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Pleiotropy in the presence of allelic heterogeneity: alternative genetic models for the influence of *APOE* on serum LDL, CSF $A\beta_{42}$, and Dementia

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Abstract

The two genetic polymorphisms, rs7412 and rs429358, that collectively form the ε2, ε3, and ε4 alleles of apolipoprotein E (*APOE)*are among the most widely studied sequence variants in the genome. The predominant model for testing *APOE* involves the haplotype combinations of ε2, ε3, and ε4 and has been basis of associations with dementia, atherosclerosis, and serum lipid levels. Here, we demonstrate the functional independence of these two component sites, with rs7412 contributing to the majority of variance in serum LDL ($p = 10^{-20}$), whereas rs429358 alone influences variance in CSF $A\beta_{42}$($p = 10^{-17}$). This latter relationship is also reflected in the association of *APOE* with dementia, where rs429358 strongly influences disease ($p = 10^{-67}$), but rs7412 does not. Models based upon ε2, ε3, and ε4 explained less variance for both dementia risk and CSF Aβ42 than did rs429358 alone. When adjusted for CSF $A\beta_{42}$, the association of rs429358 with dementia is greatly reduced but remains significant indicating that *APOE* polymorphism influences disease by additional mechanisms distinct from $A\beta_{42}$ metabolism. We reach four principal conclusion from this study; 1) rs429358 alone is responsible for the association of *APOE* with dementia 2) The association of *APOE* with dementia is substantially mediated by its effect on CNS $A\beta_{42}$ levels 3) The association of *APOE* with dementia is not mediated by its impact on peripheral lipid metabolism 4) The dichotomy of effects of rs429358 and rs7412 represents one of the best examples of genetic pleiotropy for complex traits known and illustrates the importance of allelic heterogeneity in *APOE*.

Keywords

LDL; Alzheimer Disease; amyloid; association; CSF

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Introduction

The gene encoding apolipoprotein E (*APOE*) is one of the most studied in the human genome, with several thousand genetic association studies documenting its importance in the regulation of plasma lipid metabolism and impact upon atherosclerosis and Alzheimer disease (AD) [1–3]. While multiple lipids are affected by *APOE* polymorphism, the strongest effect is upon low-density lipoprotein (LDL) (the relative importance of various genes is perhaps best captured in [4]). Importantly, an effect upon lipid metabolism is presumed to be a primary process that can contribute to AD risk and progression, though the precise molecular mechanisms remain unclear [5–7]. A key finding connecting AD and lipid metabolism is that APOE also appears to strongly affect brain β-amyloid (Aβ) deposition, which is the main component of senile plaques [8]. In support of this presumption, another prominent phenotypic consequence of *APOE* polymorphism is to contribute to a large proportion of variance in cerebrospinal fluid (CSF) levels of the 42 amino acid fragment of β-amyloid ($A\beta_{1–42}$) [9]. On the surface, these results appear to provide a plausible indication of a pathway basis for AD, with lipid and Aβ metabolism playing central roles. The extent to which the role of *APOE* has been documented in the context of lipid metabolism, cardiovascular disease, and AD provides one of the compelling cases for genetic pleiotropy (the action of a single gene on multiple phenotypes) in humans [10].

The ultimate goals of genetic association studies should be to understand the functional nature of sequence variation and to provide genetic models with the most predictive power for traits and diseases. With genome-wide association studies now well-established and with hundreds of new disease/trait polymorphisms having been discovered, a natural extension of the studies is to test identified variants across additional phenotypes. This search for pleiotropy can reveal important clues about etiological similarities across diseases as well as the evolutionary selective pressures that influence functional polymorphism. Against this background it is increasingly important to highlight peculiarities in even the most studied disease genes, to better guide and equip replication attempts. In the present study, we describe such a case for *APOE*, where the importance of allelic heterogeneity can be seen in the context of its pleiotropic effects on peripheral lipid metabolism and central nervous system β-amyloid metabolism.

