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Apolipoprotein E genotype, cardiovascular biomarkers and risk of stroke: Systematic review and meta-analysis of 14 015 stroke cases and pooled analysis of primary biomarker data from up to 60 883 individuals

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Background At the *APOE* gene, encoding apolipoprotein E, genotypes of the ε2/ε3/ε4 alleles associated with higher LDL-cholesterol (LDL-C) levels are also associated with higher coronary risk. However, the association of

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APOE genotype with other cardiovascular biomarkers and risk of ischaemic stroke is less clear. We evaluated the association of *APOE* genotype with risk of ischaemic stroke and assessed whether the observed effect was consistent with the effects of *APOE* genotype on LDL-C or other lipids and biomarkers of cardiovascular risk.

Methods We conducted a systematic review of published and unpublished studies reporting on *APOE* genotype and ischaemic stroke. We pooled 41 studies (with a total of 9027 cases and 61 730 controls) using a Bayesian meta-analysis to calculate the odds ratios (ORs) for ischaemic stroke with *APOE* genotype. To better evaluate potential mechanisms for any observed effect, we also conducted a pooled analysis of primary data using 16 studies (up to 60 883 individuals) of European ancestry. We evaluated the association of *APOE* genotype with lipids, other circulating biomarkers of cardiovascular risk and carotid intima-media thickness (C-IMT).

Results The ORs for association of *APOE* genotypes with ischaemic stroke were: 1.09 (95% credible intervals (CrI): 0.84–1.43) for $\varepsilon2/\varepsilon2$; 0.85 (95% CrI: 0.78–0.92) for $\varepsilon2/\varepsilon3$; 1.05 (95% CrI: 0.89–1.24) for $\varepsilon2/\varepsilon4$; 1.05 (95% CrI: 0.99–1.12) for $\varepsilon3/\varepsilon4$; and 1.12 (95% CrI: 0.94–1.33) for $\varepsilon4/\varepsilon4$ using the $\varepsilon3/\varepsilon3$ genotype as the reference group. A regression analysis that investigated the effect of LDL-C (using *APOE* as the instrument) on ischaemic stroke showed a positive dose-response association with an OR of 1.33 (95% CrI: 1.17, 1.52) per 1 mmol/l increase in LDL-C. In the separate pooled analysis, *APOE* genotype was linearly and positively associated with levels of LDL-C (*P*-trend: 2×10^{-152}), apolipoprotein B (*P*-trend: 8.7×10^{-06}) and C-IMT (*P*-trend: 0.001), and negatively and linearly associated with apolipoprotein E (*P*-trend: 6×10^{-26}) and HDL-C (*P*-trend: 1.6×10^{-12}). Associations with lipoprotein(a), C-reactive protein and triglycerides were non-linear.

Conclusions In people of European ancestry, *APOE* genotype showed a positive dose-response association with LDL-C, C-IMT and ischaemic stroke. However, the association of *APOE* $\varepsilon2/\varepsilon2$ genotype with ischaemic stroke requires further investigation. This cross-domain concordance supports a causal role of LDL-C on ischaemic stroke.

Keywords Stroke, lipids, apolipoprotein E, cardiovascular disease, systematic review, meta-analysis, biomarkers

Introduction

Worldwide, ischaemic stroke is the second leading cause of death after coronary heart disease (CHD) and is an important cause of disability, with a high burden of disease in low- and middle-income countries.[1,2] Ischaemic stroke and CHD share several risk factors, including increasing age, high blood pressure, smoking and diabetes. However, although low-density lipoprotein cholesterol (LDL-C) is a known risk factor for CHD, its association with ischaemic stroke is uncertain,[3] despite the fact that LDL-C-lowering statin drugs have been found to reduce both ischaemic stroke and CHD risk.[4] This has prompted speculation that the beneficial effect of statins on ischaemic stroke risk might be mediated through alternative pathways.[5]

Numerous studies have examined associations of common genetic variants of the apolipoprotein E (*APOE*) gene and cardiovascular outcomes, including ischaemic stroke and CHD. The *APOE* gene has two common non-synonymous polymorphisms, rs429358 and rs7412, that together give rise to three distinct *APOE* 'alleles' ($\varepsilon2$, $\varepsilon3$, $\varepsilon4$); and these alleles in turn form six possible *APOE* 'genotypes' ($\varepsilon2/\varepsilon2$, $\varepsilon2/\varepsilon3$, $\varepsilon2/\varepsilon4$, $\varepsilon3/\varepsilon3$, $\varepsilon3/\varepsilon4$ and $\varepsilon4/\varepsilon4$).[6]

As confirmed by recent genome-wide association studies (GWAS), variants at the *APOE* locus are one

of the strongest signals for LDL-C,[7–10] and follow a positive dose-response association (when ordered ε2/ε2, ε2/ε3, ε2/ε4, ε3/ε3, ε3/ε4 and ε4/ε4).

In addition to LDL-C, *APOE* genotype has been associated with high-density lipoprotein cholesterol (HDL-C) and triglycerides, though the magnitude and dose-response relationships appear to differ from those described for LDL-C.[11] In addition, *APOE* genotype has been reported to be associated with some inflammatory biomarker such as C-reactive protein (CRP), mainly through the ε4 allele,[12–16] but its association with other inflammatory and coagulation biomarkers is less clear.[17,18] Evidence from a large meta-analysis showed that *APOE* genotype follows a positive dose-response association with CHD concordant with that observed for LDL-C, supporting a causal role of LDL-C in CHD.[11] However, the association of *APOE* genotype with risk of ischaemic stroke is less clear and cannot be reliably extrapolated from the association with CHD. The largest published systematic review on *APOE* genotype and ischaemic stroke was inconclusive with only a tentative evidence for an association of the ε4 allele with high risk of ischaemic stroke.[19] Furthermore, this analysis was based on ε2 and ε4 carriers, whereas an analysis based on all six *APOE* genotypes is needed to estimate the genotype dose-response association. A similar limitation relates to the *APOE* association with carotid intima-media thickness (C-IMT), a non-invasive measure of atherosclerosis linked to both CHD and ischaemic stroke risk, that is used as a surrogate outcome in randomised trials.[20,21]

Genome wide-association studies of ischaemic stroke and C-IMT have not identified any associations with genetic variation at the *APOE* locus.[22,23] However, coverage of the single nucleotide polymorphisms (SNPs) contributing to or marking the APOE ε2/ε3/ε4 genotype is incomplete in arrays used in GWAS, leading to uncertainty regarding the association of *APOE* genotype with C-IMT and stroke risk. We therefore undertook a systematic review and meta-analysis to examine the association between *APOE* genotype and ischaemic stroke risk, including twice as many cases as in the previous largest meta-analysis.[19] The large data set allowed the evaluation of the risk of ischaemic stroke conferred by each of the six *APOE* genotypes individually, as well as the trend in risk across the genotype categories, which has not been possible in prior studies. In addition, to evaluate the potential mechanisms for any observed effect, we undertook a separate pooled analysis of primary data on the relationship between *APOE* genotype and a wide range of lipids and inflammation and coagulation markers as well as C-IMT.

Methods

Two separate meta-analyses were performed to test (i) the association of *APOE* genotype with cardiovascular traits and C-IMT, and (ii) association of *APOE* with ischaemic stroke using both published and unpublished studies.

