Longitudinal Serum Lipid Trends and APOE in Primary Intracerebral Hemorrhage

TABLE OF CONTENTS

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.[1-2] 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.[3-6] This burden is only expected to grow in step with increases in life expectancy as incidence rates of ICH increase dramatically with age.[7] Although several genetic and environmental risk factors have been implicated in the occurrence of ICH, including ε2 and ε4 alleles of the *APOE* gene,[8] hypertension and dyslipidemia,[9] 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.[10-12] Multiple observational and randomized-controlled studies have reported an association between hypercholesterolemia and reduced ICH risk,[13-16] and improvements in ICH-related outcomes.[17,18] 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.[13-18] 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.[19-23] In particular, serum cholesterol levels have been reported to decline precipitously within days after both acute ischemic or hemorrhagic strokes,[14,24,25] with levels subsequently rising above baseline (drawn at time of stroke) by 90 days post-event.[14] 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,[26] those hospitalized for non-cerebral diseases including sepsis and acute myocardial infarction,[27] and patients in severe pain.[28]

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:

1. Phuah CL, Raffeld M, Ayres A, Viswanathan A, Greenberg SM, Biffi A, Rosand J, Anderson CD. Subacute decline in serum lipids precedes the occurrence of primary intracerebral hemorrhage. *Neurology* 2016. (*in press*)
2. Phuah CL, Raffeld M, Ayres A, M. Edip Gurol, Viswanathan A, Greenberg SM, Biffi A, Rosand J, Anderson CD. *APOE* polymorphisms influence longitudinal lipid trends preceding primary intracerebral hemorrhage. *Neurology Genetics* 2016. (*under review*)

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

1. Rincon F, Mayer SA. The epidemiology of intracerebral hemorrhage in the United states from 1979 to 2008. *Neurocrit Care* 2013;19(1):95-102.
2. Labovitz DL, Halim A *et al*. The incidence of deep and lobar intracerebral hemorrhage in whites, blacks, and Hispanics. *Neurol* 2005;65(4):518-522.
3. Earnshaw SR, Joshi AV, Wilson MR, Rosand J. Cost-effectiveness of recombinant activated factor VII in the treatment of intracerebral hemorrhage. *Stroke* 2006;37(11):2751-2758.
4. Qureshi AI, Tuhrim S, Broderick JP, Batjer HH, Hondo H, Hanley DF. Spontaneous intracerebral hemorrhage. *NEJM* 2001;344(19):1450-1460.
5. van Asch CJ, Luitse MJ, Rinkel GJ, van der Tweel I, Algra A, Klijn CJ. Incidence, case fatality, and functional outcome of intracerebral haemorrhage over time, according to age, sex, and ethnic origin: a systematic review and meta-analysis. *Lancet Neurology* 2010;9(2):167-176.
6. Taylor TN, Davis PH, Torner JC, Holmes J, Meyer JW, Jacobson MF. Lifetime cost of stroke in the United States. *Stroke* 1996;27(9):1459-1466.
7. Broderick JP. Natural History of Primary Intracerebral Hemorrhage. In: Whisnant JP, editor. Stroke: Populations, Cohorts, and Clinical Trials. Oxford: Butterworth-Heinmann; 1993.154-173.
8. Biffi A, Sonni A, Anderson CD, Kissela B, Jagiella JM, Schmidt H, et al. Variants at *APOE* influence risk of deep and lobar intracerebral hemorrhage. *Ann Neurol* 2010;68:934-943.
9. Ariesen MJ, Claus SP, Rinkel GJE, Algra A. Risk factors for intracerebral hemorrhage in the general population. *Stroke* 2003;34:2060-2065.
10. Soljanlahti S, Raininko R, Hyttinen L, Lauerma K, Keto P, Vuorio AF, et al. Statin-treated familial hypercholesterolemia patients with coronary heart disease and pronounced atherosclerosis do not have more brain lesions than healthy controls in later middle age. *Acta Radiol* 2007;48(8):894-899.
11. Haussen DC, Henninger N, Kumar S, Selim M. Statin use and microbleeds in patients with spontaneous intracerebral hemorrhage. *Stroke* 2012;43(10):2677-2681.
12. Woo D, Sauerbeck LR, Kissela BM, Khoury JC, Szaflarski JP, Gebel J, et al. Genetic and environmental risk factors for intracerebral hemorrhage: preliminary results of a population-based study. *Stroke* 2002; 33(5): 1190-5
13. Heart Protection Study Collaborative Group. Effects of cholesterol-lowering with simvastatin on stroke and other major vascular events in 20536 people with cerebrovascular disease or other high-risk conditions. *Lancet* 2004;363:757-767.
14. Segal AZ, Chiu RI, Eggleston-Sexton PM, Beiser A, Greenberg SM. Low cholesterol as a risk factor for primary intracerebral hemorrhage: A case-control study. *Neuroepidemiol* 1999;18:185-193.

15. Woo D, Kissela BM, Khoury JC, Sauerbeck LR, Haverbusch MA, Szaflarski JP, et al. Hypercholesterolemia, HMG-CoA reductase inhibitors, and risk of intracerebral hemorrhage: a case-control study. *Stroke* 2004;35:1360-1364.
16. Baigent C, Keech A, Kearney PM, et al. Efficacy and safety of cholesterol-lowering treatment: prospective meta-analysis of data from 90,056 participants in 14 randomised trials of statins. *Lancet* 2005; 366: 1267–78.
17. Rodriguez-Luna D, Rubeira M, Ribo M, et al. Serum low-density lipoprotein cholesterol level predicts hematoma growth and clinical outcome after acute intracerebral hemorrhage. *Stroke* 2011;42(9):2447-52.
18. Mustanoja S, Strbian D, Putaala J, Meretoja A, Curtze S, Haapaniemi E, et al. Association of prestroke statin use and lipid levels with outcome of intracerebral hemorrhage. *Stroke* 2013;44:2330-2332.
19. Durrington PN. Biological variation in serum lipid concentrations. *Scand J Clin Lab Invest Suppl* 1990;198:86-91.
20. Schaefer EJ, Lamon-Fava S, Cohn SD, et al. Effects of age, gender, and menopausal status on plasma low density lipoprotein cholesterol and apolipoprotein B levels in the Framingham Offspring Study. *J Lipid Res* 1994;35(5):779-792.
21. Stevenson JC, Crook D, Godsland IF. Influence of age and menopause on serum lipids and lipoproteins in healthy women. *Atherosclerosis* 1993;98(1):83–90.
22. Ockene IS, Chiriboga DE, Stanek EJ, et al. Seasonal variation in serum cholesterol levels: treatment implications and possible mechanisms. *Arch Intern Med* 2004;164(8):863-870.
23. Craig WY, Palomaki GE, Haddow JE. Cigarette smoking and serum lipid and lipoprotein concentrations: an analysis of published data. *Br Med J* 1989;298:784-8.
24. Mendez I, Hachinski V, Wolfe B. Serum lipids after stroke. *Neurology* 1987;37:507-511.
25. Hollanders FD, Burton P, Shafar J. Serum lipid changes following completed stroke syndrome. *Postgrad Med J* 1975;51:386-389.
26. Man EB, Bettcher PG, Cameron CM, Peters JP. Plasma amino acids, nitrogen and serum lipids of surgical patients. *J Clin Invest* 1946;25:701-708.
27. Gore JM, Goldberg RJ, Matsumoto AS, Castelli WP, McNamara AB, Dalen JE. Validity of serum total cholesterol level obtained within 24 hours of acute myocardial infarction. *Am J Cardiol* 1984;54:722-725.
28. Keele KD, Stern PRS. Serum lipid changes in relation to pain. *J R Coll Physicians Lon* 1973;7:319-329.