Materials and Methods

Human Samples

In total, DNA was available for 1567 dementia cases (1275 had a possible or probable AD diagnosis) and 2203 controls. There were 990 men and 1213 women in the control group, and 598 men and 969 women in the dementia group. Average age-at-sampling for controls was 77.7 ± 8.7 (SD) years and age-at-onset for dementia/AD cases was 75.3 ± 8.3 (SD) years. All of these samples were described in detail recently [11]. Briefly, the samples were derived from four cohorts of ageing (SATSA, Octo-twin, Gender and Harmony) collected within the framework of the population-based Swedish twin registry. All participants were Swedish citizens residing in the cities of Stockholm or Jönkoping or surrounding areas. All the participants of these studies underwent detailed clinical dementia evaluation, responded to questionnaires and provided blood samples for clinical chemistry and DNA extraction. Blood lipids were measured using standardized methods on semi-automated techniques on fresh frozen samples. CSF samples were obtained in an independent clinical non-twin material consisting of AD cases and population-based controls collected from the Malmö, Piteå, and Gothenburg areas of Sweden. Diagnoses were made after a consensus conference, following DSM-IV criteria and NINCDS/ADRDA criteria for AD. This study was approved by the local Institutional Review Board of the Karolinska Institute.

Genotyping was performed using the Illumina GoldenGate assay system on Illumina Bead Station 500GX equipment, currently housed and implemented at the Uppsala University SNP Technology Platform. Prior to use on the Illumina system, all samples were subjected to Whole Genome Amplification (WGA) using standard kits involving Phi 29 DNA polymerase (Amersham). Some samples were also genotyped using Dynamic Allele Specific Hybridization as previously described [12]. *APOE* genotypes were obtained for a total of 1551 dementia cases and 2172 controls.

CSF samples were obtained from 828 individuals by lumbar puncture in the L3/L4 or L4/L5 inter-space. Further details of CSF collection can be found elsewhere [13]. CSF $A\beta_{42}$ was determined using a sandwich enzyme-linked immunosorbent assay (ELISA) (Innotest b-amyloid (1–42), Innogenetics, Ghent, Belgium) constructed to specifically measure $A\beta_{42}$.

Serum samples and blood fractions for DNA preparation were prepared from venous blood collected according to standard procedures. Blood lipids, including triglycerides (TG), low-density lipoprotein (LDL), and high-density lipoprotein (HDL) levels, were measured in fresh blood samples by routine methods on semi-automated equipment at an independent clinical chemistry laboratory. LDL was calculated using the Friedewald formula [14]. Serum lipid measures were obtained for a total of 1598 individuals.

Analyses

Both CSF $A\beta_{42}$ and serum LDL levels were log -transformed prior to analyses to achieve approximately normal distributions. Hardy-Weinberg equilibrium for individual loci was assessed using the Pearson χ^2 statistic. Tests of genetic association of *APOE* variants with CSF $A\beta_{42}$ and serum LDL were conducted using ANOVA as implemented in STATA v 11.0. Analyses were conducted in both cases and controls combined, including a diagnosis covariate to adjust for level differences between the two groups. To account for relatedeness and thus intra-class correlation for LDL analyses since some of the cases and controls were from the twin sample, multilevel mixed-effects linear regression as implemented as xtmixed in STATA v11.0 was used and models fit by full maximum likelihood. Xtmixed was used primarily to complement the significance estimates for associations with LDL levels. We applied alternating logistic regression (ALR) to the analysis of dementia risk in order to account for MZ and DZ pair correlation structures [15]. Members of the case -control samples were treated as incomplete DZ pairs for purposes of the ALR analysis. All models fitted adjusted for sex and age, reflecting the age at onset for cases or last age at follow-up for non-cases. The GENMOD procedure in SAS 9.1 was used to perform the ALR analyses (SAS Institute, Inc., Raleigh, NC).

Results

Linkage disequilibrium (LD) between rs7412 and rs429358 was found to be low in these samples (r^2= 0.021). There was no evidence of significant deviation from Hardy -Weinberg equilibrium for either marker in cases or controls (for rs429358, χ^2 = 0.08 for cases and χ^2 = 0.06 for controls; for rs7412, χ^2 = 1.2 for cases and χ^2 = 0.04 for controls). We performed first and second-order factorial ANOVA modeling to test the genetic association of markers rs7412 and rs429358 in *APOE* with serum LDL and CSF $A\beta_{42}$ levels. For each significant main effect, second-order interaction terms with disease were confirmed to be non-significant. There was a substantial difference in CSF $A\beta_{42}$ levels between dementia cases and controls and this can be seen for the diagnosis term in table 1. There was no significant difference between demented individuals and controls for serum LDL (table 2). Neither rs429358 nor rs7412 contributed to variance in TG or HDL levels (not shown)