APOE genotype, cardiovascular traits and C-IMT

We developed a collaboration of 13 studies of European ancestry with information on the association of *APOE* genotypes with a wide range of blood lipids and apolipoproteins; inflammation, coagulation and metabolic markers; and C-IMT. The studies included were the English Longitudinal Study of Ageing (ELSA),[24] the Northwick Park Heart Study II (NPHS II),[25] the Whitehall II study (WHII),[26] the Edinburgh Artery Study (EAS),[27] the British Regional Heart Study (BRHS),[28] the British Women's Heart and Health Study (BWHHS),[29] the Caerphilly and Speedwell studies (CaPS),[30] the UCL Diabetes and Cardiovascular disease Study (UDACS) study,[31] the Aspirin for Asymptomatic Atherosclerosis trial (AAAT),[32] the European Atherosclerosis Research Study (EARS) I and II,[33] the Czech sub-study from the World Health Organization (WHO) Multinational Monitoring of Trends and Determinants in Cardiovascular Disease Study (Czech-MONICA)[34] and the Uppsala Longitudinal Study of Adult Men (ULSAM).[35] These data were supplemented by published data for the European Prospective Investigation of Cancer Norfolk study (EPIC-N),[36] Perth Carotid Ultrasound Disease Assessment Study (CUDAS)[37] and the Rotterdam Study.[38] Data request forms were based on a pre-specified analysis plan and did not include adjustment for lipid-lowering treatment. In a sub-sample of nine studies (11528 individuals), adjustment for age and gender for the *APOE*-LDL-C association was available. The complete data set comprised of 60 883 individuals (Table 1).

Collectively the studies included information on several traits that were selected to interrogate the mechanisms by which variants at the *APOE* locus might exert their effects on ischaemic stroke. These were divided in two groups according to the level of evidence for their associations with *APOE* genotype. Traits with prior evidence for an association were lipids and apolipoproteins [total cholesterol, LDL-C, HDL-C, triglycerides (TG), apolipoprotein A1 (apoA1), apoB, apoE, and lipoprotein (a) (Lp(a)], inflammatory markers [CRP, fibrinogen, interleukin-6 (IL-6)] and C-IMT. Traits with unclear evidence for an association were coagulation factors [Factor VII, fibrinopeptide A, plasma viscosity, prothrombin fragment 1 + 2, tissue plasminogen activator (tPA), D-dimer, von Willebrand factor (vWF)), metabolic traits (homocysteine, insulin, glycated haemoglobin A1c (HbA1c), and creatinine], and some additional inflammatory markers (E-selectin, ICAM, VCAM and ferritin). In addition, we also included lifestyle factors (alcohol and BMI) and established risk factors for cardiovascular disease (blood pressure and glucose). For these traits, we did not anticipate an association

Table 1 Studies contributing to the analysis of APOE genotypes and cardiovascular biomarkers

Study (ref)	ELSA[24]	NPHS-II[25]	WHII[26]	UDACS[31]	EAS[27]	AAAT[32]	EARSI[33]	EARSII[33]	MONICA (CZECH)[34]	ULSAM[35]	BRHS[28]	BWHHS[54]	CaPS[30b]	EPIC Norfolk[36]	CUDAS[37b]	Rotterdam[38b]
Study design[a]	P	P	P	CS	P	P	CC	CC	P	P	P	P	P	P	CS	P
Sampling frame	Respondents of HSE	General practices	Workplace	Diabetic patients	General practices	General practices	Universities	Universities	Administrative districts	Health survey	General practices	General practices	General practices	General Practices	Community Population	Administrative district
N with DNA	5274	2775	5500	575	940	2833	1881	779	2562	879	3947	3500	1500	~22 915	1111	5401
% men	48	100	77	59	50	28	51	100	46.5	100	100	0	100	46	50	59
Year of DNA measurement[c]	2004	1989–94	2002–04	2001–02	2004	1998	1990–91	1993–94	1997–98 & 2000–01	2004	1998–2000	1999–2001	1993–94	1997–2000	1995–96	1992
Baseline year	1998, 1999, 2001	1989–94	1985–88	2001	1987	1998	1990–91	1993–94	1997	1970–73	1978–80	1999–2001	1979–83	1993	1995–96	1990–93
Physical measures																
BMI	5274	2682	5591	544	904		1891	763	2562	874	3514		1317			
Systolic BP	5274	2683	5592	547	903	2397	1891	765	2562	878	3511		1331			
Diastolic BP	5274	2682	5592	547	901	2394	1891	765	2562	878	3511		1330			
Lifestyle measures																
Alcohol		2685			904		1888	764			3464		1332			
Blood measures																
Total-C	5274	2662	5573	547	903	2385	1891	753	2562	879	3497	3240	1296	22 915		
LDL-C		1677	5097	533	898		1887	742		874	3418	3161	1295	22 915		
HDL-C	5274	1774			898		1887	741	2562	877	3386	3236	1296	22 915		
Triglycerides	5274	2664	5196	547	903		1891	753	2562	879	2433	3239	1296			
ApoAI		2287					1855	765		357			1290			
ApoB		2287		383			1853	765		357			1286			
ApoE	5195						1881	765								
Lp(a)		2230			576		1860			357			1249			
Apo-AII													1291			
Homocysteine		1311						757			3456		1291			
Glucose metabolism																
HbA1c	5274		5547	544							3471	3154				
Glucose	5274		4977	547	902		1890	738	2562	878	3495	3221	1297			
Insulin			4635				1766	707		867	2410	3303	672			
Coagulation factors/markers																
Viscosity					863	2383						3093	1286			
Tp(a)		166			767						3513	3250	1212			
vWF		171			819						3513	3251	1178			
D-Dimer		171			816						3509	3251	1178			
Factor VII		2673			578		1624				3504	3234	934			
Prothrombin		2658			572								901			
Fib peptide A		2658			899											

(continued)

Table 1 Continued

Study (ref)	ELSA[24]	NPHS-II[25]	WHII[26]	UDACS[31]	EAS[27]	AAAT[32]	EARSI[33]	EARSII[33]	MONICA (CZECH)[34]	ULSAM[35]	BRHS[28]	BWHHS[54]	CaPS[30b]	EPIC Norfolk[36]	CUDAS[37b]	Rotterdam[38b]
Inflammation markers																
Fibrinogen	5274	2670	4879		885	2185	1697			334	3511	3242	1284			
I-CAM					710											
V-CAM					709											
E-selectin					708											
IL-6		4893		537	635						2032	3253	767			
CRP	5274	4927		536	611	2027		657	2562			3156	3506			
Ferritin	5274															
Renal function																
Creatinine		2664		543						861		3240	1284			
Macrovascular function/structure																
Carotid IMT		3890			837					408				1105		5401

Empty cells denote data not applicable or not available.
[a]Study design: P, prospective; CS, cross sectional; CC, case control.
[b]From published data.
[c]Year of measurement is the year when blood for DNA was obtained. The cardiovascular biomarker measurements are in general contemporaneous with this time.

with *APOE* genotypes, but their assessment served to confirm protection from confounding of the genetic associations as a result of randomized inheritance of parental alleles (Mendel's second law).[39] Further details of the studies and measures are in Table 1 and Supplementary Table S1 (available as Supplementary data at *IJE* online).

Statistical analysis: APOE, cardiovascular traits and C-IMT

ApoB, apoE, plasma viscosity, Lp(a), homocysteine, TG, IL-6 and CRP exhibited a skewed distribution and were natural log transformed. In each study, we calculated the mean and standard deviation for each *APOE* genotype group, and used fixed effects meta-analyses to obtain the standardized mean difference (SMD), weighted mean difference (WMD) and their 95% confidence interval (CI), respectively, for each trait by genotype using the common $\varepsilon3/\varepsilon3$ genotype as the reference. Random effect meta-analysis was also conducted as a subsidiary analysis and is reported in Supplementary Table S2, available as Supplementary data at *IJE* online.[40] Data are presented in the order $\varepsilon2/\varepsilon2$, $\varepsilon2/\varepsilon3$, $\varepsilon2/\varepsilon4$, $\varepsilon3/\varepsilon3$, $\varepsilon3/\varepsilon4$ and $\varepsilon4/\varepsilon4$. I^2 (and its 95% CI) were calculated for each comparison.[41] Because of the multiple comparisons (5 genotype comparisons for 32 traits) we also estimated corrected *P*-values for each estimate using the Sidak method.[42] In order to explore the potential effect that age and gender may have on the association of *APOE* genotypes with LDL-C, we used a sub-sample of nine studies (11 708 participants) and estimated the effect of the *APOE* genotypes with and without adjustment for age and gender. Given that studies included in the analysis span a long calendar period (1990 to 2004), we also stratified the studies in two equal time periods (before and after 1998) to explore the potential effect that year of collection of blood samples from which DNA was extracted may have on the *APOE* genotype-LDL-C association.