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

Chia-Ling Phuah, MD [1,2,5], Miriam R. Raffeld, BA [2], Alison M. Ayres, BA [3,4], Anand Viswanathan, MD, PhD [3,4], Steven M. Greenberg, MD, PhD [3,4], Alessandro Biffi, MD [2,4,5], Jonathan Rosand, MD, MSc [1-5], Christopher D. Anderson, MD, MMSc [1-5]

[1] Division of Neurocritical Care and Emergency Neurology, Massachusetts General Hospital, Boston, MA

[2] Center for Human Genetic Research, Massachusetts General Hospital, Boston, MA

[3] The J. Philip Kistler Stroke Research Center, Massachusetts General Hospital, Boston, MA

[4] Hemorrhagic Stroke Research Group, Massachusetts General Hospital, Boston, MA

[5] Program in Medical and Population Genetics, Broad Institute, Cambridge, MA

Correspondence

Christopher D. Anderson, MD

Center for Human Genetic Research

Massachusetts General Hospital

185 Cambridge Street; CPZN-6818

Boston, MA 02114, USA

Tel: +1 (617) 724-2698

Fax: +1 (617) 643-3293

Email: cdanderson@mgh.harvard.edu

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

Title Word Count	12
Title Character Count	90
Abstract Word Count	250
Manuscript Word Count	3000
Introduction Word Count	237
Figures	3
Tables	2
Supplementary Data	
Word Count	1171
Figures	2
Tables	4

ACKNOWLEDGEMENTS

The authors would like to thank Dr. Daniel Woo for his helpful discussions regarding serum lipid confounders.

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.

STUDY FUNDING

The authors' work on this study was supported by funding from the National Institute for Neurologic Disorders and Stroke (R01 NS059727 and K23 NS086873). All funding entities had no involvement in study design, data collection, analysis, interpretation, writing of the report, and in the decision to submit the paper for publication.

DISCLOSURE

C. Phuah reports no disclosures relevant to the manuscript.

M. Raffeld reports no disclosures relevant to the manuscript.

A. Ayres reports no disclosures relevant to the manuscript.

A. Viswanathan is supported by NIH-NINDS K23 AG028726.

S. Greenberg is supported by NIH-NINDS U10 NS077360, R01 AG026484, R01 NS070834.

A. Biffi reports no disclosures relevant to the manuscript.

J. Rosand is supported by NIH-NINDS U01 NS069208, R01 NS073344, and R01 NS059727.

C. Anderson is supported by NIH-NINDS K23 NS 086873.

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[1-6]. Antithetical to its role in ischemic stroke, hypercholesterolemia has been associated with decreased primary intracerebral hemorrhage (ICH) risk[7-10], reduced number of cerebral microhemorrhages[11], reduced hemorrhagic conversion after ischemic strokes[12], and improved ICH outcomes[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[8]. Estimates of variation coefficients for serum lipid concentrations range from 5-25%[15], reflective of considerable variation due to biological and environmental factors[15-19]. Serum lipid levels also undergo considerable variation during acute illness[20-22], hypothesized to be due to catecholamine stress response[21,23]. As example, serum cholesterol levels decline precipitously within days after both acute ischemic or hemorrhagic strokes[8,23,24], then subsequently rising above those drawn at time of stroke by 90 days post-event[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[25]. 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. Partipants 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[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 questionaire-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 ≥ 18 years admitted to MGH within the same time period, selected via database query from a MGH-affiliated clinical data registry (RPDR)[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.

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.

Statistical methods

Continuous numeric variables were expressed as mean ± 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[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 occurence, 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 neligible 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[20-24] and are thought to be associated with active inflammation[28-29]. Previous studies of pre-stroke serum lipids association with ICH have been inconclusive[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[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[8,30-32]. Third, the use of piecewise mixed-effects model in our analysis allowed for investigation of individual, distinct, biologically-relevant time periods[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[20-24]. Acute illnesses including myocardial infarction[21] and ischemic strokes[23,24], amongst others[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[33], similar to prior observation after ICH[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)[34]. 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[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[35]. 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

1. Gordon T. Mortality experience among the Japanese in the United States, Hawaii and Japan. Public Health Rep 1957;72:543-553.
2. Kuller L, Reisler DM. An explanation for variations in distribution of stroke and arteriosclerotic heart disease among populations and racial groups. Am J Epidemiol 1971;93:1–9.
3. Yano K, Reed DM, MacLean CJ. Serum cholesterol and hemorrhagic stroke in the Honolulu Heart Program. Stroke 1989;20:1460-1465.
4. Iso H, Jacobs DR Jr, Wentworth D, Neaton JD, Cohen JD. Serum cholesterol levels and six-year mortality from stroke in 350,977 men screened for the Multiple Risk Factor Intervention Trial. N Engl J Med 1989;320:904-910.

5. Lindenstrøm E, Boysen G, Nyboe J. Influence of total cholesterol, high density lipoprotein cholesterol, and triglycerides on risk of cerebrovascular disease: the Copenhagen City Heart Study. BMJ 1994;309:11.
6. Iribarren C, Jacobs DR, Sadler M, Claxton AJ, Sidney S. Low total serum cholesterol and intracerebral hemorrhagic stroke: is the association confined to elderly men? The Kaiser Permanente Medical Care Program. Stroke 1996;27:1993-1998.
7. Collins R, Armitage J,Parish S, Sleight P, Peto R. Effects of cholesterol-lowering with simvastatin on stroke and other major vascular events in 20536 people with cerebrovascular disease or other high-risk conditions. Lancet 2004;363:757-767.
8. Segal AZ, Chiu RI, Eggleston-Sexton PM, Beiser A, Greenberg SM. Low cholesterol as a risk factor for primary intracerebral hemorrhage: A case-control study. Neuroepidemiol 1999;18:185-193.
9. Woo D, Kissela BM, Khoury JC, et al. Hypercholesterolemia, HMG-CoA reductase inhibitors, and risk of intracerebral hemorrhage: a case-control study. Stroke 2004;35:1360-1364.
10. Baigent C, Keech A, Kearney PM, et al. Efficacy and safety of cholesterol-lowering treatment: prospective meta-analysis of data from 90,056 participants in 14 randomised trials of statins. Lancet 2005;366:1267-1278.
11. Wieberdink RG, Poels MM, Vernooij MW, et al. Serum lipid levels and the risk of intracerebral hemorrhage: the Rotterdam Study. Arteriocler Thromb Vasc Biol 2011;31(12):2982-2989.

