum lipid changes in ICH and tested this hypothesis by (2) investigating APOE allele-specific effects on changes in serum lipid levels over time in a cohort of ICH patients with known APOE genotype and serial serum lipid measurements.
We identified a unique phenomenon of accelerated decline in serum total cholesterol and low-density lipoprotein levels 6 months prior to ICH, independent of measured environmental confounders. We also observed that this phenomenon is predicted by APOE epsilon allele status, providing evidence for lipid-dependent associations of APOE in ICH risk, beyond their effect on amyloid processing.

Our findings are presented in detail in the 2 manuscripts attached:

Our results further corroborate the relationship between serum lipids and ICH risk, and provide additional insight as to timing of serum lipid measurements and the nature of serum lipid levels as a biomarker, both of which are of relevance for future clinical trial designs. Further, the results provide novel insight regarding non-amyloid *APOE* mechanisms in primary ICH and have implications for ongoing efforts in dissecting the role of dyslipidemia in cerebrovascular disease risk and.

REFERENCES

Subacute Decline in Serum Lipids Precedes the Occurrence of Primary Intracerebral Hemorrhage

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Subacute Decline in Serum Lipids Precedes the Occurrence of Primary Intracerebral Hemorrhage

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AUTHOR CONTRIBUTIONS

Dr. C-L. Phuah participated in study design, data acquisition, statistical analyses, drafting and revision of the manuscript. M.R. Raffeld and A.M. Ayres was responsible for data collection. Drs. A. Viswanathan, S.M. Greenberg and Dr. J. Rosand participated in final editing of manuscript. Dr. C.D. Anderson participated in study design and revision of manuscript.

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DISCLOSURE

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ABSTRACT

Objective: We aimed to describe the temporal variation in circulating lipid levels among patients with intracerebral hemorrhage (ICH) and investigate their association with ICH risk.

Methods: This was a single-center retrospective longitudinal case-control analysis using cases drawn from an ongoing cohort study of primary ICH and controls drawn from a hospital-based clinical data registry. Piecewise linear mixed-effect random-coefficient models were used to determine the significance of changes in serum lipid trends on ICH risk.

Results: 212 ICH cases and 301 control individuals were analysed. Overall trends in serum total cholesterol (TC) and low-density lipoprotein (LDL) levels differed between ICH cases and non-ICH controls (p=0.00001 and p=0.0092, respectively). ICH patients experience accelerated decline in serum TC and LDL levels during 6 months immediately preceding ICH, compared with levels between 6 to 24 months pre-ICH (TC: -29.25 mg/dL, p=0.001; LDL: -21.48 mg/dL, p=0.0038), which was not observed in non-ICH controls. Subgroup analysis confirmed that this phenomenon cannot be attributed to statin or alcohol exposure. Serum triglycerides (TG) and high-density lipoprotein (HDL) trends did not differ between groups.

Conclusions: Longitudinal lipid levels differ between ICH cases and non-ICH controls, most notably for a decline in serum TC and LDL levels within 6 months preceding primary ICH, independent of statin or alcohol use. These changes in serum TC and LDL trends suggest a biological pathway that precipitates ICH occurrence. Further studies are needed to replicate these results and characterize rate of change in serum lipids as a potential biomarker of impending acute cerebral injury.
INTRODUCTION

Growing evidence supports a paradoxical role of dyslipidemia in cerebrovascular disease\textsuperscript{1-6}. Antithetical to its role in ischemic stroke, hypercholesterolemia has been associated with decreased primary intracerebral hemorrhage (ICH) risk\textsuperscript{7-10}, reduced number of cerebral microhemorrhages\textsuperscript{11}, reduced hemorrhagic conversion after ischemic strokes\textsuperscript{12}, and improved ICH outcomes\textsuperscript{13,14}. However, the relationship between serum lipid levels drawn at the time of ICH to individual patients’ long-term lipid levels is unclear, and likely not representative of long-term exposures\textsuperscript{8}. Estimates of variation coefficients for serum lipid concentrations range from 5-25\%\textsuperscript{15}, reflective of considerable variation due to biological and environmental factors\textsuperscript{15-19}. Serum lipid levels also undergo considerable variation during acute illness\textsuperscript{20-22}, hypothesized to be due to catecholamine stress response\textsuperscript{21,23}. As example, serum cholesterol levels decline precipitously within days after both acute ischemic or hemorrhagic strokes\textsuperscript{8,23,24}, then subsequently rising above those drawn at time of stroke by 90 days post-event\textsuperscript{8}. Given these inter- and intra-individual variabilities, understanding of temporal serum lipid trends in ICH may improve our understanding of the biology of dyslipidemia in ICH, and provide better estimates of long-term lipid exposures towards cerebrovascular disease risk.

Our objectives were (1) to describe temporal trends in serum total cholesterol (TC), low-density lipoprotein (LDL), triglycerides (TG) and high-density lipoprotein (HDL) over 48 months, before and after ICH, (2) to determine differences in serum lipid trends between ICH patients and non-ICH controls, and (3) to investigate whether changes in serum lipid trends are associated with increased ICH risk.

METHODS

Study design
We conducted a single-center retrospective longitudinal study utilizing case-control design comparing ICH patients with patients who were hospitalized for acute non-cerebral illnesses as controls (Figure 1). Serum lipid values (TC, TG, LDL and HDL) for included participants were extracted from review of hospital electronic medical records (EMR) for all available measurements falling within the interval two years before or after the acute event. In order to make comparisons between the variation in serum lipid trends before and after a random event (hospitalization for acute non-cerebral illness versus ICH), individual hospitalization events were time-locked into a balanced design with time periods before and after the acute hospitalization event subdivided into 6-monthly time intervals. Serum lipid values were segregated into their respective time intervals based on timing of the measurement in relation to the date of acute hospitalization. Means were obtained for repeated serum lipid measurements within the same time interval.

*Patient selection*

Cases: Participants (n=212) were drawn from a previously described, ongoing longitudinal cohort study of primary ICH. Briefly, study participants were patients aged ≥ 18 years presenting to the Massachusetts General Hospital (MGH) emergency department between June 1993 and June 2014 with imaging confirmed diagnosis of primary ICH. Exclusion criteria included presence of trauma, brain tumor, hemorrhagic transformation of cerebral infarction, vascular malformation, or any other cause of secondary ICH. Eligibility for participation include survival at 2 years after ICH and at least three serum lipid values for each lipid fraction of interest (TC, TG, LDL, HDL) drawn >6 months apart within 2 years before and after the date of diagnosis. Participants with recurrent ICH or other non-ICH hospitalization events during the time period of interest were excluded in order to minimize confounding from decreases in
serum lipids as part of an acute phase response\textsuperscript{8,20-24}. Clinical data recorded include information on demographics, medical history, pre-ICH medication use, and health maintenance (cigarette smoking and alcohol use). Statin use for the pre-ICH period was determined at time of ICH presentation via patient-based questionnaires and scored as ‘yes/no’ variables with constancy assumed for the duration of the study period. The validity and reliability of the questionnaire-based statin use response were internally assessed by comparisons with detailed medication history obtained from hospital EMR search. A 10% random sampling of the enrolled cohort showed 100% concordance for no statin use.

Controls: Controls (n=301) were patients aged \( \geq 18 \) years admitted to MGH within the same time period, selected via database query from a MGH-affiliated clinical data registry (RPDR)\textsuperscript{26}. Control selection utilized similar eligibility criteria with an additional criterion of including only patients with inpatient encounters of <30 days. Patients with any cerebral-related encounter diagnoses and elective admissions were excluded. Clinical data containing covariates of interest identical to cases were concurrently extracted from EMR and analyzed similarly.

\textit{Standard protocol approvals, registrations, and patient consents.}

This study was performed with approval of the MGH Institutional Review Board (IRB). Data request from RPDR containing individual-level information was obtained within the confines of the approved IRB protocol. ICH patients provided informed consent for study participation.