To evaluate a linear trend, meta-regression analysis was used on the summary estimated mean differences for each genotype using an additive model.[43,44] The standard normal distribution was used to calculate *P*-values. This analysis was conducted using Stata software (version 11.2).

Meta-analysis of the of APOE genotype with ischaemic stroke

Search strategy

Following the HuGE Review Guidelines update,[45] we searched EMBASE and MEDLINE via PubMed up to March 2011 for articles in any language restricted to 'humans' using free text and MeSH expansion of the following terms: apoe, apo e, apolipoprotein e, brain infarction, cerebrovascular accident, cerebrovascular disease, and stroke. Additional relevant studies were identified through reference lists of published

meta-analyses, review articles and the studies included in the obtained papers.

Case-control and case-cohort studies of unrelated individuals were eligible if ischaemic stroke was verified by computerised tomography (CT) or magnetic resonance imaging (MRI), or if a physician confirmed the diagnosis of stroke, but not if self-reported. Studies reporting white-matter lesions and stroke in children were excluded. We used the largest data set for studies with serial publications and verified the decision with the study authors. We used the control group that most closely resembled the cases where more than one control group was studied. Two reviewers (T.A.K. and J.P.C.) reviewed the papers and consulted a third reviewer (A.D.H.) to resolve any disagreement.

Authors of identified studies were contacted to clarify information on genotype or stroke type where details were incomplete. We requested the following: (i) updated tabular data on six *APOE* genotypes by case-control status, (ii) blinding of lab staff, (iii) duplicate data in publications, (iv) type of stroke and (v) for studies with multiple ethnic groups, genotype counts were requested separately by ethnic group. Additionally we directly approached 88 studies that had reported on ischaemic stroke, to request information on *APOE* genotype, of which 38 replied (35 studies with ischaemic stroke and 3 with haemorrhagic stroke data).

Data were extracted into a pre-specified form and entered in Microsoft Excel. Details entered for each study were: study ID, author, year of publication, study location, study name, ethnicity, number of cases, source of cases and definition and exclusion criteria, control numbers, source of control and exclusion criteria, cases and controls matching variables (age and sex), genotype data on cases and controls, genotype frequencies, blinding of genotyping staff to identity of cases and controls, genotyping method, mean age of cases and controls and percentage of males in cases and controls. Any study showing results separately for more than one ethnicity has been counted as a different data set, so total studies represent total number of data sets.

A chi-square test on control group allele frequencies or whole study allele frequencies in cohort studies was used to assess departure from Hardy–Weinberg (HW) equilibrium.

Statistical analysis

We performed a Bayesian meta-analysis to estimate the unadjusted odds ratios (ORs) and 95% credible intervals (CrI) of ischaemic stroke for each genotype ($\varepsilon2/\varepsilon2$, $\varepsilon2/\varepsilon3$, $\varepsilon2/\varepsilon4$, $\varepsilon3/\varepsilon4$ and $\varepsilon4/\varepsilon4$) against the reference genotype $\varepsilon3/\varepsilon3$. This order was decided on the basis of association of *APOE* genotypes with LDL-C. A Bayesian model was used to estimate the five ORs simultaneously in one model using Markov Chain Monte Carlo techniques[46] (for details of the model see Supplementary methods available at *IJE* online). The Bayesian approach has several advantages: It estimates simultaneously the five ORs mentioned above, taking into the fact that they are not independent from each other. It provides posterior credibility intervals that are more naturally interpreted than frequentist confidence intervals. It also allows for the use of the data coming from small studies without having to add any extra numbers to the situations when zero counts appear in an estimation of an OR.[47]

To explore the trend of these ORs (if any) we estimated a meta-logistic regression model with ischaemic stroke as the outcome and the *APOE* genotypes as the explanatory variable (for details of the model see Supplementary methods available at *IJE* online). *APOE* genotypes were coded numerically using the mean difference in LDL-C between each genotype and the $\varepsilon3/3$ group, as follows: $\varepsilon2/2 = -0.756$ mmol/l, $\varepsilon2/3 = -0.481$ mmol/l, $\varepsilon2/4 = -0.241$ mmol/l, $\varepsilon3/3 = 0$ mmol/l (reference), $\varepsilon3/4 = 0.161$ mmol/l, $\varepsilon4/4 = 0.312$ mmol/l. This mean difference for LDL-C was derived from our *APOE*-LDL-C analysis from studies in European ancestry populations. The summary OR derived from the trend analysis could be indirectly interpreted as the effect of LDL-C on ischaemic stroke through the use of *APOE* $\varepsilon2/\varepsilon3/\varepsilon4$ loci as the genetic instrument. To allow for a non-constant OR (equivalent to a non-linear trend in the log scale) we included the square of the explanatory variable (i.e. LDL-C^2) in the regression model. If the posterior 95% CrI of the coefficient of the quadratic term included the null value, we repeated the analysis simplifying the model by removing the quadratic term and estimating only a linear trend.

We undertook several sensitivity analyses by re-estimating the logistic regression in different subsets of studies considered to be at lower risk of bias. These subsets were: studies with more than 100 cases, studies with control samples in HW equilibrium, studies with no zero cells for all *APOE* genotype categories, studies with genotyping staff blinded to case-control status, studies with imaging confirmed diagnosis of ischaemic stroke and studies that used new techniques for *APOE* genotyping (e.g. Taqman genotyping assay, One-tube LightTyper assay, Heteroduplex method, Pyrosequencing) that are less susceptible to genotyping miscalls.[48,49] In order to evaluate the potential for publication bias (small-study bias) we, as a sensitivity analysis, re-estimated the effect of the *APOE* genotype on published versus unpublished studies, excluding small studies and re-quantifying the *APOE*-effect.

For the above-mentioned analyses we used fixed-effect models for the primary analysis; however, random effect models were also fitted and the results are reported as Supplementary material (available as Supplementary data at *IJE* online).

Priors

We used priors centred on the null that were non-informative in any specific direction but would

cover a reasonable range of hypotheses by restricting their variance, e.g. an OR would have a prior distribution centred on 1, with a 95% probability of being between 0.03 and 30. For parameters in the log scale [log(OR) and log(odds)] we used normal distributions. For the standard deviation between studies in the random effects model we used a uniform prior between 0 and 1.5 times the observed standard deviation between the different studies. Bayesian analyses were performed using R (version 2.13) and JAGS software (version 2.2.0).

Results

APOE genotype, LDL-C and C-IMT

Twelve studies contributed data on *APOE* genotypes and LDL-C levels (Table 1). *APOE* genotypes exhibited a positive trend with LDL-C (47 771 subjects, *P*-trend: 2.1×10^{-49}). With $\varepsilon 3/3$ as reference, the mean differences (in mmol/l) were: −0.75 (95% CI: −0.86, −0.65) for $\varepsilon 2/2$, −0.48 (95% CI −0.50, −0.46) for $\varepsilon 2/3$, −0.24 (95% CI: −0.24, −0.01) for $\varepsilon 2/4$, 0.16 (95% CI: 0.14, 0.18) for $\varepsilon 3/4$ and 0.32 (95% CI: 0.26, 0.37) for $\varepsilon 4/4$ (Figure 1a). There was a difference of 1.07 mmol/l between $\varepsilon 2/\varepsilon 2$ and $\varepsilon 4/\varepsilon 4$ individuals for LDL-C. These point estimates were used in the Bayesian meta-analysis to determine the coding of *APOE* genotypes and therefore to evaluate indirectly the effect of LDL-C on ischaemic stroke using a trend analysis. This positive dose-response between *APOE* genotypes and LDL-C remained unaltered in the sub-sample of studies that had provided data adjusted by age and gender (Supplementary Figure S1, available as Supplementary data at *IJE* online). Likewise, when the *APOE*-LDL-C analysis was stratified according to the time of DNA extraction, similar results were observed (Supplementary Figure S2, available as Supplementary data at *IJE* online).