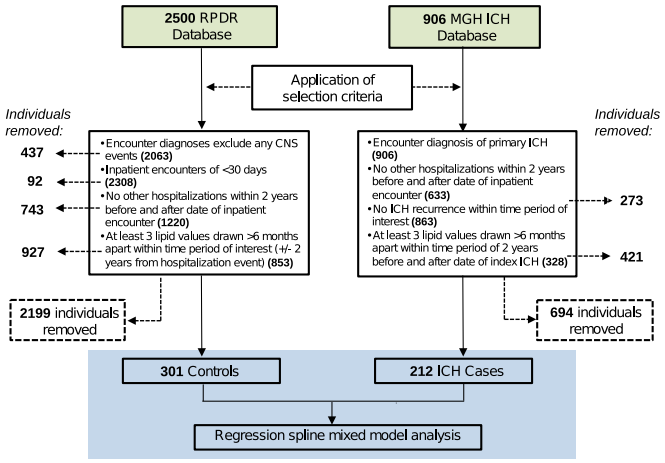
12. Bang OY, Saver JL, Liebeskind DS, et al. Cholesterol level and symptomatic hemorrhagic transformation after ischemic stroke thrombolysis. Neurology 2007;68(10):737-742.
13. Rodriguez-Luna D, Rubeira M, Ribo M, et al. Serum low-density lipoprotein cholesterol level predicts hematoma growth and clinical outcome after acute intracerebral hemorrhage. Stroke 2011;42(9):2447-2452.
14. Mustanoja S, Strbian D, Putaala J, et al. Association of prestroke statin use and lipid levels with outcome of intracerebral hemorrhage. Stroke 2013;44(8):2330-2.
15. Durrington PN. Biological variation in serum lipid concentrations. Scand J Clin Lab Invest Suppl 1990;198:86-91.
16. Schaefer EJ, Lamon-Fava S, Cohn SD, et al. Effects of age, gender, and menopausal status on plasma low density lipoprotein cholesterol and apolipoprotein B levels in the Framingham Offspring Study. J Lipid Res 1994;35(5):779-792.
17. Stevenson JC, Crook D, Godsland IF. Influence of age and menopause on serum lipids and lipoproteins in healthy women. Atherosclerosis 1993;98(1):83–90.
18. Ockene IS, Chiriboga DE, Stanek EJ, et al. Seasonal variation in serum cholesterol levels: treatment implications and possible mechanisms. Arch Intern Med 2004;164(8):863-870.
19. Craig WY, Palomaki GE, Haddow JE. Cigarette smoking and serum lipid and lipoprotein concentrations: an analysis of published data. Br Med J 1989;298:784-8.
20. Man EB, Bettcher PG, Cameron CM, Peters JP. Plasma amino acids, nitrogen and serum lipids of surgical patients. J Clin Invest 1946;25:701-708.

21. Gore JM, Goldberg RJ, Matsumoto AS, Castelli WP, McNamara AB, Dalen JE. Validity of serum total cholesterol level obtained within 24 hours of acute myocardial infarction. Am J Cardiol 1984;54:722-725.
22. Keele KD, Stern PRS. Serum lipid changes in relation to pain. J R Coll Physicians Lon 1973;7:319-329.
23. Mendez I, Hachinski V, Wolfe B. Serum lipids after stroke. Neurology 1987;37:507-511.
24. Hollanders FD, Burton P, Shafar J. Serum lipid changes following completed stroke syndrome. Postgrad Med J 1975;51:386-389.
25. Genes for Cerebral Hemorrhage on Anticoagulation (GOCHA) Collaborative Group. Exploiting common genetic variation to make anticoagulation safer. Stroke 2009;40:S64–S66.
26. Nalichowski R, Keogh D, Chueh HC, and Murphy, SN. Calculating the Benefits of a Research Patient Data Repository. AMIA Annu Symp Proc. 2006; 2006:1044.
27. Naumova EN, Must A, Laird NM. Tutorial in Biostatistics: Evaluating the impact of 'critical periods' in longitudinal studies of growth using piecewise mixed effects models. Int. J Epidemiol 2001;30:1332-1341.
28. McGullicuddy FC, de la Llera Moy M, Hinkle CC, Joshi MR, Chinquoine EH, Billheimer JT, Rothblat GH, Reilly MP. Inflammation impairs reverse cholesterol transport in vivo. Circulation 2009;119:1135–1145.
29. Esteve E, Ricart W, Fernandez-Real JM. Dyslipidemia and inflammation: an evolutionary conserved mechanism. Clin Nutr 2005;24:16–31.

30. Sturgeon JD, Folsom AR, Longstreth WT, Shahar E, Rosamond WD, Cushman M. Risk Factors for Intracerebral Hemorrhage in a Pooled Prospective Study. Stroke 2007;38:2718-2725.
31. Noda H, Iso H, Irie F, Sairenchi T, Ohtaka E, Doi M, et al. Low-Density Lipoprotein Cholesterol Concentrations and Death Due to Intraparenchymal Hemorrhage: The Ibaraki Prefectural Health Study. Circulation 2009;119:2136-2145.
32. Goldstein LB, Amarenco P, Szarek M, Callahan A, Hennerici A, Sillesen H, et al. Hemorrhagic stroke in the Stroke Prevention by Aggressive Reduction in Cholesterol Levels (SPARCL) study. Neurology 2008;24(2):2364-2370.
33. Woo J, Lam CWK, Kay R, Wong HY, Teoh R, and Nicholls MG. Acute and long-term changes in serum lipids after stroke. Stroke 1990;21:1407-1411.
34. Rincon F, Mayer SA. The epidemiology of intracerebral hemorrhage in the United States from 1979 to 2008. Neurocrit Care 2013;19(1):95-102.
35. Lee J, Koh SJ, Yoo SY, Yu JR, et al. Characteristics of Subjects with Very Low Serum Low-Density Lipoprotein Cholesterol and the Risk for Intracerebral Hemorrhage. Korean J Intern Med 2012;27(3):317–326.

FIGURES

Figure 1. Flowchart describing study cohorts and analysis plan



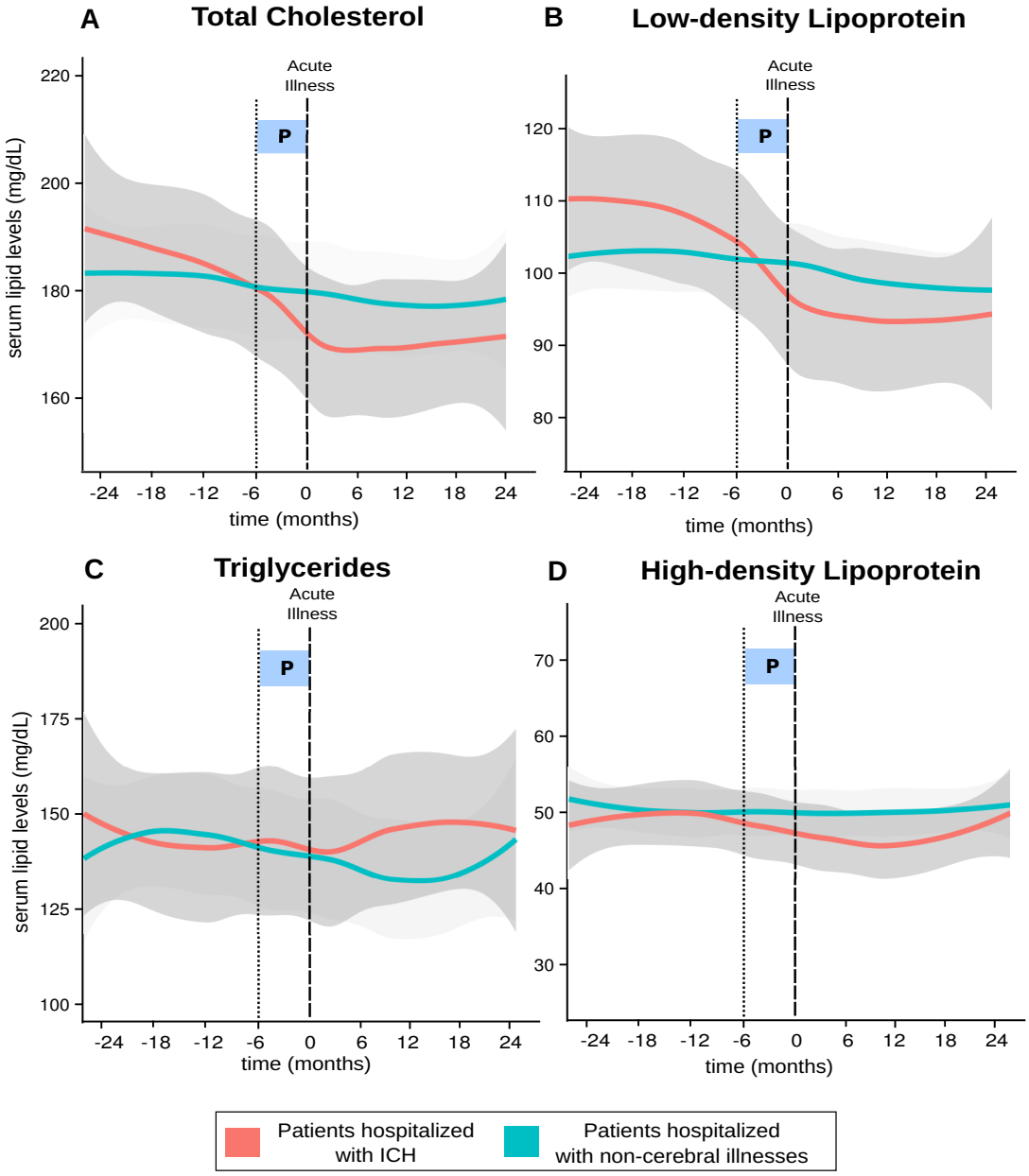
Abbreviations: **MGH ICH Database,** Massachusetts General Hospital-based patients in the Genetics of Cerebral Hemorrhage on Anticoagulation Study; **RPDR,** MGH Research Patient Data Registry**; ICH**, intracerebral hemorrhage.