\textit{Statistical methods}

Continuous numeric variables were expressed as mean \( \pm \) SD. Categorical variables were compared using Fisher exact test and continuous variables using Mann-Whitney rank-sum or unpaired \( t \) test as appropriate. We used piecewise linear mixed-effects (PLME) random-coefficient model to test the significance of changes in serum lipid trends on ICH risk using
acute illness as a critical period on temporal patterns of individual serum lipid fractions at specific time intervals which are fixed in relation to the critical period\textsuperscript{27}. This allowed modelling of rates of change in mean serum lipid levels in pre-determined time intervals of interest to differ within and between cases and controls (Supplementary Methods). With the assumption that the time interval immediately prior to acute illness is likely of most biological relevance, fixed-knots were used to mark transitions in time periods of interest corresponding to the time period 6-24 months pre-acute illness and the time period immediately prior to the acute event (0-6 months pre-acute illness) (Figure e-1). All covariates where $p<0.10$ in univariate comparisons, or with known potential to influence serum lipid levels were entered into the model. Case-control status, and covariates including demographics, clinical, and medication history were included as fixed-effects. Inter-individual and intra-individual variation in serum lipid levels were modelled as random-effects. Model validity were examined using likelihood-ratio test. Unstructured covariance was used as the covariance structure model. Comparisons of the difference in rate of change in serum lipid levels (slope) between time intervals of interest in cases and controls were made using Wald test. Significance threshold was set at $p<0.05$ (2-tailed) for univariate analysis, and $p<0.0125$ (Bonferroni-correction for 4 tests) for individual serum lipid fraction mixed-model analyses. All statistical analyses were performed using STATA 10.0 (StataCorp LP, USA).

RESULTS

Cohort Characteristics

A total of 212 ICH cases and 301 controls with non-cerebral acute illnesses were available for analysis (Figure 1). 351 ICH individuals were removed due to absence of serum lipid data. There were no significant differences in demographics, pre-ICH medical history or medication
use between included and excluded ICH individuals to suggest significant bias from study selection criteria (Table e-1). There were significant differences for age, sex, race, smoking status, diabetes and hypertension between ICH patients and controls. Risk of coagulopathy represented by rates of aspirin and warfarin use were similar in both groups (Table 1). Among cases, ICH location was lobar in 47.6%, with the remainder located in the deep hemispheres and brainstem. Measures of ICH severity were available in 190 ICH patients. There was a preponderence of mild-moderate ICH cases with admission ICH volume of <30cc (77.9%), and Glasgow Coma Scale of >8 (81.6%). Statin exposure pre-ICH did not differ between ICH cases and controls, as well as across ICH subgroups (p=0.97).

Temporal trends in serum lipid levels in ICH and acute non-cerebral illness

The number of serum lipid measurements per participant were similar for each time interval in the pre-acute illness period between ICH cases and controls. Conversely, the number of serum lipid measurements were unbalanced in the post-acute illness period, with 25-50% fewer measurements per individual in ICH patients compared with controls (Table e-2). Temporal trends in the mean serum lipid levels between participants hospitalized for ICH and controls hospitalized for acute non-cerebral illnesses were assessed visually using Loess-smoothed curves (Figure 2) fitted from scatterplots of serum lipid variation over time (Figure e-2). Serum TC and LDL trends over the 24-month period pre-acute illness differ between ICH cases and controls (TC: p=0.0003; LDL: p=0.0029). In particular, ICH patients experienced overall declines in mean serum TC and LDL levels in the 24 months preceding ICH occurrence, with notable acceleration in rates of decline of both lipid fractions beginning 6 months prior to ICH event. Following ICH, serum TC and LDL trends became flat with levels remaining depressed over 24 months post-ICH. In contrast, non-ICH controls demonstrated less
variability in mean serum TC and LDL levels, which remained largely stable throughout the 4-year period. There were no significant changes in serum TG and HDL trends for all participants regardless of their acute illness diagnoses within the time period examined.

Change in serum lipid trends in the final 6 months prior to acute illness and association with ICH risk

Estimates of rates of change in serum lipid levels in the immediate 0-6 month interval pre-acute illness compared with the antecedent interval (6-24 months pre-acute illness) in ICH cases and controls with acute non-cerebral illness are presented in Table 2A. The rates of decline of mean serum TC and LDL levels in the 6 months immediately preceding ICH increased compared with trends in the antecedent 18-month period (TC: -4.89 mg/dL/month, \( p=0.001 \); LDL: -3.58 mg/dL/month, \( p=0.0038 \)). In contrast, non-ICH controls experienced negligible change in serum TC and LDL trends within the same time period immediately preceding acute illness (TC: -0.72 mg/dL/month, \( p=0.41 \); LDL: -0.26 mg/dL/month, \( p=0.85 \)). Serum TG and HDL trends remained unchanged during 0-6 months immediately preceding acute illness for both ICH and non-ICH participants.

Serum lipid trends pre-ICH are unrelated to environmental exposures

The observed changes in serum TC and LDL trends in the 6 months immediately preceding ICH remained significant even with the inclusion of variables known to affect serum lipids (Table 2A and Table e-3). Subgroup analysis of ICH patients stratified by statin-use pre-ICH demonstrated accelerated declines in serum TC and LDL levels in the 6-month interval immediately preceding ICH only in those who were statin-naïve (\( p=0.0001 \) and \( p=0.0002 \), respectively) (Figure 3 and Table 2B). In contrast, ICH patients who were on statin medication showed stable serum TC and LDL trends, with gradual decline in serum levels of both lipid
fractions over 48 months consistent with known statin effects, and did not experience a more
dramatic drop in levels prior to the ICH. Similar analysis of ICH patients by cigarette smoking
and alcohol use also demonstrated no significant change in serum lipid trends in the 6-month
time period immediately prior to ICH.

DISCUSSION

We identified a novel behavior in serum lipid trends in primary ICH in which serum TC and LDL
levels decline precipitously within a 6-month time interval immediately preceding the
occurrence of primary ICH. This observation was distinct for ICH as similar changes in serum
lipid trends were not demonstrated in the same time period prior to non-cerebral acute
illnesses which served as controls. Furthermore, the change in serum TC and LDL trends
preceding primary ICH is independent of measured environmental exposures known to
influence serum lipid variability, and raises the question of whether a systemic process may
contribute towards the occurrence of ICH.

Rapid declines in serum lipid levels have been observed in the context of acute illnesses\textsuperscript{20-24}
and are thought to be associated with active inflammation\textsuperscript{28-29}. Previous studies of pre-stroke
serum lipids association with ICH have been inconclusive\textsuperscript{30-32}, being dependent on serum lipid
concentrations measured at a single time-points. Given our observations of a temporal
association, we speculate that the decline in serum lipids may represent an increase in
systemic inflammatory response preceding the occurrence of ICH.

There are several strengths to our study. First, we constructed a unique dataset of longitudinal
lipid data to describe temporal lipid patterns before ICH. The relatively large sample size of the
cohort and the inclusion of serial serum lipid measurements both prior to and after ICH in the
present cohort allows for more accurate representation of longitudinal lipid trends in ICH in
comparison with previous studies which lack data on pre-stroke serum lipid measurements\textsuperscript{8}.

Second, our results derived from biannual serum lipid measurements over 4 years are focused on understanding the broader temporal variation in lipid trends in ICH which potentially improves biological relevance in lipids-disease risk associations, in contrast with short-term lipid variance which may be more unstable\textsuperscript{8,30-32}. Third, the use of piecewise mixed-effects model in our analysis allowed for investigation of individual, distinct, biologically-relevant time periods\textsuperscript{27}, as separate slopes can be fitted to observations representing periods before and after the time period of interest. Also, the broad inclusion criteria for inpatient diagnoses (Table e-4) in control participants attempted to best reflect the mixed biology of acute illnesses which are known to influence serum lipid variability\textsuperscript{20-24}. Acute illnesses including myocardial infarction\textsuperscript{21} and ischemic strokes\textsuperscript{23,24}, amongst others\textsuperscript{20,22} have all been associated with rapid and large declines in serum lipids immediately following the event. Hence, the selection of individuals who are severely ill including individuals with cardiac arrest or required cardiac surgeries, mainly due to acute myocardial infarction in our cohort, have the additional benefit of building an inherent bias towards the null hypothesis of no difference in serum lipid trends between ICH cases and controls, and thus allow for robust comparisons of ICH-specific effects on changes in serum lipid trends.