Five studies contributed data on *APOE* genotypes and C-IMT (Table 1). We found a positive dose-response association between *APOE* genotypes and C-IMT (11 641 subjects, *P*-trend: 0.001) that strongly resembled the association between *APOE* genotype and LDL-C. There was a mean difference of 0.043 mm of C-IMT between $\varepsilon 2/\varepsilon 2$ and $\varepsilon 4/\varepsilon 4$ individuals (Figure 1b).

Association of APOE genotype with other lipids and apolipoproteins

Sixteen studies contributed data on *APOE* genotypes and cardiovascular phenotypes. *APOE* genotype exhibited a positive trend with total cholesterol (54 377 subjects, *P*-trend: 2×10^{-152}) and apoB (6931 individuals, *P*-trend: 8.7×10^{-06}), similar to the association with LDL-C described above (Figure 2). We observed a negative trend of *APOE* genotype with levels of apoE (7841 individuals, *P*-trend: 6×10^{-26}) and a weak negative trend association with HDL-C (50 571 individuals, *P*-trend: 1.6×10^{-49}) and apoAI (6554 individuals, *P*-trend: 5.3×10^{-8}). Associations of *APOE* genotype with other lipids and lipoproteins were more complex in shape. There was a non-linear relationship between *APOE* genotype and triglycerides (27 637 individuals), with both $\varepsilon 2/\varepsilon 2$ and $\varepsilon 4/\varepsilon 4$ individuals having higher concentrations of triglycerides, compared with the $\varepsilon 3/\varepsilon 3$ reference group. We also identified an association between *APOE* genotype and Lp(a) among 6272 individuals (Figure 2).

Association of APOE genotype with inflammation markers

We found a marked association of *APOE* genotype with CRP concentration (26 401 individuals). With reference to the $\varepsilon 3/\varepsilon 3$ group, individuals with the $\varepsilon 4/\varepsilon 4$ genotype had the lowest concentration of CRP, whereas individuals with the $\varepsilon 2/\varepsilon 4$ genotype and the $\varepsilon 3/\varepsilon 4$ genotype had CRP levels approximately midway between the $\varepsilon 3/\varepsilon 3$ and $\varepsilon 4/\varepsilon 4$ groups. There was no difference in CRP levels among individuals with the $\varepsilon 2/\varepsilon 2$ or $\varepsilon 2/\varepsilon 3$ genotypes. We did not find any association of *APOE* genotype with another hepatocyte-derived inflammation marker, fibrinogen (25 961 individuals, *P*-trend: 0.09), with the macrophage/adipocyte-derived inflammation marker IL-6 (12 117 individuals, *P*-trend: 0.9) (Figure 3), or with a range of other inflammation markers (Supplementary Figure S3, available as Supplementary data at IJE online).

Association of APOE with coagulation markers, metabolic traits and other variables related to cardiovascular risk

We found no evidence for an association of *APOE* genotype with the coagulation markers factor VII (12 547 individuals), fibrinopeptide A (3 557 individuals), D-dimer (8925 individuals) or tPA (58 908 individuals), the endothelial activation markers vWF (8932 individuals) and E-selectin (708 individuals), systolic blood pressure (28 334 individuals), BMI (25 916 individuals), alcohol consumption (11 037 individuals) or indices of glycaemic control including HbA1c (17 990 individuals), fasting glucose (25 781 individuals) and insulin (14 360 individuals) (Figure S4).

Mean difference (95%CI), I^2 (95% CI), unadjusted and Sidak-corrected *P*-values for each of the *APOE* genotype associations with cardiovascular traits are provided in Supplementary Table S2 (available as Supplementary data at *IJE* online).

APOE genotype and ischaemic stroke

Search results

We screened 1395 abstracts from the primary search and identified 249 potentially eligible articles. Of these, 2 studies included self-reported stroke, 7 contained duplicate data and a further 163 reported incomplete data or were not ischaemic stroke, and were excluded, leaving 77 studies reporting stroke that

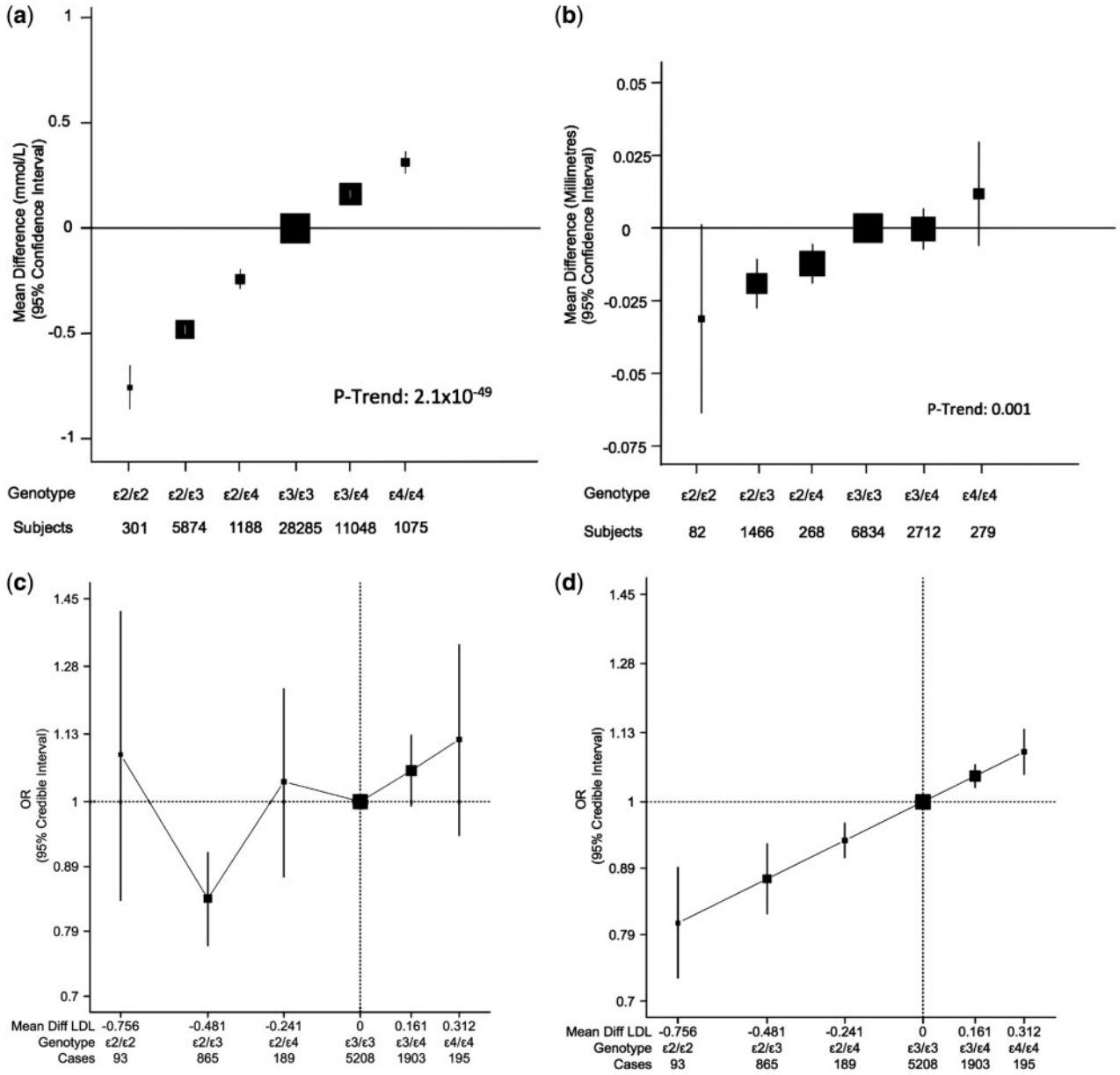

Figure 1 Association of APOE genotypes in studies of European ancestry individuals with (a) LDL-cholesterol (b) carotid intima-media thickness, (c) ischaemic stroke and (d) ischaemic stroke (trend analysis). Black boxes indicate summary estimates with their size proportional to weight. For (c) and (d), the x-axis is plotted in the log-scale with distance between APOE genotypes equal to mean difference of LDL-C in mmol/l. For (d), the effect estimate of trend analysis indicates an OR of 1.33 (95% CrI: 1.17, 1.52) per 1 mmol/l increase in LDL-C