Table 1. Cohort characteristics and univariate analyses

	Controls (n=301)	ICH (n=212)	*P* Value
Age, y (mean +/- SD)	76.8 +/- 10.4	73.3 +/- 11.3	0.0004[a]
Females, n (%)	88 (29.2)	95 (44.8)	<0.0001[b]
White, n (%)	301 (100.0)	186 (87.7)	<0.0001[b]
HTN, n (%)	114 (37.9)	178 (84.0)	<0.0001[b]
DM, n (%)	60 (19.9)	49 (35.8)	0.001[b]
Alcohol Use, n (%)	177 (58.8)	93 (43.9)	0.47[b]
Smokers, n (%)	163 (54.2)	25 (14.3)	<0.0001[b]
Statin Use, n (%)	153 (50.8)	92 (43.6)	0.11[b]
ASA Use, n (%)	167 (55.5)	106 (50.0)	0.22[b]
Warfarin Use, n (%)	71 (23.6)	58 (27.4)	0.33[b]

Abbreviations: **HTN,** hypertension; **DM**, diabetes mellitus; **ASA**, aspirin. Statistical significance at $p<0.05$, [a] t test, [b] Fisher exact test.

Figure 2. Serum lipid trends in ICH cases and non-ICH controls



E

Serum Lipids	ICH		Non-ICH	
	Wald Test	*P* Value	Wald Test	*P* Value
TC	10.74(1)	**0.001**	0.69(1)	0.41
LDL	8.38(1)	**0.0038**	0.04(1)	0.85
TG	1.07(1)	0.30	0.15(1)	0.70
HDL	6.05(1)	0.02	0.91(1)	0.34

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).

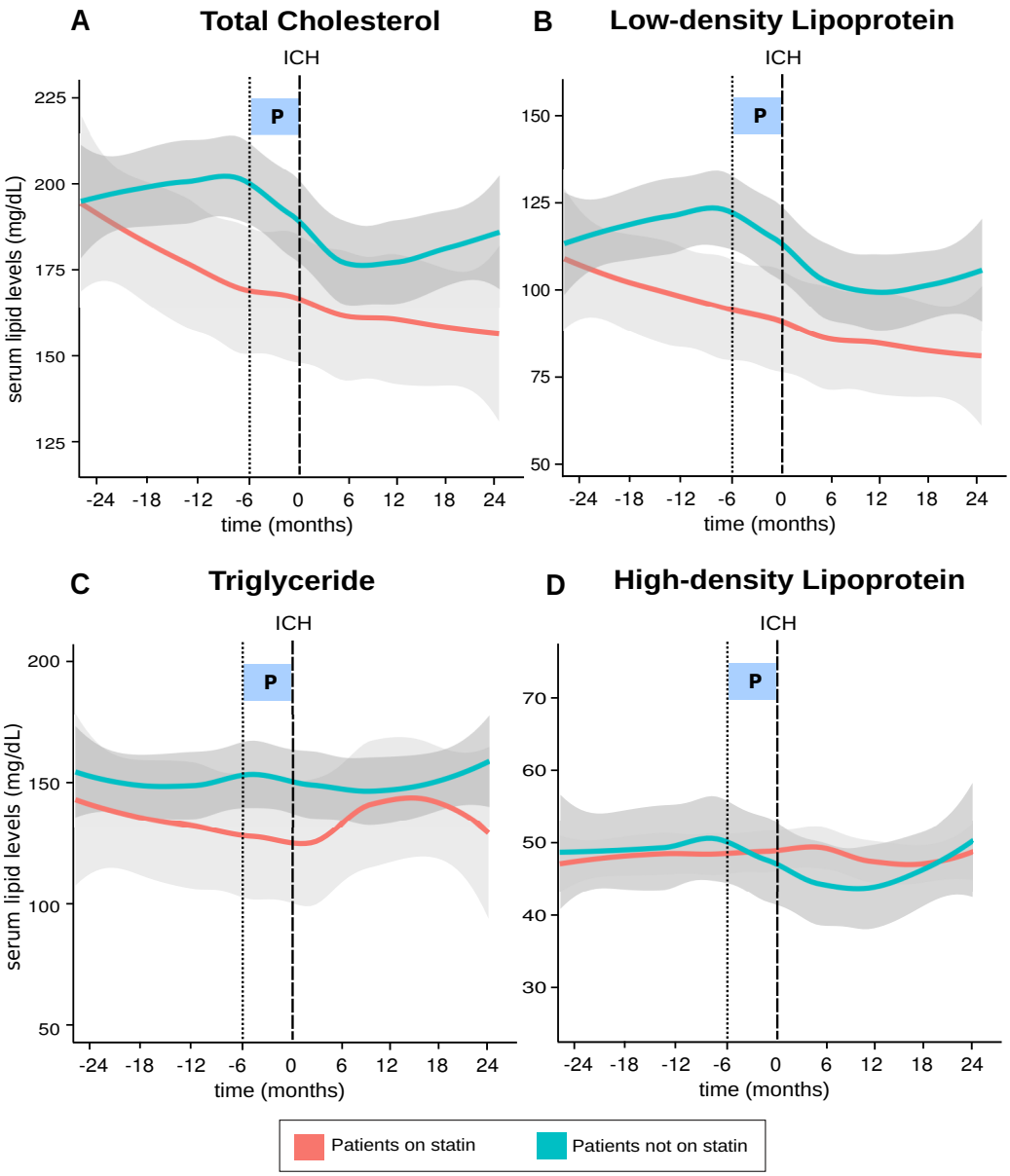
Table 2. Estimated rates of change in serum lipid levels prior to acute illness

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

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-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:

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

	Excluded ICH Cohort (n=694)	*P* Value
Age, y (mean +/- SD)	74.3+/- 11.6	0.35[a]
Females, n (%)	349 (50.3)	0.18[b]
White, n (%)	629 (90.6)	0.38[b]
HTN, n (%)	575 (84.5)	0.9[b]
DM, n (%)	179 (27.5)	0.26[b]
Alcohol Use, n (%)	282 (48.7)	0.19[b]
Smokers, n (%)	56 (11.7)	0.46[b]
Statin Use, n (%)	269 (41.4)	0.72[b]
Lobar ICH, n (%)	321 (46.2)	0.86[b]
ASA Use, n (%)	341 (49.4)	0.93[b]
Warfarin Use, n (%)	204 (32.9)	0.15[b]

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-2. Number of serum lipid measurements available at each time interval