The full sets of results for the effects of rs7412 and rs429358 on CSF A β_{42} and serum LDL levels can be found in tables 1 and 2. The central finding was that rs429358 acts independently upon CSF Aβ_{42} levels, whereas there is no independent main effect for rs7412 (table 1). For serum LDL, the strongest effect is found for rs7412, but there was evidence that both variants act independently, albeit with association of rs429358 being much weaker (table 2). We also tested for interaction (epistasis) between rs7412 and rs429358 in regression models taking the basic form $y = b_0 + b_1X_1 + b_2X_2 + b_3X_1X_2 + c$, where X_1 and X_2 are the genotypes of rs7412 and rs429358, respectively. Full models including diagnosis, age, sex, rs7412, rs429358, and the rs7412*rs429358 interaction term were tested for both traits. For CSF Aβ_{42} and LDL, p-values for the interaction term were 0.029 and 0.19, respectively. In sum, there was no strong evidence that the two variants were interacting (the marginal effect for CSF Aβ_{42} was not regarded as significant given multiple testing). Thus, the best genetic model for CSF Aβ_{42} is one in which rs429358 acts alone, with no contribution from rs7412. In contrast, the best model for serum LDL is involves additivity of the two markers, where rs7412 contributes the most to trait variance.

We also contrasted the single locus results with standard ε2, ε3, and ε4 diplotype models (that is genotypes based upon the combination of extended 2-locus haplotypes that can be inferred unambiguously due to the absence of one of the 4 possible haplotypes) for both the LDL and CSF Aβ_{42} traits. For CSF A β_{42} in the combined sample adjusting for case-control status, the variance explained using the diplotype model was 11% versus 14% when using rs429358 alone. This was similar in cases and controls when analyzed separately (not shown). For LDL, the variance explained in the diplotype model was 5.7% versus 5.1% using rs7412 alone and 0.9% using rs429358 alone. We do note that the rs7412*rs429358 interaction term provides a perfect representation of the standard ε2/ε3/ε4 model. For LDL and CSF Aβ42, the effect estimates of the interaction term alone were ($F_{4,822} = 23.97$, $p = 8.9 \times 10^{-19}$) and ($F_{5,1484} = 21.23$, $p = 1.4 \times 10^{-20}$), respectively. Thus there is no strong evidence that the interaction term provides information for LDL or CSF Aβ_{42} beyond what is obtained by examining rs7412 or rs429358 alone, respectively.

The genetic architecture of the association of *APOE* with dementia in this sample reflects most closely the association with CSF A β_{42} in that rs429358 is the main predictor of disease risk, with very modest association with rs7412 (table 3). Inclusion of CSF A β_{42} level as a covariate in logistic regression models for dementia risk reduces the strength of the association with rs429358. This was done using only the 819 individuals that had both CSF Aβ_{42} and *APOE* genotypes. For rs429358, the effect size of the association with dementia without CSF Aβ_{42} in the model was OR 4.41, 95% CI 2.89–6.73, $p = 5.5 \times 10^{-12}$. Inclusion of CSF Aβ_{42} in the model gave OR 2.71, 95% CI 1.72–4.26, $p = 1.7 \times 10^{-5}$, indicating that the association of rs429358 with dementia is substantially, but not entirely mediated by its impact on CSF Aβ_{42}. For the sample with LDL serum levels (1598 individuals), the effect size of the association with dementia without LDL was OR 1.98, 95% CI 1.63–2.41, $p = 8.4 \times 10^{-12}$. Adjustment for LDL provided an OR estimate of 2.06, 95% CI 1.69–2.52, $p = 1.0 \times 10^{-12}$. In the full set of dementia cases we again tested the standard diplotype model that includes the ε2/ε2, ε2/ε3, ε2/ε4, ε3/ε3, ε3/ε4, and ε4/ε4 groups to evaluate the relative variance explained compared to the single marker analyses. For the model with rs429358 alone, the proportion of variance explained was 10.3%, in contrast to the diplotype model which explained 9.1%. We also tested the formal interaction model for rs7412 and rs429358. This interaction term was non-significant when tested together with rs7412 and rs429358 (OR 1.07, 95% CI 0.68–1.70, p = 0.38). Genotype counts for both rs7412 and rs429358 in dementia cases and controls are also shown in table 4.