Our analysis has important limitations. As our case cohort included only patients with ICH, we cannot speculate that these effects are unique to ICH. Declining serum lipid trends have been observed following acute cerebrovascular diseases including transient ischemic attacks (TIAs) and ischemic strokes\textsuperscript{33}, similar to prior observation after ICH\textsuperscript{8}. It remains a possibility that this phenomenon of declining serum lipids heralding ICH may instead reflect more general processes influencing the occurrence of acute cerebral illnesses which may be addressed by
further studies. However, the inclusion of control individuals with increased likelihood of ischemic cerebral insults such as patients with cardiac arrest or who underwent cardiac surgeries for acute myocardial infarction failed to demonstrate the phenomenon of declining serum lipids preceding the acute event in the control group, which would be expected of a non ICH-specific phenomena. Second, causal inference is limited by the retrospective design. A similar study in a prospective cohort will be ideal but extremely challenging and unfeasible in practice given low ICH event rates (17.0 per 100,000 persons/year)\(^3\). Third, in our case-control comparison of lipid trends, serum lipid measurements during the post-acute illness period were unbalanced between case-control cohorts, which increases the risk of asymptotic bias in estimates of serum lipid trends during that same period. This may account for the discrepancy in our observation of depressed serum TC and LDL levels up to 2 years post-discharge in ICH cases compared with prior report of serum TC elevations by 90 days post-ICH\(^8\). However, we attempted to minimize such biases using complete case analysis for multivariable comparisons in order to preserve comparability at the expense of reduction in statistical power. Furthermore, serum lipid measurements during the pre-acute illness period related to our primary finding were balanced across both case-control cohorts and amongst individual time intervals. Fourth, our case-control cohorts were similarly unbalanced for the majority of clinical characteristics due to prioritization in matching by statin-use status. However, we adjusted for these covariates as fixed-effects in or final multivariate model to account for the differences. Fifth, selection biases are present both from subject-specific indications for serial lipid measurements and inclusion of predominantly small to moderate sized ICH cases which were necessitated by the study question and design. Hence, our findings of decline in serum TC and LDL preceding ICH occurrence cannot be generalized
towards all primary ICH patients although the likelihood of significant differences in dysplipidemia in ICH pathophysiology by ICH size or severity would seem less biologically plausible. Future validation and confirmation of these results in larger, prospective cohorts will be needed to surmount these sampling biases and current limitations in relevance towards predominantly mild-moderately severe ICH.

We attempted to address confounding of serum lipid levels by including variables known to influence serum lipid variability. Contrary to known effects of statin on lowering serum TC and LDL levels, subgroup analysis of ICH cases by statin-use status demonstrated that serum lipid declines preceding ICH were unrelated to statin exposure. Only individuals who were statin-naïve experienced a more pronounced decline in trends of both TC and LDL. Due to limitations in information collected through the study protocol, we could not account for the additional effects of statin dose or non-statin lipid lowering agents. However, as the latter are typically less potent than statin in their lipid-lowering effects, they are unlikely to behave as major confounders in the absence of statin effects on serum lipid declines. Due to the broad range of environmental exposures that can influence serum lipid variations, we were unable to exhaustively address additional potential confounders. We did not have nutritional data for our cohorts but severe acute malnutrition would not be expected based on our selection criteria and we would also expect serum TG to be affected by dietary changes\textsuperscript{15}. Fluctuations in menstrual cycles, pregnancy, menopause and seasonal variations, which can influence serum lipid levels, are unlikely to play a predominant role in serum lipid trends observed given the elderly age of our cohort, while the long study duration of 48 months would be expected to limit the effect of seasonal variations in serum lipid levels.
Our results have implications for ongoing efforts in dissecting the role of dyslipidemia in cerebrovascular disease risk. Our finding of significant declines in serum lipid levels within 6 months preceding primary ICH suggests an association between accelerations in serum lipid decline and ICH risk, and that temporal lipid trends may augur a generalized process that precipitates ICH. Our observations also suggest that absolute levels of serum lipid measurements may not be biologically relevant for stroke risk associations. This may be of relevance in informing designs of future prospective clinical trials for lipid biomarkers or lipid-lowering agents in stroke. Given our study limitations, these findings should be considered hypothesis-generating at present and future studies are needed to replicate these results in prospective cohorts, probe for potential mediation effects between TC and LDL, and further characterize changes in serum lipid trends as a potential biomarker of impending acute cerebral injury.

REFERENCES


FIGURES

Figure 1. Flowchart describing study cohorts and analysis plan

Abbreviations: MGH ICH Database, Massachusetts General Hospital-based patients in the Genetics of Cerebral Hemorrhage on Anticoagulation Study; RPDR, MGH Research Patient Data Registry; ICH, intracerebral hemorrhage.

Table 1. Cohort characteristics and univariate analyses

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<th>Controls (n=301)</th>
<th>ICH (n=212)</th>
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<td>Age, y (mean +/- SD)</td>
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<td>Females, n (%)</td>
<td>88 (29.2)</td>
<td>95 (44.8)</td>
<td>&lt;0.0001&lt;sup&gt;b&lt;/sup&gt;</td>
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<td>White, n (%)</td>
<td>301 (100.0)</td>
<td>186 (87.7)</td>
<td>&lt;0.0001&lt;sup&gt;b&lt;/sup&gt;</td>
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<td>HTN, n (%)</td>
<td>114 (37.9)</td>
<td>178 (84.0)</td>
<td>&lt;0.0001&lt;sup&gt;b&lt;/sup&gt;</td>
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<td>DM, n (%)</td>
<td>60 (19.9)</td>
<td>49 (35.8)</td>
<td>0.001&lt;sup&gt;b&lt;/sup&gt;</td>
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<td>Alcohol Use, n (%)</td>
<td>177 (58.8)</td>
<td>93 (43.9)</td>
<td>0.47&lt;sup&gt;b&lt;/sup&gt;</td>
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<td>Smokers, n (%)</td>
<td>163 (54.2)</td>
<td>25 (14.3)</td>
<td>&lt;0.0001&lt;sup&gt;b&lt;/sup&gt;</td>
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<td>Statin Use, n (%)</td>
<td>153 (50.8)</td>
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<td>0.11&lt;sup&gt;b&lt;/sup&gt;</td>
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<td>ASA Use, n (%)</td>
<td>167 (55.5)</td>
<td>106 (50.0)</td>
<td>0.22&lt;sup&gt;b&lt;/sup&gt;</td>
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<td>Warfarin Use, n (%)</td>
<td>71 (23.6)</td>
<td>58 (27.4)</td>
<td>0.33&lt;sup&gt;b&lt;/sup&gt;</td>
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Abbreviations: HTN, hypertension; DM, diabetes mellitus; ASA, aspirin. Statistical significance at p<0.05, <sup>a</sup> t test, <sup>b</sup> Fisher exact test.
Figure 2. Serum lipid trends in ICH cases and non-ICH controls

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<th>Serum Lipids</th>
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<th>Non-ICH</th>
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<td>Wald Test</td>
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<td>TC</td>
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<td>LDL</td>
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<td>TG</td>
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<td>HDL</td>
<td>6.05(1)</td>
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**A-D** Loess smoothed curves of serum lipid levels (mg/dL) against time (in months), for ICH cases and patients who were hospitalized for acute non-cerebral illnesses (controls). Light grey areas indicate standard error (SE) for controls. Dark grey areas indicate standard error (SE) for ICH cases. **E** Comparison of difference in rates of change of serum lipid levels (slope) between time interval 6-24 months pre-acute illness and 0-6 months pre-acute illness in ICH and non-ICH controls. Test statistic, Wald test; degree of freedom in parentheses; **TC**, total cholesterol; **LDL**, low-density lipoprotein; **TG**, triglyceride; **HDL**, high-density lipoprotein; **P**, time interval 0-6 months prior to acute illness; statistical significance at $p<0.0125$ (Bonferonni-corrected).

**Table 2. Estimated rates of change in serum lipid levels prior to acute illness**

<table>
<thead>
<tr>
<th></th>
<th>Time (months)</th>
<th>TC</th>
<th>LDL</th>
<th>TG</th>
<th>HDL</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>A</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ICH</td>
<td>6 - 24</td>
<td>-0.19*</td>
<td>-0.07*</td>
<td>-0.12</td>
<td>-0.02</td>
</tr>
<tr>
<td></td>
<td>0 - 6</td>
<td>-4.89*</td>
<td>-3.58*</td>
<td>-3.84</td>
<td>-1.03</td>
</tr>
<tr>
<td>Non-ICH</td>
<td>6 - 24</td>
<td>-0.02</td>
<td>-0.04</td>
<td>-0.14</td>
<td>+0.01</td>
</tr>
<tr>
<td></td>
<td>0 - 6</td>
<td>-0.72</td>
<td>-0.26</td>
<td>+0.43</td>
<td>-0.19</td>
</tr>
<tr>
<td>Statin</td>
<td>6 - 24</td>
<td>-0.34</td>
<td>-0.23</td>
<td>-0.28</td>
<td>+0.07</td>
</tr>
<tr>
<td></td>
<td>0 - 6</td>
<td>-1.78</td>
<td>-1.12</td>
<td>-3.29</td>
<td>-0.64</td>
</tr>
<tr>
<td>No Statin</td>
<td>6 - 24</td>
<td>+0.20*</td>
<td>+0.28*</td>
<td>+0.22</td>
<td>+0.25*</td>
</tr>
<tr>
<td></td>
<td>0 - 6</td>
<td>-4.14*</td>
<td>-3.53*</td>
<td>-2.87</td>
<td>-1.17*</td>
</tr>
</tbody>
</table>

Comparisons of rates of change in serum lipid levels (in mg/dL/month) prior to acute illness event by time periods 6-24 months and 0-6 months pre-event using covariate-adjusted PLME model in **A** ICH cases (ICH) versus controls with non-cerebral acute illnesses (Non-ICH) and **B** ICH cases on statin (Statin) versus ICH cases not on statin (No Statin). **TC**, total cholesterol; **LDL**, low-density lipoprotein; **TG**, triglyceride; **HDL**, high-density lipoprotein; *
Statistical significance by Wald test for comparisons between time periods, \( p < 0.0125 \) (Bonferroni-corrected).