		Pre-Event				Post-Event			
Time Interval		1	2	3	4	5	6	7	8
Serum Lipids		Non-cerebral Acute Illnesses							
	TC	0.42	0.45	0.52	0.60	0.56	0.61	0.49	0.52
	TG	0.39	0.39	0.43	0.51	0.49	0.52	0.46	0.45
	LDL	0.36	0.38	0.40	0.51	0.46	0.52	0.46	0.42
	HDL	0.40	0.39	0.43	0.53	0.49	0.53	0.47	0.47
		ICH							
	TC	0.43	0.40	0.49	0.60	0.38	0.33	0.22	0.32
	TG	0.38	0.38	0.44	0.57	0.37	0.32	0.21	0.30
	LDL	0.36	0.36	0.42	0.52	0.39	0.31	0.21	0.30
	HDL	0.39	0.37	0.46	0.56	0.37	0.32	0.21	0.32

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

$$E(lipid|RE) = \beta 1 + \beta 2\ P1 + \beta 3\ P2 + \beta 4\ Group\ x\ P1 + \beta 5\ Group\ x\ P2 + \beta 6\ P3 + age + sex + race + hypertension + diabetes + smoker + RE\ (individual) + RE\ (time)$$

A

		TC		LDL		TG		HDL	
FE	Variable	Estimate	SE	Estimate	SE	Estimate	SE	Estimate	SE
	Intercept	225.91	11.70	137.78	10.59	147.75	26.35	60.53	4.33
	β2	-0.41	0.91	-0.70	0.84	-2.48	2.39	0.26	0.29
	β3	-3.90	4.70	-0.85	4.33	5.05	13.11	-1.40	1.47
	β4	-3.05	1.29	-0.52	1.18	0.41	3.26	-0.57	0.44
	β5	-21.89	8.34	-19.41	7.53	-26.02	21.46	-4.49	2.57
	β6	-0.59	0.84	-1.79	0.78	-0.10	2.26	0.76	0.26
	Age	-16.81	4.08	-11.91	3.75	-16.32	9.48	1.65	1.57
	Male	-27.63	3.75	-12.47	3.50	-11.05	8.80	-13.82	1.45
	White	-2.20	10.67	-5.10	9.54	16.34	23.64	-3.33	3.94
	HTN	-9.55	3.73	-10.41	3.46	6.94	8.71	-2.30	1.42
	DM	-3.30	4.19	-6.29	3.76	18.59	9.47	-1.94	1.57
	Smoking	-3.48	3.68	-3.01	3.44	-8.18	8.65	2.11	1.41
RE	Time	22.65	4.24	21.37	3.88	80.71	20.24	2.51	0.48
	ID	1670.61	184.75	1452.79	165.23	6075.82	790.82	210.71	22.18
	Residual	522.11	25.10	358.39	19.23	3556.41	170.48	43.12	2.29
LR		-7838.26		-6490.63		-8244.75		-5393.82	

B

	ICH		Controls	
Lipid	P1	P2	P1	P2
TC	-0.19*	-4.89*	-0.02	-0.72
LDL	-0.07*	-3.58*	-0.04	-0.26
TG	-0.12	-3.84	-0.14	+0.43
HDL	-0.02	-1.03	+0.01	-0.19

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

Inpatient Diagnoses include:	n
Shock	20
Sepsis, SIRS	4
Cardiac arrest	3
AMI, Angina	38
Cardiac surgeries	26
Trauma	15
Acute Renal Failure	9
GI Hemorrhage	10
Pneumonia	9
Respiratory Failure	6
Orthopedic Surgeries	28

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:

$$E(lipid|RE) = \beta 1 + \beta 2\ P1 + \beta 3\ P2 + \beta 4\ Group\ x\ P1 + \beta 5\ Group\ x\ P2 + \beta 6\ P3 + age + sex + race + hypertension + diabetes + smoker + RE\ (individual) + RE\ (time)$$

where the variables P1, P2, and P3 each form separate linear spline basis functions (P1=time(24-6 months pre-event); P2=time(0-6 months pre-event); P3=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

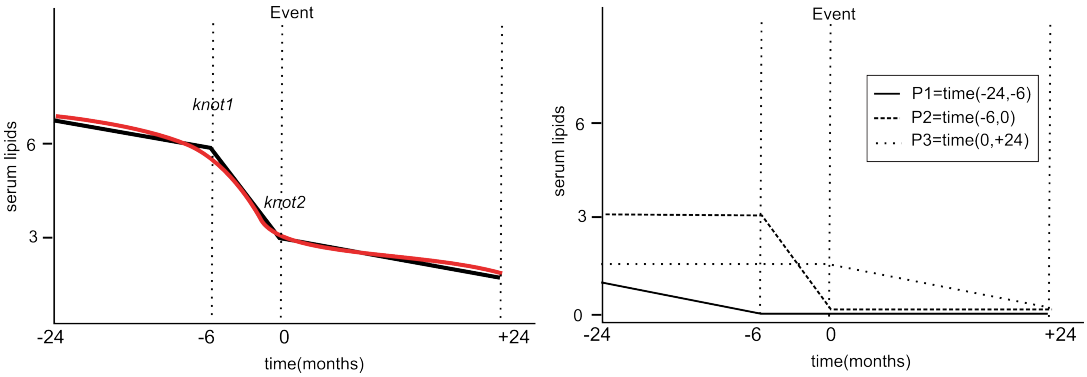
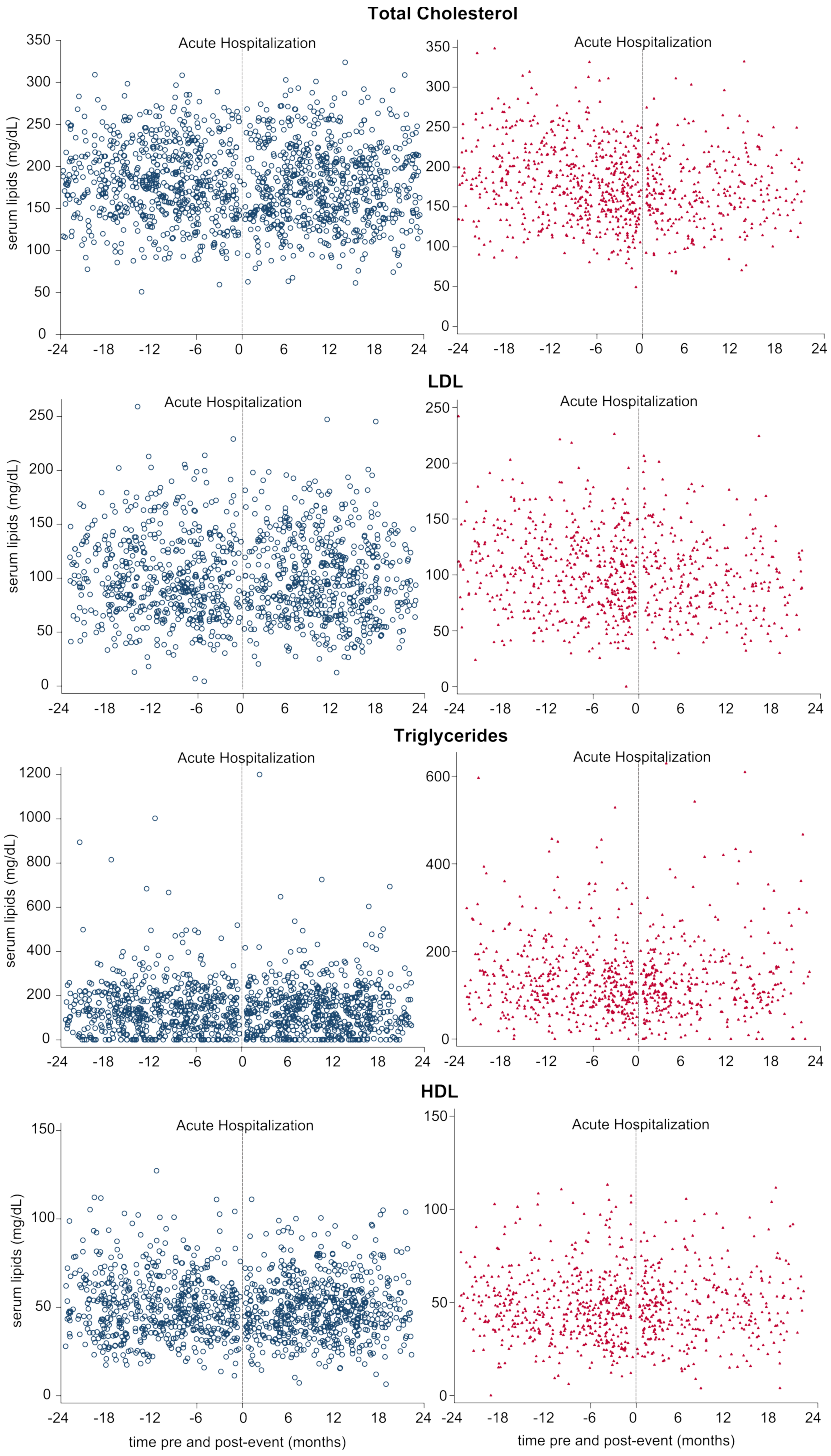