Discussion

In the present study, we highlight the importance of allelic heterogeneity in *APOE* exhibited by the independent effects of common sequence variants on CNS and peripheral phenotypes. This has implications for extrapolating gene -specific associations across phenotypes (pleiotropy) and possibly across tissues. This is important given that one of the traditions in genetic association studies is to take a single commonly studied sequence variant that has been suggested to be associated with one trait, and to test that variant across numerous distinct phenotypes. Examples include the common Alu insertion/deletion polymorphism in *ACE*, Val66Met (rs6265) in *BDNF*, and C677T (rs1801133) in *MTHFR* [16–18]. In terms of the present study, testing of rs7412 alone against dementia risk or CSF $A\beta_{42}$ would (depending on sample size) likely return only a marginal or negative result. Extended studies of *APOE* variation have not exposed markers with better evidence of association than rs429358 for AD risk [19].

So how might rs7412 and rs429358 differ functionally to explain the discrepancies that have been observed? The structural differences between APOE isoforms based upon knowledge of the rs7412 and rs429358 polymorphisms have been described in detail (e.g. [20]), but there remain numerous unanswered questions. The alternative alleles at each locus both encode for either arginine or cysteine residues. At position rs429358 (amino acid position 130) the arginine residue is associated with lower CSF $A\beta_{42}$ levels and increased dementia risk. At rs7412 (amino acid position 176), the cysteine residue is associated with increased serum LDL levels and increased cardiovascular disease risk [2]. Polymorphism at rs7412 clearly affects LDL binding, but its effects on Aβ metabolism are unknown, and results here suggest rs7412 has no impact at all on CSF $A\beta_{42}$. In contrast rs429358 is well known to affect molten globule formation [20], but why that would impact CSF $A\beta_{42}$ and not serumlipids is unknown. Another intriguing finding is that the levels of APOE dimers have been found to differ in CSF and serum dependent upon which isoform is present [21].

Tissue specificity is another area that the present results have bearing upon. As an example, studies at the genome-wide level have provided strong evidence of genetic association of SNPs with mRNA expression traits. These studies have been conducted in various tissues, including liver [22], brain [23], and transformed lymphocytes [24]. A key observation across those studies was that there was little overlap in the most significant findings, where the most highly associated markers failed to replicate [23]. Comparisons done to date have primarily centered on single markers, and so there remains a question of whether additional regulatory polymorphism does exist in associated genes. A weakness of our study is the lack of measures of lipids in CSF or Aβ in serum/plasma. We have however previously reported that CSF Aβ and total cholesterol are correlated [25], though sample sizes were in our view too small to test for *APOE* association with CSF cholesterol. Monitoring multiple lipid traits in addition to other Aβ fragments in both serum and CSF would help to further resolve these issues.

In our view, there are two important implications of the present results. The first is the finding that the association of rs429358 with dementia risk is substantially, but not entirely, mediated by CSF $A\beta_{42}$ metabolism. This suggests that there are other biological traits that are affected by *APOE* polymorphism that in turn contribute to dementia risk. Peripheral lipid metabolism has long been suspected as a likely candidate for this process, but our results discount that possibility in that LDL levels are equivalent between cases and controls and that rs429358 has no effect on serum LDL levels. The other serum lipid traits, HDL and triglycerides, are also unlikely candidates. Amyloid metabolism in the CNS is complex and the measurement of additional CSF or post-mortem brain molecules in the context of a broader amyloid pathway or network might help to explain the missing variance. Several

studies have shown an inverse relation between CSF $A\beta_{42}$ levels and both post-mortem plaque load in the brain [26–27], and positron emission tomography (PET) measurement of brain $A\beta_{42}$ load using the amyloid ligand Pittsburgh Compound B (PIB) [28–29]. These data suggest that CSF $A\beta_{42}$ reflects fibrillar $A\beta_{42}$ and plaque load in the brain. The most widely accepted explanation for this is that the aggregation of Aβ into plaques (and thus retention in the brain parenchyma) results in less Aβ available to diffuse into the CSF. At present however, we cannot discount the possibility that another CNS mechanism for *APOE*, distinct from amyloid metabolism, is at play.