**Figure 3. Serum lipid trends by statin use in ICH patients**

**A** Total Cholesterol

**B** Low-density Lipoprotein

**C** Triglyceride

**D** High-density Lipoprotein

A-D Loess smoothed curves of serum lipid levels (mg/dL) against time (in months), for comparison between ICH cases that were either on statin medication or statin-naive. Dark grey areas indicate standard error (SE) for statin-naïve ICH cases. Light grey areas indicate standard error (SE) for ICH cases on statin.
SUPPLEMENTARY DATA

Table of Contents:
Page 2 – Table e-1: Characteristics of ICH cohort excluded by selection criteria
Page 3 – Table e-2: Number of serum lipid level measurements available at each time interval
Page 4 – Table e-3: Estimates for covariate-adjusted PLME model of serum lipid levels (mg/dL) and covariate-adjusted serum lipid trends (mg/dL/month) in ICH cases and non-ICH controls
Page 6 – Table e-4: Inpatient diagnoses of control participants with non-cerebral acute illness
Page 7 – Supplementary Methods
Page 8 – Figure e-1: Illustration of piecewise linear function with knots at -6 and 0 for modeling of nonlinear curve (in red) representing change in serum lipid levels over time
Page 9 – Figure e-2: Scatterplot of serum lipids (mg/dL) against time (months) relative to date of acute hospitalization for non-ICH controls (blue open circle) and ICH cases (red triangles)
Comparison of baseline clinical characteristics between excluded vs. included ICH individuals by study selection criteria. Abbreviations: **HTN**, hypertension; **DM**, diabetes mellitus; **ASA**, aspirin. Statistical significance at $p<0.05$, $^a$ t test, $^b$ Fisher exact test.

### Table e-1. Characteristics of ICH cohort excluded by selection criteria

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Excluded ICH Cohort (n=694)</th>
<th>$P$ Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, y (mean +/- SD)</td>
<td>74.3 +/- 11.6</td>
<td>0.35$^a$</td>
</tr>
<tr>
<td>Females, n (%)</td>
<td>349 (50.3)</td>
<td>0.18$^b$</td>
</tr>
<tr>
<td>White, n (%)</td>
<td>629 (90.6)</td>
<td>0.38$^b$</td>
</tr>
<tr>
<td>HTN, n (%)</td>
<td>575 (84.5)</td>
<td>0.9$^b$</td>
</tr>
<tr>
<td>DM, n (%)</td>
<td>179 (27.5)</td>
<td>0.26$^b$</td>
</tr>
<tr>
<td>Alcohol Use, n (%)</td>
<td>282 (48.7)</td>
<td>0.19$^b$</td>
</tr>
<tr>
<td>Smokers, n (%)</td>
<td>56 (11.7)</td>
<td>0.46$^b$</td>
</tr>
<tr>
<td>Statin Use, n (%)</td>
<td>269 (41.4)</td>
<td>0.72$^b$</td>
</tr>
<tr>
<td>Lobar ICH, n (%)</td>
<td>321 (46.2)</td>
<td>0.86$^b$</td>
</tr>
<tr>
<td>ASA Use, n (%)</td>
<td>341 (49.4)</td>
<td>0.93$^b$</td>
</tr>
<tr>
<td>Warfarin Use, n (%)</td>
<td>204 (32.9)</td>
<td>0.15$^b$</td>
</tr>
</tbody>
</table>
Table e-2. Number of serum lipid measurements available at each time interval

<table>
<thead>
<tr>
<th>Serum Lipids</th>
<th>Time Interval</th>
<th>Pre-Event</th>
<th>Post-Event</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>TC</td>
<td></td>
<td>0.42</td>
<td>0.45</td>
</tr>
<tr>
<td>TG</td>
<td></td>
<td>0.39</td>
<td>0.39</td>
</tr>
<tr>
<td>LDL</td>
<td></td>
<td>0.36</td>
<td>0.38</td>
</tr>
<tr>
<td>HDL</td>
<td></td>
<td>0.40</td>
<td>0.39</td>
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</table>

Non-cerebral Acute Illnesses

<table>
<thead>
<tr>
<th>Serum Lipids</th>
<th>Time Interval</th>
<th>Pre-Event</th>
<th>Post-Event</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>TC</td>
<td></td>
<td>0.43</td>
<td>0.40</td>
</tr>
<tr>
<td>TG</td>
<td></td>
<td>0.38</td>
<td>0.38</td>
</tr>
<tr>
<td>LDL</td>
<td></td>
<td>0.36</td>
<td>0.36</td>
</tr>
<tr>
<td>HDL</td>
<td></td>
<td>0.39</td>
<td>0.37</td>
</tr>
</tbody>
</table>

Comparisons of the proportion of serum lipid measurements available in each 6-month time interval for ICH cases and non-ICH controls. **TC**, total cholesterol; **TG**, triglycerides; **LDL**, low-density lipoprotein; **HDL**, high-density lipoprotein.
Table e-3. Estimates for covariate-adjusted PLME model of serum lipid levels (mg/dL) and covariate-adjusted serum lipid trends (mg/dL/month) in ICH cases and non-ICH controls

\[ E(\text{l lipid}|RE) = \beta_1 + \beta_2 P1 + \beta_3 P2 + \beta_4 \text{Group} \times P1 + \beta_5 \text{Group} \times P2 + \beta_6 P3 + \text{age} + \text{sex} + \text{race} + \text{hypertension} + \text{diabetes} + \text{smoker} + RE (\text{individual}) + RE (\text{time}) \]

<table>
<thead>
<tr>
<th>A</th>
<th>TC</th>
<th>LDL</th>
<th>TG</th>
<th>HDL</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>FE</td>
<td>Variable</td>
<td>Estimate</td>
<td>SE</td>
</tr>
<tr>
<td>Intercept</td>
<td>225.91</td>
<td>11.70</td>
<td>137.78</td>
<td>10.59</td>
</tr>
<tr>
<td>β2</td>
<td>-0.41</td>
<td>0.91</td>
<td>-0.70</td>
<td>0.84</td>
</tr>
<tr>
<td>β3</td>
<td>-3.90</td>
<td>4.70</td>
<td>-0.85</td>
<td>4.33</td>
</tr>
<tr>
<td>β4</td>
<td>-3.05</td>
<td>1.29</td>
<td>-0.52</td>
<td>1.18</td>
</tr>
<tr>
<td>β5</td>
<td>-21.89</td>
<td>8.34</td>
<td>-19.41</td>
<td>7.53</td>
</tr>
<tr>
<td>β6</td>
<td>-0.59</td>
<td>0.84</td>
<td>-1.79</td>
<td>0.78</td>
</tr>
<tr>
<td>Age</td>
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<td>4.08</td>
<td>-11.91</td>
<td>3.75</td>
</tr>
<tr>
<td>Male</td>
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<td>3.75</td>
<td>-12.47</td>
<td>3.50</td>
</tr>
<tr>
<td>White</td>
<td>-2.20</td>
<td>10.67</td>
<td>-5.10</td>
<td>9.54</td>
</tr>
<tr>
<td>HTN</td>
<td>-9.55</td>
<td>3.73</td>
<td>-10.41</td>
<td>3.46</td>
</tr>
<tr>
<td>DM</td>
<td>-3.30</td>
<td>4.19</td>
<td>-6.29</td>
<td>3.76</td>
</tr>
<tr>
<td>Smoking</td>
<td>-3.48</td>
<td>3.68</td>
<td>-3.01</td>
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</tr>
<tr>
<td>RE Time</td>
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<tr>
<td>ID</td>
<td>1670.61</td>
<td>184.75</td>
<td>1452.79</td>
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<td>Residual</td>
<td>522.11</td>
<td>25.10</td>
<td>358.39</td>
<td>19.23</td>
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<tr>
<td>LR</td>
<td>-7838.26</td>
<td>-6490.63</td>
<td>-8244.75</td>
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</table>