Figure e-2. Scatterplot of serum lipids (mg/dL) against time (months) relative to date of acute hospitalization for non-ICH controls (blue open circle) and ICH cases (red triangles)



LDL, low-density lipoprotein; **HDL**, high-density lipoprotein.

APOE Polymorphisms Influence Longitudinal Lipid Trends Preceding Intracerebral Hemorrhage

Chia-Ling Phuah, MD[1,2,5], Miriam R. Raffeld, BA[2], Alison M. Ayres, BA[3,4], M. Edip Gurol, MD, MSc[3,4], Anand Viswanathan, MD, PhD[3,4], Steven M. Greenberg, MD, PhD[3,4], Alessandro Biffi, MD[2,5,6,7], Jonathan Rosand, MD, MSc[1-5], Christopher D. Anderson, MD, MMSc[1-5]

[1] Division of Neurocritical Care and Emergency Neurology, Massachusetts General Hospital, Boston, MA
[2] Center for Human Genetic Research, Massachusetts General Hospital, Boston, MA
[3] The J. Philip Kistler Stroke Research Center, Massachusetts General Hospital, Boston, MA
[4] Hemorrhagic Stroke Research Group, Massachusetts General Hospital, Boston, MA
[5] Program in Medical and Population Genetics, Broad Institute, Cambridge, MA
[6] Division of Behavioral Neurology, Massachusetts General Hospital, Boston, MA
[7] Division of Psychiatry, Department of Psychiatry, Massachusetts General Hospital, Boston, MA

Title Character Count	89
Abstract Word Count	250
Manuscript Word Count	3111
Figures	3
Tables	2
Number of References	38
Supplementary Data	Table e-1
Search Terms	[7]Intracerebral hemorrhage,[54]Cohort studies,[91]All Genetics, Lipids

Correspondence
Christopher D. Anderson, MD
Center for Human Genetic Research
Massachusetts General Hospital
185 Cambridge Street; CPZN-6818
Boston, MA 02114, USA
Tel: +1 (617) 724-2698
Fax: +1 (617) 643-3293
Email: cdanderson@mgh.harvard.edu

Chia-Ling Phuah	cphuah@partners.org
Miriam R. Raffeld	mraffeld@partners.org
Alison M. Ayres	aayres@mgh.harvard.edu
M. Edip Gurol	edip@mgh.harvard.edu
Anand Viswanathan	aviswanathan1@partners.org
Steven M. Greenberg	sgreenberg@partners.org
Alessandro Biffi	abiffi@mgh.harvard.edu
Jonathan Rosand	jrosand@partners.org

DISCLOSURES

Dr. C-L. Phuah, M. Raffeld , A.M. Ayres, Dr. M. Edip Gurol, and Dr. A. Biffi report no disclosures. Dr. A. Viswanathan is supported by NIH-NINDS K23 AG028726. Dr. S.M. Greenberg is supported by NIH-NINDS U10 NS077360, R01 AG026484, R01 NS070834. Dr. J.Rosand is supported by NIH-NINDS U01 NS069208, R01 NS073344, and R01 NS059727. Dr. C.D. Anderson is supported by NIH-NINDS K23 NS 086873.

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 *APOE* are needed to improve understanding of *APOE* biology in cerebrovascular disease risk.

INTRODUCTION

Primary intracerebral hemorrhage (ICH) accounts for 10-15% of all strokes,[1] 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.[2,3] Previous studies have established ε2/ε4 alleles of the *APOE* gene as potent determinants of ICH risk, severity and outcome.[4-6] *APOE* ε2 and ε4 are associated with increased risk of ICH occuring in the lobar regions of the brain, whereas *APOE* ε4, but not ε2, is associated with risk of nonlobar ICH.[4,7] Separately, several epidemiological studies have also observed an association between serum lipid levels and ICH risk and outcome.[8-16] Hypercholesterolemia has been associated with reduced risk of ICH,[8-13] fewer cerebral microbleeds, and improved outcome following ICH.[15,16] However, despite known functions of *APOE* gene products in lipid transport and regulating circulating lipid levels[17] 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[7] 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.[18] 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 from an ongoing prospective longitudinal cohort study of primary ICH at Massachusetts General Hospital (MGH)[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.[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 occurence. 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.[4] 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.[22] 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.[18] 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 (P_1), the time period 0-6 months immediately pre-ICH (P_2), and time period 0-24 months post-ICH (P_3).

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 ε2 and ε4 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 chacteristics 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.[23]

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.[17] 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 occurence consistent with observed trends seen previously in a larger cohort[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* ε4 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-ε4 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 ε4 carriers compared to non-carriers ($p=0.049$ and $p=0.014$, respectively), while no significant changes were observed in ε2 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 ε4 carriers (Figure 3). This accelerated decline in the 6 month period prior to ICH is consistent with our prior report in a larger cohort.[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* assocations with steady-state serum lipid levels.[17] *APOE* ε4 carriers experienced drops in TC and LDL levels in the 24 month period prior to their ICH, in comparison with non-*APOE* ε4 carriers. Furthermore, in the 6 month period immediately preceding ICH, *APOE* ε4 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 ε2 and ε4 are risk factors for lobar ICH in part through their effects on amyloid processing.[4] *APOE* ε4 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.[24] 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 ε4 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 ε4 genotype. Furthermore, our demonstration of *APOE* allele-specific effects on temporal serum lipid trends in ICH corroborates the notion of divergent mechanistic pathways between ε2 and ε4 alleles in the pathophysiology of ICH.[34,35] 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[36] while *APOE* genotype-specific elevation in pro-inflammatory response has been observed in *APOE* ε4 carriers compared with *APOE* ε2 and *APOE* ε3 carriers in transgenic murine models.[37] 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* allelles 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.[18] A third limitation was our relatively small sample size, particularly with regard to the total number of *APOE* □2 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.[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[38] 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,[18] 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

1. Krishnamurthi RV, Moran AE, Forouzanfar MH, Bennett DA, Mensah GA, Lawes CM, et al. The global burden of hemorrhagic stroke: a summary of findings from the GBD 2010 study. Glob Heart 2014;9:101–106.
2. Poon MT, Fonville AF, Al-Shahi Salman R. Long-term prognosis after intracerebral haemorrhage: systematic review and meta-analysis. J Neurol Neurosurg Psychiatry 2014;85:660–667.