The second implication of the present results is for disease/trait gene discovery in general where further genotyping of associated regions may be necessary to define the full spectrum of functional variation. Hundreds of new disease markers are now emerging from genome-wide association studies but for each identified region only a few functional variants have yet been identified. For *APOE*, two highly plausible functional polymorphisms are known (rs7412 and rs429358) with at present little evidence of additional functional variation, but even after over a decade of research, there remain gaps in understanding the biology and genetics of *APOE*. Allelic heterogeneity will be an important parameter to consider as new variants are identified in association studies and taken forward for testing in additional diseases and traits.

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Table 1

Association of *APOE* variants rs7412 and rs429358 with CSF Abeta42

term	F-statistic (crude)	P (crude)	F-statistic (full)	P (full)
rs7412	F(1,825) = 10.08	1.55E-03	F(1,822) = 2.74	9.82E-02
rs429358	F(2,822) = 42.61	2.47E-18	F(2,822) = 42.65	2.38E-18
diagnosis	F(1,828) = 276.68	8.06E-54	F(1,822) = 135.20	4.86E-29
sex	F(1,828) = 0.08	7.78E-01	F(1,822) = 0.29	5.90E-01
age	F(1,828) = 4.37	3.70E-02	F(1,822) = 1.38	2.40E-01

F-statistics were estimated using ANOVA. Diagnosis, sex, and age were included as covariates in crude models for rs7412 and rs429358.

Table 2

Association of *APOE* variants rs7412 and rs429358 with serum LDL

term	F-statistic (crude)	P (crude); P xtmixed	F-statistic (full)	P (full); P xtmixed
rs7412	F(2,1489) = 46.10	3.75E-20; 1.69E-20	F(2,1484) = 39.54	1.86E-17; 6.69E-18
rs429358	F(2,1516) = 11.76	8.55E-06; 4.65E-06	F(2,1484) = 7.48	5.85E-04; 2.83E-04
diagnosis	F(1,1563) = 3.01	8.32E-02; 1.13E-01	F(1,1484) = 10.11	1.51E-03; 4.51E-03
sex	F(1,1596) = 36.48	1.91E-09; 6.59E-09	F(1,1484) = 49.74	2.68E-12; 1.39E-11
age	F(1,1597) = 8.24	414E-03; 5.11E-03	F(1,1484) = 10.18	1.37E-03; 9.66E-04

F-statistics were estimated using ANOVA. Diagnosis, sex, and age were included as covariates in crude models for rs7412 and rs429358. Statistics were complemented with xtmixed to account for twin dependencies.

Table 3

Association of rs7412 and rs429358 in *APOE* with dementia risk

term	OR (crude)	P (crude)	OR (full)	P (full)
rs7412	0.59, 0.48–0.72	1.87E-07	0.77, 0.63–0.95	1.40E-02
rs429358	0.34, 0.30–0.39	1.51E-67	0.34, 0.31–0.39	3.09E-64
sex	NE	1.49E-04	NE	1.59E-04
age	NE	1.81E-04	NE	1.78E-04

Significance was determined using alternating logistic regression to account for twin dependencies. Odds ratios (ORs) are presented along with 95% confidence intervals. NE = not estimated. Sex and age were included as covariates in crude models.

Table 4

Genotype counts for rs7412 and rs429358 in dementia cases and controls

	rs429358			
	A/A	**A/G**	**G/G**	**OR; 95%CI**
cases	654 (0.42)	709 (0.46)	186 (0.12)	0.32; 0.29–0.36
controls	1578 (0.73)	545 (0.25)	49 (0.02)	
	rs7412			
	A/A	A/G	G/G	OR; 95%CI
cases	2 (0.001)	160 (0.10)	1390 (0.90)	0.57; 0.47–0.69
controls	18 (0.008)	351 (0.16)	1802 (0.83)	

Genotype counts (frequencies) in dementia cases and controls. Allelic odds ratios are presented without accounting for twin structure (see table 3 for corrected ORs). ORs are with respect to the “A” allele for both sites.