B

<table>
<thead>
<tr>
<th>ICH</th>
<th>Controls</th>
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</thead>
<tbody>
<tr>
<td>Lipid</td>
<td>P1</td>
</tr>
<tr>
<td>TC</td>
<td>-0.19*</td>
</tr>
<tr>
<td>LDL</td>
<td>-0.07*</td>
</tr>
<tr>
<td>TG</td>
<td>-0.12</td>
</tr>
<tr>
<td>HDL</td>
<td>-0.02</td>
</tr>
</tbody>
</table>

A Estimated regression coefficients and standard errors (SE) for case-control comparison of changes in serum lipid levels using separate covariate-adjusted PLME model for serum lipid fractions TC, LDL, TG and HDL. B Estimated rates of change in serum lipid trends (mg/dL/month) for 6-24 month period pre-acute illness (P1) and 0-6 month period pre-acute
illness (P2) in ICH patients and non-ICH controls (adapted from Table 2A). PLME, piecewise linear mixed-effects; TC, total cholesterol; LDL, low-density lipoprotein; TG, triglyceride; HDL, high-density lipoprotein; Group, case control status with ICH patients designated as 1 and controls designated as 0; P3, time interval post-acute illness; FE, fixed-effects; RE, random-effects; β2, change in serum lipids (mg/dL) for non-ICH controls during P1; β2+β4, change in serum lipids (mg/dL) for ICH patients during P1; β2+β3, change in serum lipids (mg/dL) for non-ICH controls during P2; β2+β4+β3+β5, change in serum lipids (mg/dL) for ICH patients during P2; HTN, hypertension; DM, diabetes mellitus; LR, likelihood-ratio. * Statistical significance by Wald test for comparisons between time periods, p<0.0125 (Bonferroni-corrected).
**Table e-4. Inpatient diagnoses of control participants with non-cerebral acute illness**

<table>
<thead>
<tr>
<th>Inpatient Diagnoses include:</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>Shock</td>
<td>20</td>
</tr>
<tr>
<td>Sepsis, SIRS</td>
<td>4</td>
</tr>
<tr>
<td>Cardiac arrest</td>
<td>3</td>
</tr>
<tr>
<td>AMI, Angina</td>
<td>38</td>
</tr>
<tr>
<td>Cardiac surgeries</td>
<td>26</td>
</tr>
<tr>
<td>Trauma</td>
<td>15</td>
</tr>
<tr>
<td>Acute Renal Failure</td>
<td>9</td>
</tr>
<tr>
<td>GI Hemorrhage</td>
<td>10</td>
</tr>
<tr>
<td>Pneumonia</td>
<td>9</td>
</tr>
<tr>
<td>Respiratory Failure</td>
<td>6</td>
</tr>
<tr>
<td>Orthopedic Surgeries</td>
<td>28</td>
</tr>
</tbody>
</table>

**SIRS**, systemic inflammatory response syndrome; **AMI**, acute myocardial infarction; **GI**, gastrointestinal.
SUPPLEMENTARY METHODS

Piecewise Linear Mixed-Effects (PLME) model uses generalized linear mixed-effects for regression analysis of correlated data with time as a linear function comprising of separate ‘pieces’ of time intervals corresponding to pre-specified time intervals. This method allows separate slopes to be fitted to observations representing specific time periods before and after a ‘critical period’ or event in order to analyze the hypothesis that changes in serum lipid trends are dependent upon time of the event (ICH or non-cerebral acute illness). Selection of the PLME model allows for lack of independence in the outcome variable (correlated serum lipids in individual subjects), incomplete or unbalanced data, and incorporation of a priori determined, distinct, biologically-relevant time periods before and after a critical event (index ICH in cases, non-cerebral acute illness in controls) which is itself limited by uncertainty as to timing of the event.

Using PLME, the nonlinear relationships between serum lipid levels and time were modelled by splitting time pre- and post-event (ICH or non-cerebral acute illness) into intervals, at knots, and straight line-segments were fitted between the knots, which is equivalent of linear splines. With the assumption that the time interval immediately prior to acute illness is likely of most biological relevance, knots were placed at the date of acute illness and the date 6 months prior to acute illness representing the time point at which the function of the response variable (serum lipids) transitions from time period before (6-24 months pre-acute illness) to time period representing the time interval immediately prior to the acute event (0-6 months pre-acute illness), and subsequently to the time period after the acute event (0-24 months post-acute illness). The PLME function is therefore a linear model resulting from the combination of individual linear spline basis functions as separate covariates:

\[
E(\text{lipid}|RE) = \beta_1 + \beta_2 P_1 + \beta_3 P_2 + \beta_4 \text{Group} \times P_1 + \beta_5 \text{Group} \times P_2 + \beta_6 P_3 + \text{age} + \text{sex} + \text{race} + \text{hypertension} + \text{diabetes} + \text{smoker} + RE\ (\text{individual}) + RE\ (\text{time})
\]

where the variables \(P_1\), \(P_2\), and \(P_3\) each form separate linear spline basis functions (\(P_1=\text{time(24-6 months pre-event)})\); \(P_2=\text{time(0-6 months pre-event)})\); \(P_3=\text{time(0-24 months post-event)})\). This is further illustrated below.
Figure e-1. Illustration of piecewise linear function with knots at -6 and 0 for modeling of nonlinear curve (in red) representing change in serum lipid levels over time.
Figure e-2. Scatterplot of serum lipids (mg/dL) against time (months) relative to date of acute hospitalization for non-ICH controls (blue open circle) and ICH cases (red triangles).

LDL, low-density lipoprotein; HDL, high-density lipoprotein.
APOE Polymorphisms Influence Longitudinal Lipid Trends Preceding Intracerebral Hemorrhage

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Manuscript Word Count 3111
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Tables 2
Number of References 38
Supplementary Data Table e-1
Search Terms [7]Intracerebral hemorrhage,[54]Cohort studies,[91]All Genetics, Lipids

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Alessandro Biffi abiffi@mgh.harvard.edu
Jonathan Rosand jrosand@partners.org
DISCLOSURES

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INTELLECTUAL CONTRIBUTIONS

Dr. C-L. Phuah participated in study design, data acquisition, statistical analyses, drafting and revision of manuscript. M.R. Raffeld and A.M. Ayres was responsible for data collection. Drs. A. Viswanathan, M. Edip Gurol, S.M. Greenberg and Dr. J. Rosand participated in final editing of manuscript. Dr. C.D. Anderson participated in study design and funding, and revision of manuscript.

ACKNOWLEDGEMENTS

None.

CONFLICTS OF INTERESTS

None.

FUNDING

The authors' work on this study was supported by funding from the National Institutes of Health (see Disclosures). No funding entities were involved in study design, data collection, analysis, interpretation, writing of the report, or in the decision to submit the results for publication.
ABSTRACT

Objective: We sought to determine whether APOE genotype influences a previously observed decline in serum total cholesterol (TC) and low-density lipoprotein (LDL) levels preceding primary intracerebral hemorrhage (ICH), as a potential demonstration of non-amyloid mechanisms of APOE in ICH risk.

Methods: We performed a single-center retrospective longitudinal analysis using patients with known APOE genotype drawn from an ongoing cohort study of ICH. Serum lipid measurements for TC, triglycerides (TG), LDL, and high-density lipoprotein (HDL) collected within two-years before and after index ICH were extracted from electronic medical records. Piecewise linear mixed-effects models were used to compare APOE allele-specific effects on temporal serum lipid trends in ICH. Demographics, medical history, medications, and health maintenance data were included as fixed-effects. Inter- and intra-individual variations in lipid levels were modeled as random-effects.

Results: 124 ICH cases were analyzed. APOE ε4 carriers had greater rates of decline in serum TC and LDL within 6 months preceding ICH (TC:-7.30mg/dL/month, p=0.0035; LDL:-8.44mg/dL/month, p=0.0001). Conversely, serum TC and LDL levels in APOE ε2 carriers were unchanged within the same time period. APOE genotype had no associations with serum HDL or TG trends.