3. van Asch CJ, Luitse MJ, Rinkel GJ, van der Tweel I, Algra A, Klijn CJ. Incidence, case fatality, and functional outcome of intracerebral haemorrhage over time, according to age, sex, and ethnic origin: a systematic review and meta-analysis. Lancet Neurol 2010;9:167-176.
4. Biffi A, Sonni A, Anderson CD, Kissela B, Jagiella JM, Schmidt H, et al. Variants at APOE influence risk of deep and lobar intracerebral hemorrhage. Ann Neurol 2010;68:934–943.
5. Devan WJ, Falcone GJ, Anderson CD, Jagiella JM, Schmidt H, Hansen BM, et al. Heritability estimates identify a substantial contribution to risk and outcome of intracerebral hemorrhage. Stroke 2013;44:1578-1583.
6. Brouwers HB, Biffi A, Ayres AM, Schwab K, Cortellini L, Romero JM, et al. Apolipoprotein E genotype predicts hematoma expansion in lobar intracerebral hemorrhage. Stroke 2012;43:1490-1495.
7. Raffeld MR, Biffi A, Battey TWK, Ayres AM, Viswanathan A, Greenberg SM, et al. APOE ε4 and lipid levels affect risk of recurrent nonlobar intracerebral hemorrhage. Neurology 2015;85:1-8.
8. Woo D, Deka R, Falcone GJ, Flaherty ML, Haverbusch M, Martinin SR, et al. Apolipoprotein E, statins, and risk of intracerebral hemorrhage. Stroke 2013;44:3013-3017.
9. Wang X, Dong Y, Qi X, Huang C, Hou L. Cholesterol levels and risk of hemorrhagic stroke: a systematic review and meta-analysis. Stroke 2013;44:1833–1839.

10. Collins R, Armitage J,Parish S, Sleight P, Peto R. Effects of cholesterol-lowering with simvastatin on stroke and other major vascular events in 20536 people with cerebrovascular disease or other high-risk conditions. Lancet 2004;363:757-767.
11. Segal AZ, Chiu RI, Eggleston-Sexton PM, Beiser A, Greenberg SM. Low cholesterol as a risk factor for primary intracerebral hemorrhage: A case-control study. Neuroepidemiol 1999;18:185-193.
12. Woo D, Kissela BM, Khoury JC, Sauerbeck LR, Haverbusch MA, Szaflarski JP, et al. Hypercholesterolemia, HMG-CoA reductase inhibitors, and risk of intracerebral hemorrhage: a case-control study. Stroke 2004;35:1360-1364.
13. Baigent C, Keech A, Kearney PM, Blackwell L, Buck G, Pollicino C, et al. Efficacy and safety of cholesterol-lowering treatment: prospective meta-analysis of data from 90,056 participants in 14 randomised trials of statins. Lancet 2005;366:1267-1278.
14. Wieberdink RG, Poels MM, Vernooij MW, Koudstaal PJ, Hofman A, van der Lugt A, et al. Serum lipid levels and the risk of intracerebral hemorrhage: the Rotterdam Study. Arteriocler Thromb Vasc Biol 2011;31:2982-2989.
15. Rodriguez-Luna D, Rubeira M, Ribo M, Coscojuela P, Pagola J, Pineiro S, et al. Serum low-density lipoprotein cholesterol level predicts hematoma growth and clinical outcome after acute intracerebral hemorrhage. Stroke 2011;42:2447-2452.
16. Mustanoja S, Strbian D, Putaala J, Meretoja A, Curtze S, Haapaniemi E, et al. Association of prestroke statin use and lipid levels with outcome of intracerebral hemorrhage. Stroke 2013;44:2330-2332.

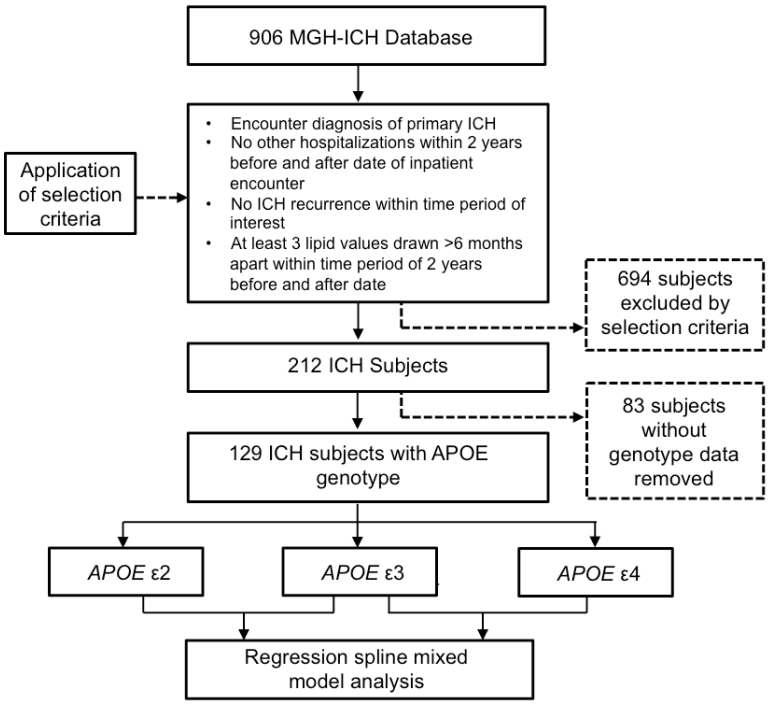
17. Teslovich TM, Musunuru K, Smith AV, Edmondson AC, Stylianou IM, Koseki M, et al. Biological, clinical and population relevance of 95 loci for blood lipids. Nature 2010;466:707-713.
18. Phuah C, Raffeld MR, Ayres AM, Viswanathan A, Greenberg SM, Biffi A, et al. Subacute decline in serum lipids preceds the occurrence of primary intracerebral hemorrhage. Neurology 2016 (in press)
19. O'Donnell HC, Rosand J, Knudsen KA, Furie KL, Segal AZ, Chiu RI, et al. Apolipoprotein E genotype and the risk of recurrent lobar intracerebral hemorrhage. N Engl J Med 2000;342:240-245.
20. Man EB, Bettcher PG, Cameron CM, Peters JP. Plasma amino acids, nitrogen and serum lipids of surgical patients. J Clin Invest 1946;25:701-708.
21. Gore JM, Goldberg RJ, Matsumoto AS, Castelli WP, McNamara AB, Dalen JE. Validity of serum total cholesterol level obtained within 24 hours of acute myocardial infarction. Am J Cardiol 1984;54:722-725.
22. Naumova EN, Must A, Laird NM. Tutorial in Biostatistics: Evaluating the impact of 'critical periods' in longitudinal studies of growth using piecewise mixed effects models. Int. J Epidemiol 2001;30:1332-1341.
23. Ordovas JM, Litwack-Klein L, Wilson PWF, Schaefer MM, Schaefer EJ. Apolipoprotein E isoform phenotyping methodology and population frequency with identification of Apo El and Apo E5 isoforms. J Lipid Res 1987;28:371-380.
24. Woo D, Sauerbeck LR, Kissela BM, Khoury JC, Szaflarski JP, Gebel J, et al. Genetic and Environmental Risk Factors for Intracerebral Hemorrhage: Preliminary Results of a Population-Based Study. Stroke 2002;33:1190-1196.