were included in this analysis (Supplementary Figure S5, available as Supplementary data at *IJE* online). Of these, 64 included information on ischaemic stroke and 19 gave information on haemorrhagic stroke (there was some overlap as 16 studies gave information on both). Thirteen studies reported strokes that were not classified as ischaemic or haemorrhagic. Of these, 10 were studies in European ancestry subjects[28,35,36,50–54] (2265 stroke events) exclusively and these were counted as ischaemic stroke since 80% of stroke in European ancestry is ischaemic in nature.[2] This took the total number of ischaemic stroke studies to 74 with total of 14 015 cases and 77 888 controls. This included data from 39 published studies 3635 cases and 42 024 controls from previously unpublished studies from 35 from complete unpublished data sets plus data sets updated from previous published studies.

A description of study design, source of controls, use of neuroimaging, genotyping method, blinding, and number of cases and controls, observed and expected genotype and allele frequencies (by ethnic groups) is

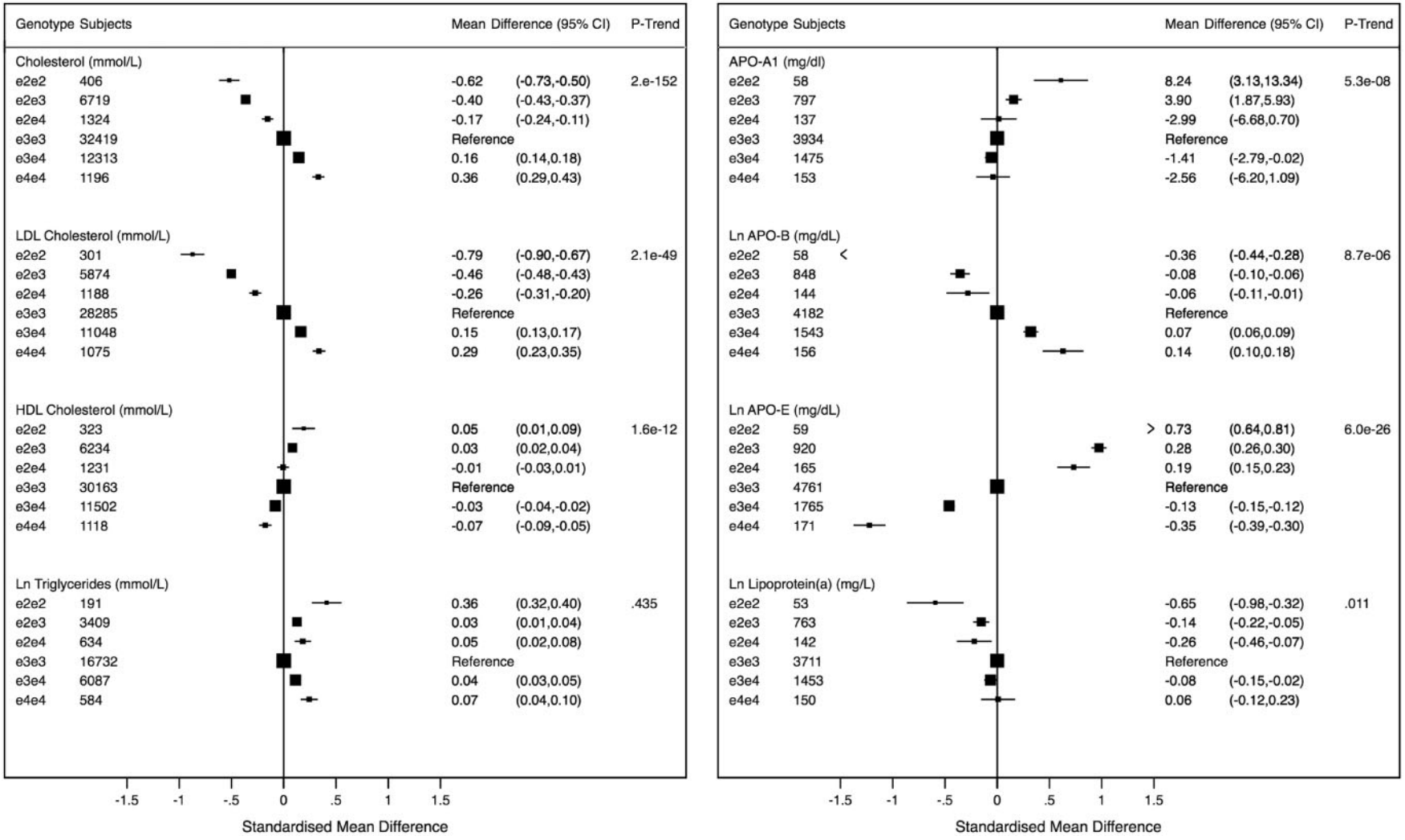

Figure 2 APOE genotypes and lipid and apolipoprotein traits. The graphs are displayed in standardized scale to allow comparability and show standardized mean differences of biomarker levels with *APOE* genotypes with $\varepsilon 3/\varepsilon 3$ as reference. The values (on the right) correspond to absolute weighted mean difference. Black boxes indicate estimates proportional to counts and horizontal lines represent 95% CI. Ln, natural log transformed