Conclusions: APOE allele status predicts serum TC and LDL changes preceding acute ICH. Our results have implications for ongoing efforts in dissecting the role of dyslipidemia in cerebrovascular disease risk. APOE-genotype specific influence on lipid trends provides a clue for one mechanism by which APOE may influence risk of ICH. Further characterization of the
metabolic roles of \textit{APOE} are needed to improve understanding of \textit{APOE} biology in cerebrovascular disease risk.
INTRODUCTION

Primary intracerebral hemorrhage (ICH) accounts for 10-15% of all strokes, but is the most severe form of acute cerebrovascular disease, with 90-day mortality rates of 40-50%, and with fewer than a third of survivors regaining functional independence by 12 months. Previous studies have established ε2/ε4 alleles of the APOE gene as potent determinants of ICH risk, severity and outcome. APOE ε2 and ε4 are associated with increased risk of ICH occurring in the lobar regions of the brain, whereas APOE ε4, but not ε2, is associated with risk of nonlobar ICH. Separately, several epidemiological studies have also observed an association between serum lipid levels and ICH risk and outcome. Hypercholesterolemia has been associated with reduced risk of ICH, fewer cerebral microbleeds, and improved outcome following ICH. However, despite known functions of APOE gene products in lipid transport and regulating circulating lipid levels the biological mechanisms mediating the roles of APOE and serum lipids on ICH risk remain unclear. A previous finding that serum low-density lipoprotein (LDL) mediates APOE ε4-associated nonlobar ICH risk suggests that the effect of APOE on ICH may be at least in part, due to its effect on lipids.

We have recently demonstrated that ICH is preceded by declines in serum total cholesterol (TC) and LDL levels. We hypothesized that APOE genotype may influence these temporal lipid trends in ICH and tested this hypothesis by investigating APOE allele-specific effects on changes in serum lipid trends over time in a cohort of ICH patients with longitudinal lipid data.
METHODS

Study Design

Patients were drawn from an ongoing prospective longitudinal cohort study of primary ICH at Massachusetts General Hospital (MGH)\textsuperscript{19} (Figure 1). All aspects of this study were approved by the MGH Institutional Review Board (IRB), and written informed consent was obtained from all patients or their legal guardians prior to study participation.

Patient Selection

Individuals enrolled in the MGH longitudinal ICH study presenting to the MGH Emergency Department between June 1993 and June 2014 were screened for eligibility for the present study based on the following: (1) availability of APOE genotype, (2) survival up to 2 years following ICH, and (3) possessing at least three serum lipid values for each lipid fraction of interest including TC, LDL, triglycerides (TG) and high-density lipoprotein (HDL) drawn >6 months apart within 24 months before and after the date of acute ICH. Patients with recurrent ICH or other non-ICH hospitalization events during time period of interest were excluded to minimise confounding by variations in serum lipid levels during periods of acute illness.\textsuperscript{20,21}

Data Collection

All included individuals had serum lipid values (TC, TG, LDL and HDL) extracted via semi-automated review of hospital electronic medical records (EMR). As ICH events are observed at random, in order to make comparisons for variations in serum lipid trends before and after ICH, individual ICH events were time-locked into a balanced design with equal time periods of 24 months before and after the acute event. Serum lipid values were obtained for 6-monthly time intervals (four intervals before and four intervals after ICH event). Serum lipid values were
segregated into their respective time intervals based on timing of the measurement in relation to the date of ICH occurrence. Means were obtained for multiple serum lipid measurements within the same time interval.

Clinical data were recorded at the time of index presentation including information on demographics, medical history (including hypertension and diabetes), pre-ICH medication use, and health maintenance (including cigarette smoking and alcohol use). Statin use within 24 months pre-ICH was determined at time of ICH presentation using a patient-based questionnaire and scored as ‘yes/no’ binary variables. Validity and reliability of statin use response in the questionnaire were internally assessed by comparisons with medication history obtained from hospital EMR search. A 10% random sampling of the enrolled cohort demonstrated 100% concordance in patients who were statin-naïve. ICH location was assigned as either lobar or nonlobar based on admission CT scans. Nonlobar ICH was restricted to hemorrhage locations involving the brainstem, thalamus, or basal ganglia whereas lobar ICH included hemorrhages originating from cortical-subcortical regions.

**Genotyping**

Study individuals had *APOE* genotype determined previously from blood samples.\(^4\) Briefly, peripheral whole blood was collected from study individuals at time of consent. DNA was isolated from fresh or frozen blood, quantified using a quantification kit (Qiagen, Valencia, CA, USA) and normalized to a concentration of 30ng/ul. *APOE* genotypes were determined by genotyping two variants in *APOE*, rs7412 and rs429358 via two separate assays\(^4\) with the subsequent allelic reads from both assays then combined for translation to *APOE* genotypes (ε3ε3, ε3ε4, ε4ε4, ε3ε2, ε2ε2 and ε2ε4).

*Standard protocol approach, registrations, and patient consents.*
This study was performed with approval of the MGH Institutional Review Board (IRB). ICH patients provided informed consent for study participation.

Statistical methods

Study individuals were grouped by APOE ε2 and ε4 carrier status (having either 1 or 2 allelic copies of ε2 and ε4, respectively) with APOE ε3ε3 individuals serving as a reference cohort as the ε3 allele is not associated with ICH risk. Patients with ε2ε4 APOE genotype (n=5) were excluded due to an inability to assign a single carrier status. Comparisons of differences in cohort characteristics between ε2 or ε4 carriers and reference ε3ε3 group were made by univariate analyses using unpaired t test, Mann-Whitney rank-sum or Fisher exact test, as appropriate. Continuous numeric variables were expressed as mean ± SD.

Piecewise linear mixed-effects (PLME) random-coefficient models were used to evaluate APOE allele-specific effects on temporal variation in serum lipid trends in ICH patients within pre-specified time periods which are fixed in relation to acute ICH occurence. This allowed for modelling of change in serum lipid trends in pre-determined time intervals of interest to differ within and between ε2 or ε4 carriers and non-carriers. In a previous case-control analysis comparing ICH patients and non-ICH controls, we demonstrated significant decline in serum lipid trends in the 6-month interval immediately preceding the occurrence of ICH which was not observed in non-ICH controls. Accordingly, fixed-knots were placed at the date of acute ICH and the date 6 months prior to acute ICH to mark transitions in time periods of interest corresponding to the time period 6-24 months pre-ICH (P₁), the time period 0-6 months immediately pre-ICH (P₂), and time period 0-24 months post-ICH (P₃).

Separate multivariate linear mixed-models were constructed for ε2 and ε4 carriers, with carrier status and covariates whose p value were <0.20 on univariate analyses, or with known
potential to influence serum lipid levels included as fixed-effects. The final multivariate model was adjusted for variables: age, sex, race, pre-ICH history of hypertension, statin use (yes/no), smoking history (ever smoked), and ICH location. Inter-individual and intra-individual variation in serum lipid levels were modelled as random-effects. Model validity was examined using a likelihood-ratio test. Unstructured covariance was used as the covariance model. Comparisons of the significance in change in serum lipid trends (slope) at time period of interest, $P_2$ (0-6 months pre-ICH) by APOE allele carrier status were made using the Wald test. Subgroup analyses stratified by ICH location (lobar and nonlobar) for $\varepsilon^2$ and $\varepsilon^4$ carrier status were separately performed but not shown due to insufficient statistical power. Significance threshold was set at $p<0.05$ (2-tailed) for univariate analyses, and $p<0.0125$ (Bonferroni-correction for 4 tests) for individual serum lipid fraction mixed-model analyses. All statistical analyses were performed using STATA 10.0 (StataCorp LP, USA).
RESULTS

Cohort Characteristics

A total of 212 ICH patients enrolled between June 1993 and June 2014 with longitudinal serum lipid levels measured met our inclusion criteria, of whom 129 patients were genotyped for APOE. The 83 ICH patients removed due to absence of APOE genotype (Figure 1) did not differ in clinical characteristics from the group of patients who ultimately were included in our analyses (Table e-1). After removing the 5 patients with APOE ε2/ε4, we analyzed 124 individuals including 19 ε2 carriers, 39 ε4 carriers and 66 ε3/ε3 patients (Table 1). Compared with the reference group (APOE ε3ε3), ε2 carriers were less likely to have a pre-ICH history of hypertension, while ε4 carriers were more likely to be smokers and have ICH located in the lobar region (all p<0.05). There were no significant difference in rates of statin use between the three groups. APOE allelic frequencies in our analysis cohort were consistent with previously observed population estimates for North American Caucasians.  

APOE alleles and serum lipid levels in ICH patients

We first sought to confirm previously observed effects of APOE on serum lipid levels, as seen in prior population-level genome-wide association studies (GWAS) of lipids. Comparisons of mean serum levels of TC, TG, LDL and HDL prior to ICH by APOE-allele status revealed an expected allelic dose-dependent increase in mean serum TC and LDL levels in ε4 carriers compared to non-carriers whereas levels of both lipid fractions were decreased in ε2 carriers compared to non-carriers (TC: 217.21±58.37 mg/dL in ε4 carriers, 157.67±41.12 mg/dL in ε2 carriers; LDL: 130.43±48.67 mg/dL in ε4 carriers, 69.90±19.86 mg/dL in ε2 carriers). No
associations were observed for serum TG and HDL levels, consistent with known absence of
APOE effects on these lipid fractions.