25. Romero JR, Preis SR, Beiser A, DeCarli C, Viswanathan A, Martinez-Ramirez S, et al. Risk factors, stroke prevention treatments, and prevalence of cerebral microbleeds in the Framingham Heart Study. Stroke 2014;45:1492-1494.
26. Stables MJ, Gilroy DW. Old and new generation lipid mediators in acute inflammation and resolution. Prog Lipid Res 2011;50:35-51.
27. Phillips MC. Molecular mechanisms of cellular cholesterol efflux. J Biol Chem 2014;289:24020-24029.
28. Ledeen RW, Wu G. Nuclear sphingolipids: metabolism and signaling. J Lipid Res 2008;49:1176-1186.
29. Ohanian J, Ohanian V. Sphingolipids in mammalian cell signalling. Cell Mol Life Sci 2001;58:2053-2068.
30. Ooneda G, Yoshida Y, Suzuki K, Sekiguchi T. Morphogenesis of plasmatic arterionecrosis as the cause of hypertensive intracerebral hemorrhage. Virchows Arch A Pathol Pathol Anat 1973;361:31-38.
31. Russell RW. How does blood pressure cause stroke? Lancet 1974;2:1283-1285.
32. Konishi M, Terao A, Doi M, Iida M, Kozima S, Ito M, et al. Osmotic resistance and cholesterol content of the erythrocyte membrane in cerebral hemorrhage. Igaku No Ayumi 1982;120:30-32.
33. Kroes J, Ostwald R. Eryrthocyte membranes – effect of increased cholesterol content on permeability. Biochem Biophys Acta 1971;249:647-650.
34. McCarron MO, Nicoll JA, Stewart J, Ironside JW, Mann DM, Love S, et al. The apolipoprotein E epsilon2 allele and the pathological features in cerebral amyloid angiopathy-related hemorrhage. J Neuropathol Exp Neurol 1999;58:711-718.

35. Greenberg SM, Vonsattel JP, Segal AZ, Chiu RI, Clatworthy AE, Liao A, et al. Association of apolipoprotein E epsilon2 and vasculopathy in cerebral amyloid angiopathy. Neurology 1998;50:961-965.
36. McGullicuddy FC, de la Llera Moy M, Hinkle CC, Joshi MR, Chinquoine EH, Billheimer JT, et al. Inflammation impairs reverse cholesterol transport in vivo. Circulation 2009;119:1135-1145.
37. Jofre-Monseny L, Loboda A, Wagner AE, Huebbe P, Boesch-Saadatmandi C, Jozkowicz A, et al. Effects of apoE genotype on macrophage inflammation and heme oxygenase-1 expression. Biochem Biophys Res Commun 2007;357(1-3):319-324.
38. Durrington PN. Biological variation in serum lipid concentrations. Scand J Clin Lab Invest Suppl 1990;198:86-91.

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.

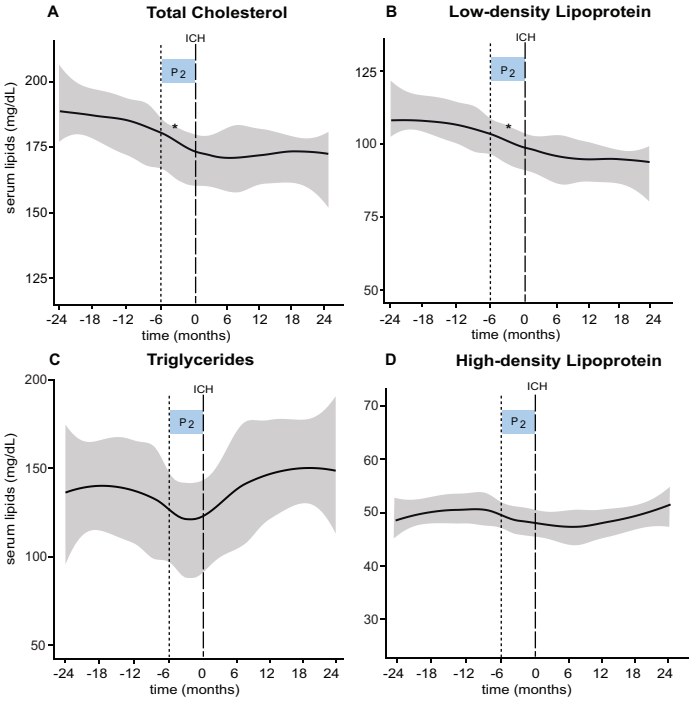
Table 1. Baseline characteristics of study cohort.

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

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

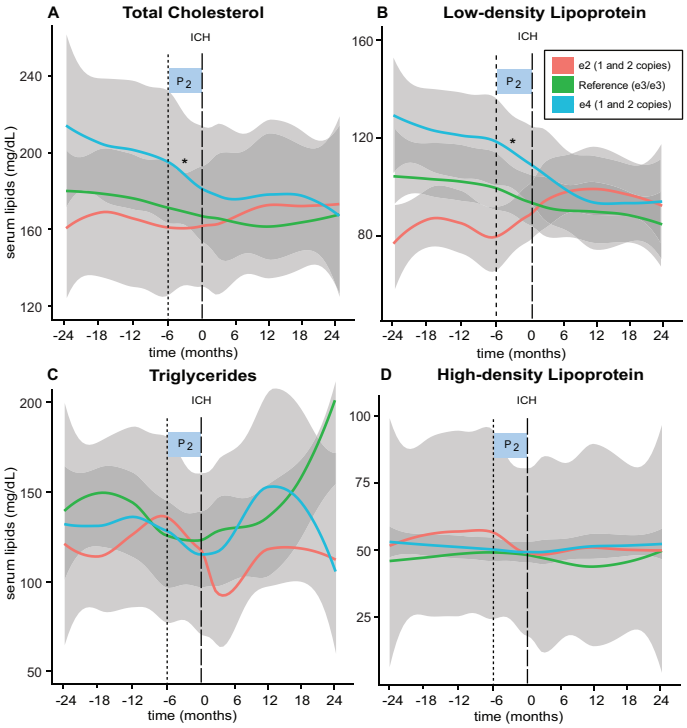
Abbreviations: **HTN**, hypertension; **DM**, diabetes mellitus; **ICH**, intracerebral hemorrhage.

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, (P_2 0-6 months pre-ICH). * p<0.0125, rate of change of serum lipids by Wald test for time period P_2.

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, (P_2 0-6 months pre-ICH). * p<0.0125, rate of change of serum lipids by Wald test for time period P_2 in *APOE* □2 or □4 carrier status compared to reference (*APOE* □3/□3).

Table 2. Estimated rates of change in serum lipid levels pre-ICH by APOE genotype status.

Rate of change in serum lipids (mg/dL/month)			
	Time interval Pre-ICH (months)		*p* Value
	6-24	0-6	
Total Cholesterol			
APOE ε4	-0.05	-7.30	0.0035*
APOE ε3†	-0.19	-3.79	Ref.
APOE ε2	-0.40	+1.72	0.43
Low-density Lipoprotein			
APOE ε4	+0.09	-8.44	0.0001*
APOE ε3†	-0.02	-2.39	Ref.
APOE ε2	-0.40	+5.16	0.07
Triglycerides			
APOE ε4	-0.24	-6.86	0.26
APOE ε3†	-0.10	-6.10	Ref.
APOE ε2	+0.46	-16.06	0.08
High-density Lipoprotein			
APOE ε4	-0.01	-0.06	0.88
APOE ε3†	+0.03	-1.11	Ref.
APOE ε2	-0.02	-0.69	0.65

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* ε3 consist of individuals with *APOE* ε3/ε3 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

	Excluded ICH Cohort (n=83)	*P* Value
Age, y (mean +/- SD)	73.7+/-12.0	0.71[a]
Females, n (%)	39 (47.0)	0.67[b]
White, n (%)	74 (89.2)	0.67[b]
HTN, n (%)	68 (81.9)	0.57[b]
DM, n (%)	14 (25.9)	0.07[b]
Alcohol Use, n (%)	28 (53.9)	0.87[b]
Smokers, n (%)	11 (20.4)	0.16[b]
Statin Use, n (%)	39 (47.0)	0.48[b]
Lobar ICH, n (%)	39 (47.0)	0.97[b]

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