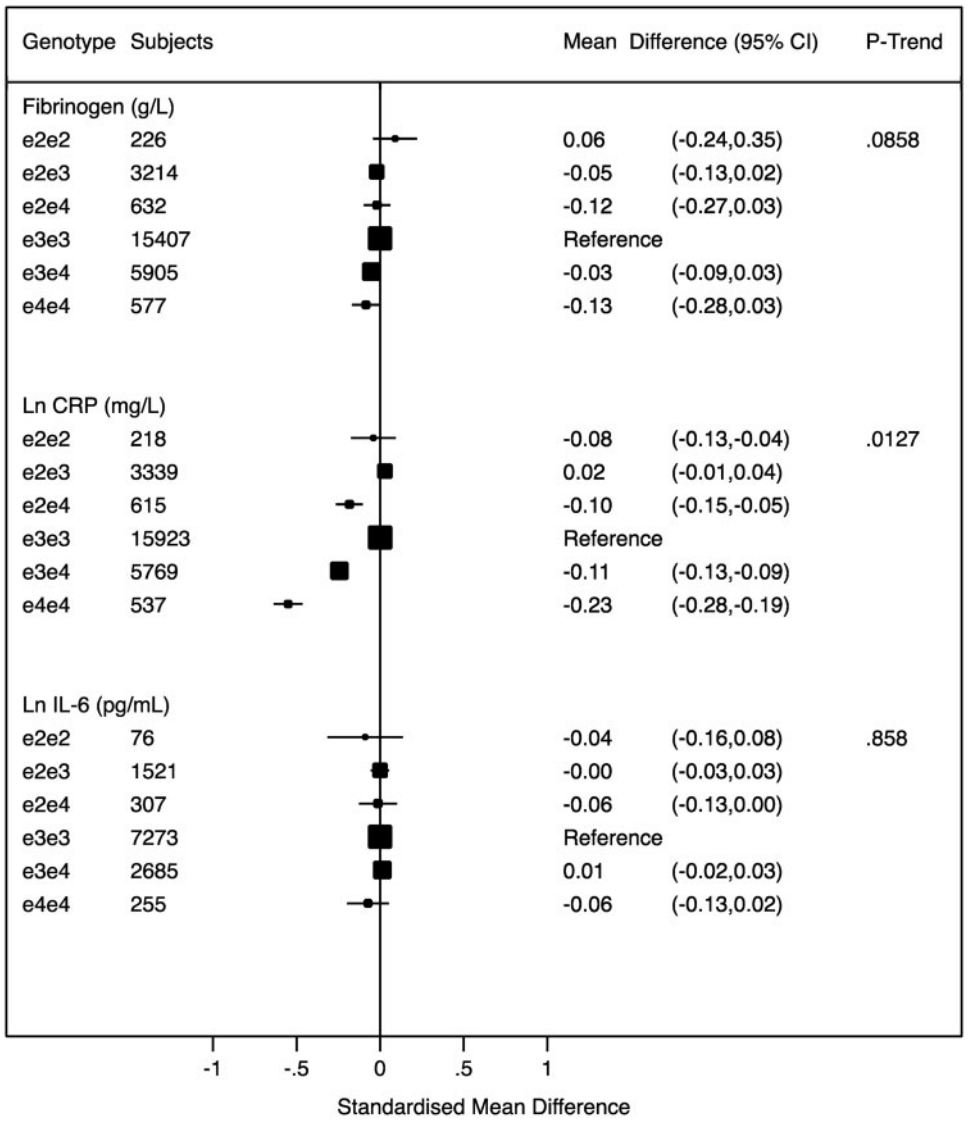

Figure 3 APOE genotypes and mean differences for inflammatory traits. The graphs are displayed in standardized scale to allow comparability and show standardised mean differences of biomarker levels with APOE genotypes with $\varepsilon 3/\varepsilon 3$ as reference. The values (on the right) correspond to absolute weighted mean difference. Black boxes indicate estimates proportional to counts and horizontal lines represent 95% CI. Ln, natural log transformed

given in Supplementary Tables S3, S4 and S5, available as Supplementary data at *IJE* online.

Association of APOE genotype with ischaemic stroke in European ancestry individuals

In 41 studies (14 prospective and 27 case-control studies), with a total of 9027 cases and 61 730 controls of European ancestry using the $\varepsilon 3/\varepsilon 3$ genotype as the reference group, the ORs for ischaemic stroke were 1.09 (95% CrI: 0.84, 1.43) for $\varepsilon 2/\varepsilon 2$, 0.85 (95% CrI: 0.78, 0.92) for $\varepsilon 2/\varepsilon 3$, 1.05 (95% CrI: 0.89, 1.24) for $\varepsilon 2/\varepsilon 4$, 1.05 (95% CrI: 0.99, 1.12) for $\varepsilon 3/\varepsilon 4$, and 1.12 (95% CrI: 0.94, 1.33) for $\varepsilon 4/\varepsilon 4$ (Figure 1c). We note the large uncertainty in OR estimates around the less common genotypes ($\varepsilon 2/\varepsilon 2$, $\varepsilon 2/\varepsilon 4$ and $\varepsilon 4/\varepsilon 4$), and in particular the $\varepsilon 2/\varepsilon 2$ genotype which only contributed 1% of the total cases.

We initially fitted a model that included a quadratic variable (LDL-C^2) to allow for a non-linear trend, but the coefficient for this variable had a posterior credibility interval that included the null and we decided to remove this term and estimate a simpler model only with a linear trend. The results of the linear trend of the log-odds of *APOE* $\varepsilon 2/\varepsilon 3/\varepsilon 4$ genotypes (coded in terms of the *APOE* effect on LDL-C) on ischaemic stroke are described in Figure 1d. In this regression model, the OR of ischaemic stroke per 1 mmol/l increase in LDL-C was 1.33 (95% CrI: 1.17, 1.52), equivalent to an OR of 0.75 (95% CrI: 0.66, 0.85) per 1 mmol/l reduction in LDL-C. An exploratory sensitivity analysis showed that this positive linear trend remained largely

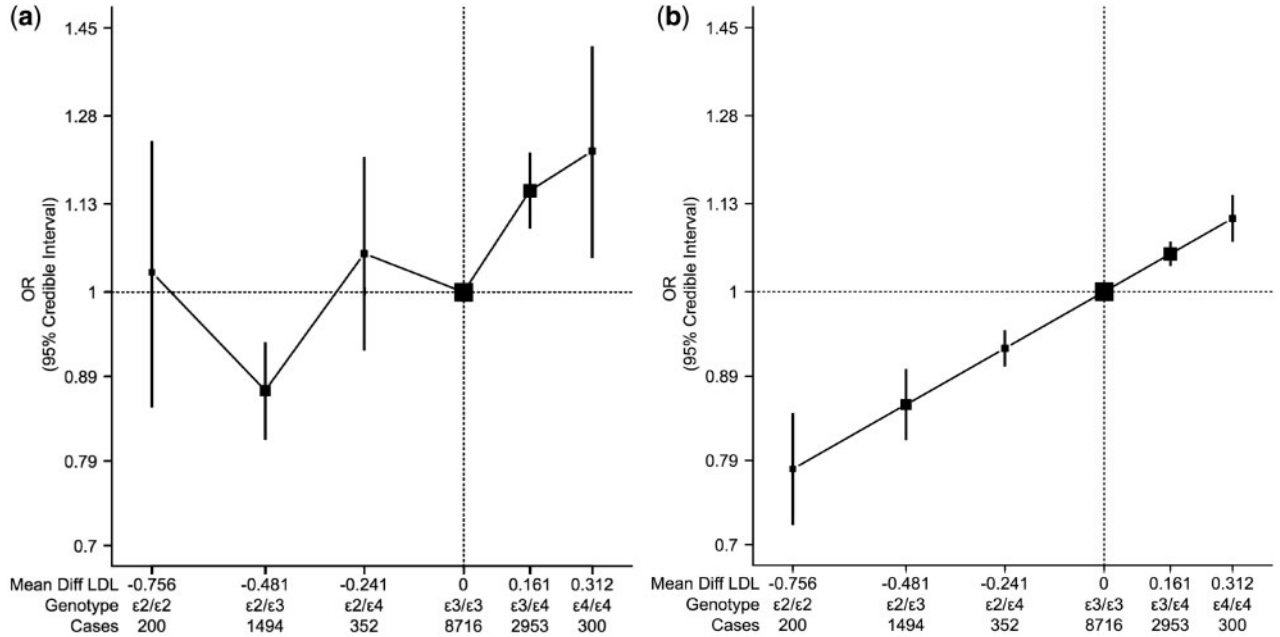

Figure 4 Association of APOE genotypes with ischaemic stroke in all ethnicities with (a) ischaemic stroke, (b) ischaemic stroke (trend analysis). Black boxes indicate summary estimates with their size proportional to weight. The x-axis is plotted in the log-scale with distance between APOE genotypes equal to mean difference of LDL-C in mmol/l. For (b), the effect estimate of trend analysis indicates an OR of 1.39 (95% CrI: 1.25, 1.54) per 1 mmol/l increase in LDL-C

unaltered in all the subgroups (Supplementary Figure S6, available as Supplementary data at *IJE* online), with none of them showing strong evidence of a quadratic trend (the coefficient of the quadratic term had a posterior interval that included the null).

The random effects model showed very similar results to the fixed effects analysis despite the greater uncertainty in the effect estimates for less frequent *APOE* genotypes. The trend analysis was also concordant with the fixed effects model (Supplementary Figure S7, available as Supplementary data at *IJE* online).

Association of APOE genotype with ischaemic stroke in all ethnicities

In 74 studies (16 prospective and 58 case-control studies), with a total of 14 015 cases and 77 888 controls in all populations using the $\varepsilon 3/\varepsilon 3$ genotype as the reference group, the ORs for ischaemic stroke were 1.05 (95% CrI: 0.87, 1.26) for $\varepsilon 2/\varepsilon 2$, 0.88 (95% CrI: 0.82, 0.94) for $\varepsilon 2/\varepsilon 3$, 1.06 (95% CrI: 0.93, 1.21) for $\varepsilon 2/\varepsilon 4$, 1.15 (95% CrI: 1.09, 1.21) for $\varepsilon 3/\varepsilon 4$, and 1.22 (95% CrI: 1.05, 1.41) for $\varepsilon 4/\varepsilon 4$ (Figure 4a).