*APOE alleles influence 24-month pre-ICH serum lipid trends*

Temporal lipid patterns in our analysis cohort revealed decline in both serum TC and LDL
levels beginning several months preceding acute ICH occurrence consistent with observed
trends seen previously in a larger cohort\(^\text{18}\) (Figure 2). Subgroup analysis by APOE carrier
status revealed distinct differences in temporal serum lipid trends, visualised using Loess
smoothed curves, during this time period. APOE \(\varepsilon\)4 carriers experienced an overall decline in
serum TC and LDL levels in the 24 months pre-ICH. In contrast, both serum TC and LDL
trends remained relatively flat in non-\(\varepsilon\)4 carriers during the same time period preceding ICH
(Figure 3). Comparisons of serum lipid trends by APOE allele demonstrated an overall decline in
serum TC and LDL levels during the pre-ICH time period in \(\varepsilon\)4 carriers compared to non-
carriers (\(p=0.049\) and \(p=0.014\), respectively), while no significant changes were observed in \(\varepsilon\)2
carriers.

*APOE alleles influence differential change in serum lipid trends immediately preceding ICH
occurrence*

Visual inspection of the Loess smoothed curves also revealed distinct differences in temporal
serum lipid trends in the 6-month time period immediately preceding ICH occurrence by
APOE-allele status. Subacute decline in mean serum TC and LDL levels beginning around 6
months pre-ICH were observed only in \(\varepsilon\)4 carriers (Figure 3). This accelerated decline in the 6
month period prior to ICH is consistent with our prior report in a larger cohort.\(^\text{18}\) Covariate-
adjusted PLME was used to compare differences in serum lipid trends by APOE genotype in
the immediate 0-6 month interval pre-ICH and in the antecedant interval (6-24 months pre-ICH) (Table 2). APOE ε4 carriers experienced acceleration in rates of decline in serum TC and LDL levels in the 6 months prior to acute ICH event compared to trends in the antecedant 18-month time interval. The observed association remained significant after the inclusion of potential confounders in multivariate analysis, including hypertension (Table 2). Comparatively, serum TC trends were unchanged in the same 6-month time period immediately pre-ICH in both APOE ε2 carriers and APOE ε3/ε3 individuals. In contrast, APOE ε2 carriers experienced a non-significant increase in serum LDL levels in the immediate 0-6 month interval pre-ICH while serum LDL levels remained flat in APOE ε3/ε3 individuals within the same time period. Neither temporal serum HDL nor TG trends differed by APOE genotype.

APOE alleles do not influence temporal serum lipid trends post-ICH

Serum lipid levels remained largely depressed for TC, LDL and HDL in the 48 months post-ICH with no significant difference in serum lipid trends by APOE carrier status during that time. Serum TG trends post-ICH demonstrate similar variability as in the pre-ICH period but no significant difference were observed between APOE ε2 and ε4 carriers, or individuals with APOE ε3/ε3 genotype within the time period examined (Figure 3).
DISCUSSION

Our results demonstrate that temporal variation in serum lipids in ICH patients are influenced by APOE allele status, and differ from APOE associations with steady-state serum lipid levels.\(^1\) APOE \(\varepsilon4\) carriers experienced drops in TC and LDL levels in the 24 month period prior to their ICH, in comparison with non-APOE \(\varepsilon4\) carriers. Furthermore, in the 6 month period immediately preceding ICH, APOE \(\varepsilon4\) carriers displayed increased rates of decline in serum TC and LDL levels. This observation of genotype-specific differences in temporal serum lipid trends builds upon our prior observation of subacute decline in serum TC and LDL levels prior to acute ICH, and suggests that APOE gene products may exert at least some of their effect on risk of ICH through modulation of serum lipids.

Demonstration of APOE epsilon allele specific effects on serum lipid trends in ICH patients raises several hypotheses regarding the role of APOE in ICH risk. Both \(\varepsilon2\) and \(\varepsilon4\) are risk factors for lobar ICH in part through their effects on amyloid processing.\(^4\) APOE \(\varepsilon4\) is also independently associated with increased risk of nonlobar ICH,\(^4\) presumably through non-amyloid related mechanisms given that cerebral amyloid angiopathy is almost universally absent from the deeper small vessels.\(^2\) A growing body of evidence supports the association of hypocholesterolemia with elevated risk of ICH,\(7-14\) and with progression of ICH-related phenotypes such as cerebral microbleeds.\(14,25\) This association has been hypothesized to be the result of loss in vasular integrity in low circulating cholesterol states, which predisposes towards vessel rupture in ICH, although the complex role of lipids in cellular biology, inflammation, and signalling,\(26-29\) in addition to cell membrane integrity, make it difficult to attribute the observed associations to any one mechanism.\(30-33\)
Known $APOE$ effects in lipid metabolism and vascular amyloid deposition raise the possibility that $APOE$ may influence ICH risk through amyloid and non-amyloid effects. Our results appear to support the hypothesis of a non-amyloid role of $APOE$ in ICH risk through the observed $APOE$-genotype specific associations with subacute serum lipid changes before primary ICH. Given that $APOE\ \varepsilon4$ associates with higher average TC and LDL levels, our results also raise the hypothesis that the rate of change in serum levels, rather than the baseline average, is an important determinant of ICH risk imparted by the $APOE\ \varepsilon4$ genotype. Furthermore, our demonstration of $APOE$ allele-specific effects on temporal serum lipid trends in ICH corroborates the notion of divergent mechanistic pathways between $\varepsilon2$ and $\varepsilon4$ alleles in the pathophysiology of ICH. Our observations of similar $APOE$-allele specific serum TC and LDL trends pre-ICH in subgroup analyses stratified by ICH location (lobar and nonlobar) likewise support such a notion, but our study was insufficiently powered to detect significant differences due to the small sample size.

However, caution must be exercised in making mechanistic links due to biological complexity of the underlying disease, as well as $APOE$ pleiotropy. Based on our observation, we can only speculate as to whether serum lipid declines directly drive the process of increased vessel wall vulnerability leading ultimately to vessel rupture and ICH, or serve as a surrogate marker of a separate process affecting cerebral small vessels. Active inflammation is associated with lower serum TC and LDL levels while $APOE$ genotype-specific elevation in pro-inflammatory response has been observed in $APOE\ \varepsilon4$ carriers compared with $APOE\ \varepsilon2$ and $APOE\ \varepsilon3$ carriers in transgenic murine models. Thus, it is possible that the influence of $APOE$ polymorphisms on temporal lipid trends in ICH may instead reflect $APOE$ genotype-specific differences in innate inflammatory processes in the cerebral small vessels.
A strength of this study is the use of a unique dataset combining both APOE genotype and longitudinal lipid data in a rigorously phenotyped ICH cohort. The additional information conferred by serum lipid variations over time both before and after primary ICH revealed APOE-genotype specific associations distinct from known steady-state relationships. This in turn allowed for the dissection of lipid-dependent associations of APOE in primary ICH risk.

There are limitations to our study. First, we had to exclude almost 40% of eligible cases identified due to the absence of APOE genotype data. It should be noted, however, that the analysis cohort remained representative of the larger ICH cohort, with no significant difference in all covariates of interest to suggest a sampling bias. Furthermore, distribution of the APOE alleles in our small study was also consistent with frequency estimates in the population at large. We were also unable to account completely for selection bias arising from subject-specific indications for serial serum lipid measurements although in our particular study cohort, prior analysis showed no differences in clinical characteristics between ICH patients with and without serum lipid data.\(^\text{18}\) A third limitation was our relatively small sample size, particularly with regard to the total number of APOE \(\varepsilon2\) individuals which may influence our ability to more accurately assess association with serum lipid trends in those individuals. We attempted to address this by utilizing mixed effect modeling to increase statistical power through additional use of inter-individual change with time. Nevertheless, future studies incorporating a longitudinal design with available APOE genotypes and relatively frequent recorded lipid levels will be necessary to validate and confirm these results. Fourth, we were unable to exhaustively address the broad range of all the possible external environmental factors that can influence biological variation in serum lipid levels.\(^\text{37}\) We did attempt to minimize potential confounding by including these measures where available by including covariates of age, statin and alcohol
use in our models, and using a longitudinal trial design of sufficiently long duration (4 years) which limits the impact of seasonal variations in serum lipids. Additionally, we were also unable to account for either statin dose or type, as well as potential intermittent use. However, as the decline in both serum TC and LDL levels immediately preceding ICH occurrence were previously noted to be independent of statin use, the differential degree of lipid-lowering conferred by nuances in statin use is unlikely to contribute significant confounding. Fifth, although there is a high likelihood that LDL may be a major mediator of TC effects seen in APOE ε4 carriers, our study design prohibits formal mediation analysis due to violation of several assumptions needed for establishing a correctly specified mediation model. Finally, although the demonstration of temporal changes in serum lipids preceding ICH strongly suggest a correlation between serum lipid changes and ICH development, we are unable to confirm a causal relationship due to the retrospective study design.