A visual inspection of the trend analysis of the log-odds of *APOE* on ischaemic stroke indicated the presence of a positive dose-response over the greater part of the LDL-C range (indirectly measured by the *APOE* genotypes). Under the assumption of a common model across ethnic groups for the LDL-C–ischaemic stroke association, we fitted a linear model and estimate that the OR of ischaemic stroke for 1 mmol/l of increase in LDL-C was 1.39 (95% CrI: 1.25, 1.54) (Figure 4b). However, we also allow for the possibility of alternative models (i.e. quadratic model), and these results are included in our sensitivity analysis (Supplementary Figure S8, available as Supplementary data at *IJE* online). The random effects model produced very similar findings to the fixed effects model but with wider credible intervals (Supplementary Figure S9, available as Supplementary data at *IJE* online).

Assessment of the cumulative evidence, using the Venice criteria, suggest that the epidemiological credibility for the *APOE*-ischaemic stroke association is strong in European population studies and moderate or weak in all other ethnic groups (Supplementary Table S6, available as Supplementary data at *IJE* online).

The results of the sensitivity analysis that excluded small studies and the of one that re-estimated the effect for published and unpublished studies independently did not differ from the overall result, reducing the possibility that small-study bias influenced our results (Supplementary Figures S6 and S8, available as Supplementary data at *IJE* online).

In 11 studies with a total of 759 cases and 22 193 controls, using the $\varepsilon 3/\varepsilon 3$ genotype as the reference, no clear effect for any of the *APOE* genotypes with haemorrhagic stroke was observed (Figure S10, available as Supplementary data at *IJE* online).

Discussion

Using the largest collection of studies yet accumulated, our analysis of European populations confirmed the

presence of a positive dose-response association between *APOE* genotype and LDL-C, and clarified the genetic effect on C-IMT, which followed the same dose-response trend observed for LDL-C. The pattern was concordant in the meta-analysis of European ancestry studies on ischaemic stroke that also showed a positive dose-response association when the *APOE* genotypes were ordered and coded to reflect their effects on LDL-C (Figure 1). The large degree of consistency across the different outcome domains evaluated, [LDL-C (intermediate phenotype), C-IMT (surrogate end-point) and ischaemic stroke (clinical event)] argues for a causal effect of LDL-C on ischaemic stroke, and provides an adequate explanation for the beneficial effects on ischaemic stroke observed with statins in randomized trials.

In this respect, it is important to note that the estimate derived from our regression model in European ancestry studies that used *APOE* genotypes as instrument for LDL-C showed an OR of ischaemic stroke of 0.75 (95% CrI: 0.66, 0.85) per 1 mmol/l LDL-C reduction, which is consistent with the rate ratio of 0.79 (95% CI: 0.74, 0.85) for ischaemic stroke for the same LDL-C reduction derived from randomized trials of statins.[55]

The large size of the data set synthesised in this report that included 2392 more cases of ischaemic stroke (including 6362 cases from unpublished and updated collections) than previous meta-analyses,[19] permitted for the first time a reliable evaluation of the risk of ischaemic stroke in each of the six *APOE* genotype groups separately. Also, using *APOE* genotype as an instrument for LDL-C enabled the presence of a positive dose-response trend of LDL-C on ischaemic stroke to be determined. This is the largest study examining association of *APOE* genotypes with C-IMT to date, and shows that the genotypes are associated with atherosclerosis in a positive dose-response manner. This was similar in magnitude to the results of a previous meta-analysis examining this association.[20] This current study also suggests that the *APOE* effect on ischaemic stroke is mediated via LDL-C, providing further evidence of a causal role for this lipid fraction, which has been a point of conflict over several decades of research.

The finding of a positive dose-response effect of LDL-C (indexed by *APOE* genotype) on ischaemic stroke in European ancestry individuals was supported by the results from our sensitivity analysis that was restricted to the subset of studies considered to be at low risk of bias according to study level characteristics, such as study size, outcome diagnosis, blinding of genotyping staff, or genotyping technique. However, it is important to note that despite the large sample size included in this analysis, we still observed considerable uncertainty on the effect estimates for ischaemic stroke for the less frequent *APOE* genotypes, in particular the $\varepsilon2/\varepsilon2$ genotype.

When we extended our analysis of the *APOE* loci on ischaemic stroke to genetic studies from all ethnic groups, a similar dose-response was observed for most of the association. However, a slight increase on ischaemic stroke for the section of the regression analysis associated with lower LDL-C levels, indexed by the genotypes $\varepsilon2/\varepsilon3$ and $\varepsilon2/\varepsilon2$, was observed. Our sensitivity analysis, however, indicated that a potential explanation for this apparent increase in risk could be due to genotyping errors as showed by the analysis restricted to the subset of studies in HW equilibrium, with blinding of the genotyping staff or those who used recent genotyping techniques. In this subset, the slight increase in risk of ischaemic stroke for $\varepsilon2/\varepsilon2$ genotypes was not seen and instead a positive dose-response relationship emerged (Supplementary Figure S6, available as Supplementary data at *IJE* online). A concern that some of the *APOE* genotypes may be prone to miscalling has been previously shown by some researchers,[48] indicating that studies that used Hixson and Vernier's PCR-RFLP method of genotyping or similar methods[56,57] are more likely to have an excess of $\varepsilon2/\varepsilon2$ genotypes.[48,58] This limitation is overcome by the newer *APOE* genotyping techniques (e.g. LightTyper assay and Taqman).[49]

A major limitation to this study is that we could not reliably assess the possibility of differential effects on the sub-types of ischaemic stroke. It has been postulated that different ischaemic stroke sub-types exhibit different associations with cholesterol and *APOE* genotype, suggesting differences in the underlying pathologies.[19] Although information on haemorrhagic stroke was limited in our study, it was concordant with the lack of effect seen in observational studies (see Supplementary data at *IJE* online). However, our haemorrhagic stroke analysis had considerably less statistical power than our ischaemic stroke analysis. Larger studies are needed to answer this question with more confidence.[59] The $\varepsilon4$ allele already has a validated association with Alzheimer's disease;[60] however, misclassification of Alzheimer's with ischaemic stroke is very unlikely as the two diseases have very distinct clinical and imaging profiles, and thus we believe this misclassification did not play a role in our results.

Small-study bias can be a major limitation in any meta-analysis. The analysis of *APOE* genotypes and cardiovascular traits was entirely based on *de novo* data and included a sample size of 60 883 individuals. This avoids the impact of small-study bias which is mainly observed in meta-analyses based on published studies. With regard to the analysis of *APOE* genotypes with stroke, our two sensitivity analyses, i.e. excluding small studies and re-quantifying the *APOE*-effect and comparing published versus unpublished studies, did not show any evidence of small-study bias.

Mechanisms linking APOE genotype to ischaemic stroke risk

To evaluate the mechanism by which *APOE* genotype might alter ischaemic stroke risk, we undertook a

detailed analysis of the association of *APOE* genotypes with a wide range of potential intermediate phenotypes including lipid, inflammation, coagulation, endothelial cell activation and metabolic markers measured in population-based and cross-sectional studies from European ancestry. Since genotype is determined at random at conception, intermediate phenotypes residing off the causal pathway from SNPs to disease, should be balanced evenly among the different genotypic groups, as they are in a randomized trial. In contrast, biomarkers that mediate the effect of genomic variation on disease risks should differ by genotype and the shape of the associations should be concordant.[61,62] We identified robust associations of *APOE* genotype with total-, LDL- and HDL-C and triglycerides as reported in a recent systematic review.[11] However, we extended the analysis to apolipoproteins E (the protein encoded by the *APOE* gene), apoA1, apoB, Lp(a), and 18 other biomarkers, including the well-studied inflammation marker CRP, among several thousand individuals. An exploratory, but biologically interesting finding was the fact that the shape of the *APOE* association with these traits varied considerably. Whereas a dose-response association was clearly observed with total-C, LDL-C, apoB, apoE and HDL-C, the effect on other traits appeared to be allele-specific, with lower Lp(a) and higher apoA1 being associated with the ε2 allele, and lower CRP with the ε4 allele. Although it is possible that these effects are due to a different biological mechanism of the *APOE* ε2 and ε4 variants on lipoprotein particle metabolism, it is also possible that these allele-specific associations could be due to different LD patterns between rarer, untyped, functional SNPs and the two genetic variants that comprise *APOE* ε2/ε3/ε4. It is often argued that except for rare variants, most of the observed phenotypic changes in lipoprotein biology can be explained by differences in the ε2/ε3/ε4 genotypes. We believe this view is simplistic and does not take into account the recent discoveries at this locus. Whole genome and dense gene-centric SNP arrays (e.g. the Illumina Cardiochip) have identified associations of SNPs in the *APOE* gene region with LDL-C[8,10,63–65] and Alzheimer's disease that encompass non-coding variants in the flanking genes and appear to be independent of the rs429358 and rs7412 SNPs (the two SNPS that encode ε2/ε3/ε4) in multivariate analysis.[63] These include variants in *PVRL2, APOC1* and *TOMM40*.[66,67] This suggests possible functional roles for one or more of the flanking genes (and their encoded proteins), mechanisms relating to *APOE* expression (not just function), or both. However, the capture of both common and rare SNPs in the *APOE* gene cluster is not yet exhaustive, so it is uncertain which of the implicated variants is causal, which mark as yet unidentified causal variation or whether causal SNPs differ for the different associated traits and disease outcomes. Not only are low-frequency variants (<5% minor allele frequency) under-represented on whole genome arrays but surprisingly, there is also sparse coverage of the common alleles in the *APOE* region (including the omission of rs429358 and rs7412 SNPs) (Supplementary Figure S11 and Supplementary Table S7, available as Supplementary data at *IJE* online). The low degree of LD at this locus also makes it difficult to impute untyped variants, which means additional SNP-disease/trait association signals may have been overlooked by the prior GWAS. Early re-sequencing studies and isoelectric focusing analysis of *APOE* isoforms[68–73] indicate the existence of many low-frequency, non-synonymous coding variants in this region, but these studies were narrowly focused on the *APOE* gene itself, rather than the flanking genes, and were neither exhaustive nor systematic.

The shape of the association of *APOE* genotype with an intermediate phenotype on the causal pathway should be consistent in the direction of the association with the disease end-point. Thus, the reported association of *APOE* genotype with CHD appears to be consistent with its effect on LDL-C. This is further supported by similar concordant associations of SNPs in the *HMGCR*, *PCSK9* and *SORT1* genes with both LDL-C and CHD risk.[8,10,64,74] Taken together with data from observational studies and randomized trials, the findings endorse the role of LDL-cholesterol in the pathogenesis of CHD. The positive dose-response association of *APOE* genotype with ischaemic stroke risk in European population studies could be consistent with an almost complete mediating effect of LDL-C because both associations are positive and linear. None of the other biomarkers studied, including other lipids and apolipoproteins, as well as Lp(a), or inflammation markers (including CRP) exhibited associations that were consistent in shape or direction with that seen for ischaemic stroke. Indeed, it is important to note that the ε4 allele associated with high values of LDL-C and C-IMT as well as high stroke and CHD risk showed low CRP levels. Although these results appear to be in contradiction with observational studies, we considered that findings of *APOE* on CRP could be explained by differential pattern of LD, as described above. Moreover, recent large-scale Mendelian randomization studies have excluded CRP as causal factor in cardiovascular disease.[75]

The concordance in shape of the *APOE* ε2/ε3/ε4 with LDL-C and ischaemic stroke risk observed in European ancestry received further support from the *APOE* ε2/ε3/ε4 effects on C-IMT (derived from European ancestry studies) that also revealed a positive dose-response association (Figure 1). However, to confirm that the positive dose-response association of *APOE* loci on ischaemic stroke is mainly due to its effect on LDL-C would require access to large-scale cohorts with genotype information, LDL-C measures and ischaemic stroke outcomes in sufficiently large numbers to undertake appropriate Mendelian randomization/instrumental variables analyses.

C-IMT represents the structural change in arterial intima that strongly associates with both cardiovascular risk factors (LDL-C) and cardiovascular events, and it is also used as an intermediate marker of atherosclerosis.[21] Overviews of statin trials have noted a linear relationship between the degree of LDL-C lowering and the change in C-IMT during follow-up, with modest LDL-C reductions being associated with reduced progression and more extreme LDL-C reductions being associated with regression of C-IMT.[76] The linear dose-response association of *APOE* genotypes with C-IMT was concordant in shape with the association of *APOE* with LDL-C, and endorses C-IMT as a valid surrogate marker of LDL-C–mediated atherosclerosis.[77,78] Similar to our study, a recent meta-analysis of *APOE* and C-IMT showed lower levels of C-IMT for $\varepsilon 2$ carriers and higher values for $\varepsilon 4$ carriers when compared with $\varepsilon 3/3$.[79] In contrast, the only GWAS of C-IMT[23] reported a lack of association with *APOE* variant rs7412 that marks the $\varepsilon 4$ allele (*P*-value: 0.7). Our study has the advantage of reporting the full range of *APOE* genotypes that might better represent this association.

Conclusions

In the most comprehensive study to date, we found a positive dose-response association of *APOE* genotype with ischaemic stroke in European populations. Concordance between the effects of the *APOE* genotype on LDL-C, C-IMT and ischaemic stroke risk was observed. Although this argues for a causal role of LDL-C in ischaemic stroke, and also helps to clarify the benefit of statins in ischaemic stroke, our finding must be interpreted with caution and it does not exclude the possibility that additional risk factors may be involved.

The use of the custom genotyping array Metabochip,[80] which includes both *APOE* SNPs and a greater coverage around the *APOE* locus, will provide an opportunity to replicate our findings on C-IMT, but also to explore in further detail the allele-specific associations of *APOE* locus with certain cardiovascular traits.

Supplementary Data

Supplementary data are available at *IJE* online.

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T.A.K., T.S., A.D.H. and J.P.C. designed the experiment and the analysis plan. T.A.K., T.S. and D.P. did the statistical analysis and drafted the report with A.D.H. and J.P.C. All authors provided critical revisions. All contributors had shared summary data and contributed to the interpretation of the results and to the redrafting of the report. All members of the coordinating centre contributed to the collection, standardization, analysis and interpretation of the data. T.A.K., T.S., D.P., J..PC. and A.D.H. had full access to all data in the study and had final responsibility to submit the report for publication. The study was conducted and analysed independently from its funders.

Conflict of interest: J.W. is 90% employed at GlaxoSmithKline while retaining a 10% appointment at London School of Hygiene & Tropical Medicine. A.D.H. is a member of the editorial board of *Drug and Therapeutics Bulletin* (a BMJ group publication) and has received honoraria for speaking at educational meetings most or all of which have been donated to charity. There are no other conflicts of interest.

KEY MESSAGES

- The six *APOE* genotypes when ordered from ε2/ε2, ε2/ε3, ε2/ε4, ε3/ε3, ε3/ε4 and ε4/ε4 have a linear association with LDL-C and CIMT thickness.
- The largest meta-analysis of *APOE* genotype with ischaemic stroke shows a positive linear association of increasing risk when ordered from ε2/ε2, ε2/ε3, ε2/ε4, ε3/ε3, ε3/ε4 and ε4/ε4 in European ancestry populations.
- The concordance in the dose-response associations of *APOE* genotype with LDL-C, CIMT and ischaemic stroke provides strong support for a causal role of LDL-C in ischaemic stroke.

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