APOE ε4 strongly predicts pre-ICH trends in serum TC and LDL levels, as well as the acute decline in serum TC and LDL in the 6 month period prior to acute ICH. Our results have implications for ongoing efforts in dissecting the role of dyslipidemia in cerebrovascular disease risk and provide novel insight regarding non-amyloid APOE mechanisms in ICH risk.

REFERENCES


FIGURES

Figure 1. Study cohort and analysis plan.

Abbreviations: MGH-ICH Database, Massachusetts General Hospital-based patients in the Genetics of Cerebral Hemorrhage on Anticoagulation Study; ICH, intracerebral hemorrhage.
### Table 1. Baseline characteristics of study cohort.

<table>
<thead>
<tr>
<th>Variable</th>
<th>APOE ε2 (n=19)</th>
<th>APOE ε3/ε3 (n=66)</th>
<th>APOE ε4 (n=39)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, y (mean +/- SD)</td>
<td>75.2 +/- 9.7</td>
<td>73.6 +/- 10.7</td>
<td>70.7 +/- 11.5</td>
</tr>
<tr>
<td>Females, n (%)</td>
<td>8 (42.1)</td>
<td>25 (37.9)</td>
<td>22 (56.4)</td>
</tr>
<tr>
<td>White, n (%)</td>
<td>17 (89.5)</td>
<td>59 (89.4)</td>
<td>33 (84.6)</td>
</tr>
<tr>
<td>HTN, n (%)</td>
<td>12 (63.2)†</td>
<td>57 (86.4)</td>
<td>38 (97.4)</td>
</tr>
<tr>
<td>DM, n (%)</td>
<td>3 (25.0)</td>
<td>22 (44.0)</td>
<td>9 (47.4)</td>
</tr>
<tr>
<td>Alcohol Use, n (%)</td>
<td>10 (62.5)</td>
<td>33 (56.9)</td>
<td>20 (54.1)</td>
</tr>
<tr>
<td>Smokers, n (%)</td>
<td>1 (5.9)</td>
<td>3 (5.0)</td>
<td>7 (18.0)†</td>
</tr>
<tr>
<td>Statin Use, n (%)</td>
<td>8 (42.1)</td>
<td>27 (41.5)</td>
<td>17 (43.6)</td>
</tr>
<tr>
<td>Lobar ICH, n (%)</td>
<td>8 (42.1)</td>
<td>25 (37.9)</td>
<td>24 (61.5)†</td>
</tr>
</tbody>
</table>

† p<0.05 in comparison to reference group (APOE ε3/ε3).

Abbreviations: HTN, hypertension; DM, diabetes mellitus; ICH, intracerebral hemorrhage.
Figure 2. Temporal trends in individual serum lipid fractions in ICH patients.

A-D Loess smoothed curves of serum lipid levels (mg/dL) against time (in months) before and after ICH. Grey areas indicate standard error (SE). Time period of interest indicated by shaded boxes, (P₂ 0-6 months pre-ICH). * $p<0.0125$, rate of change of serum lipids by Wald test for time period P₂.
Figure 3. Temporal trends in individual serum lipid fractions in ICH patients by APOE allele carrier status.

**A-D** Loess smoothed curves of serum lipid levels (mg/dL) against time (in months) before and after ICH. Grey areas indicate standard error (SE). Time period of interest indicated by shaded boxes, (P2 0-6 months pre-ICH). * p<0.0125, rate of change of serum lipids by Wald test for time period P2 in APOE ε2 or ε4 carrier status compared to reference (APOE ε3/ε3).
Table 2. Estimated rates of change in serum lipid levels pre-ICH by APOE genotype status.

<table>
<thead>
<tr>
<th>Rate of change in serum lipids (mg/dL/month)</th>
<th>Time interval Pre-ICH (months)</th>
<th>p Value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>6-24</td>
<td>0-6</td>
</tr>
<tr>
<td>Total Cholesterol</td>
<td></td>
<td></td>
</tr>
<tr>
<td>APOE $\varepsilon$4</td>
<td>-0.05</td>
<td>-7.30</td>
</tr>
<tr>
<td>APOE $\varepsilon$3†</td>
<td>-0.19</td>
<td>-3.79</td>
</tr>
<tr>
<td>APOE $\varepsilon$2</td>
<td>-0.40</td>
<td>+1.72</td>
</tr>
<tr>
<td>Low-density Lipoprotein</td>
<td></td>
<td></td>
</tr>
<tr>
<td>APOE $\varepsilon$4</td>
<td>+0.09</td>
<td>-8.44</td>
</tr>
<tr>
<td>APOE $\varepsilon$3†</td>
<td>-0.02</td>
<td>-2.39</td>
</tr>
<tr>
<td>APOE $\varepsilon$2</td>
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<td>+5.16</td>
</tr>
<tr>
<td>Triglycerides</td>
<td></td>
<td></td>
</tr>
<tr>
<td>APOE $\varepsilon$4</td>
<td>-0.24</td>
<td>-6.86</td>
</tr>
<tr>
<td>APOE $\varepsilon$3†</td>
<td>-0.10</td>
<td>-6.10</td>
</tr>
<tr>
<td>APOE $\varepsilon$2</td>
<td>+0.46</td>
<td>-16.06</td>
</tr>
<tr>
<td>High-density Lipoprotein</td>
<td></td>
<td></td>
</tr>
<tr>
<td>APOE $\varepsilon$4</td>
<td>-0.01</td>
<td>-0.06</td>
</tr>
<tr>
<td>APOE $\varepsilon$3†</td>
<td>+0.03</td>
<td>-1.11</td>
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<tr>
<td>APOE $\varepsilon$2</td>
<td>-0.02</td>
<td>-0.69</td>
</tr>
</tbody>
</table>

Comparisons of rates of change in serum lipid levels (in mg/dL/month) pre-ICH between time periods 6-24 months pre-ICH and 0-6 months pre-ICH using covariate-adjusted PLME model by APOE allele carrier status. †APOE $\varepsilon$3 consist of individuals with APOE $\varepsilon$3/$\varepsilon$3 genotype serving as the control group for comparison. * $p<0.0125$ by Wald test for comparisons between time periods.
SUPPLEMENTARY DATA

Table e-1. Baseline characteristics of ICH cohort without APOE genotype data

<table>
<thead>
<tr>
<th></th>
<th>Excluded ICH Cohort (n=83)</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, y (mean +/- SD)</td>
<td>73.7 +/-12.0</td>
<td>0.71\textsuperscript{a}</td>
</tr>
<tr>
<td>Females, n (%)</td>
<td>39 (47.0)</td>
<td>0.67\textsuperscript{b}</td>
</tr>
<tr>
<td>White, n (%)</td>
<td>74 (89.2)</td>
<td>0.67\textsuperscript{b}</td>
</tr>
<tr>
<td>HTN, n (%)</td>
<td>68 (81.9)</td>
<td>0.57\textsuperscript{b}</td>
</tr>
<tr>
<td>DM, n (%)</td>
<td>14 (25.9)</td>
<td>0.07\textsuperscript{b}</td>
</tr>
<tr>
<td>Alcohol Use, n (%)</td>
<td>28 (53.9)</td>
<td>0.87\textsuperscript{b}</td>
</tr>
<tr>
<td>Smokers, n (%)</td>
<td>11 (20.4)</td>
<td>0.16\textsuperscript{b}</td>
</tr>
<tr>
<td>Statin Use, n (%)</td>
<td>39 (47.0)</td>
<td>0.48\textsuperscript{b}</td>
</tr>
<tr>
<td>Lobar ICH, n (%)</td>
<td>39 (47.0)</td>
<td>0.97\textsuperscript{b}</td>
</tr>
</tbody>
</table>

Comparison of baseline characteristics between excluded vs. included ICH individuals by APOE genotype availability. Statistical significance at p<0.05, \textsuperscript{a} t test, \textsuperscript{b} Fisher exact test. Abbreviations: HTN, hypertension; DM, diabetes mellitus; ICH, intracerebral hemorrhage.