Human Molecular Genetics, 2007, Vol. 16, No. 18 **2199–2208**
doi:10.1093/hmg/ddm171
Advance Access published on July 5, 2007

Regulatory region single nucleotide polymorphisms of the apolipoprotein E gene and the rate of cognitive decline in Alzheimer's disease

Olivia Belbin[1], Janette L. Dunn[2], Yan Ling[1], Linda Morgan[1], Sally Chappell[1], Helen Beaumont[3], Donald Warden[3], David A. Smith[3], Noor Kalsheker[1] and Kevin Morgan[1,*]

[1]Division of Clinical Chemistry, Institute of Genetics, Queen's Medical Centre, University of Nottingham, Nottingham NG7 2UH, UK, [2]School of Physics and Astronomy, University of Nottingham, University Park, Nottingham NG7 2RD, UK and [3]OPTIMA, Oxford Centre for Gene Function, Department of Physiology, Anatomy and Genetics, Oxford OX1 3PT, UK

Received May 11, 2007; Revised and accepted June 28, 2007

The aim of this study was to investigate whether single nucleotide polymorphisms (SNPs) in the regulatory regions of the apolipoprotein E (*APOE*) gene modify the well-established ε4-associated risk for Alzheimer's disease (AD). Sequencing of the *APOE* gene regulatory regions revealed four previously reported promoter SNPs and one novel SNP in the previously described macrophage enhancer (ME.1). In addition, we also studied the two classic allelic missense SNPs that define ε2/ε3/ε4 status in a case–control association study. Analysis of pair-wise linkage disequilibrium (LD) of the five regulatory region SNPs with classic *APOE* SNPs revealed a previously unreported 7 kb LD block covering the entire *APOE* gene, part of the promoter and 3′ enhancer region. We report here that in a case–control association study ($N = 719$) of the seven SNPs, the genotype at codon 112 captures all the information required to assess disease risk. To explore correlations with quantitative traits, 169 patients were studied in whom rates of cognitive decline were available. In addition to the ε4 allele, two regulatory region SNPs were associated with the rate of cognitive decline in AD patients. This study highlights the effect of *APOE* gene variation on risk of AD and rate of cognitive decline and demonstrates that a single SNP, which confers ε4 status, captures all of the risk of developing AD but two SNPs in the regulatory region may affect the rate of cognitive decline in AD patients.

INTRODUCTION

Familial early-onset Alzheimer's disease (AD) accounts for ~5–10% of AD cases and most can be explained by inheritance of known mutations in amyloid precursor protein (*APP)* and presenilins (PSEN) 1 and 2 genes in an autosomal dominant fashion (1). The remaining AD cases are associated with a later age at onset and represent 'sporadic' AD, although these sporadic cases may also have a significant genetic component. Several genes have been implicated as potential candidates for this late-onset AD (LOAD) but the apolipoprotein (*APOE*) gene is the only one to date that has been replicated in numerous studies (2–4). The protein exists in three major isoforms apoE2, apoE3 and apoE4 that arise from three *APOE* alleles: *APOE* ε2, *APOE* ε3 and *APOE* ε4 as a result of two missense single nucleotide polymorphisms (SNPs), rs429358 and rs7412, which result in amino acid changes at residues 112 and 158, respectively (5). More detail on these two SNPs is shown in Table 1. Henceforth in this manuscript, these two polymorphisms are designated 112 and 158. Approximately 50% of LOAD cases are accounted for by individuals possessing the *APOE* ε4 allele (3), although possession of this allele is not essential for predisposition to the disease. Possession of this allele not only increases the risk of developing AD (lifetime risk increases from 20% when no *APOE* ε4 alleles are possessed to 90% when two copies

*To whom correspondence should be addressed. Tel: +44 1158230724; Fax: +44 1159709167; Email: kevin.morgan@nottingham.ac.uk

© *The Author 2007. Published by Oxford University Press. All rights reserved.*
For Permissions, please email: journals.permissions@oxfordjournals.org

Downloaded from https://academic.oup.com/hmg/article/16/18/2199/713403 by guest on 16 August 2022

Table 1. Genotypes and the resulting amino acid changes associated with APOE allelic status

E2/E3/E4 status	112 (rs429358)		158 (rs7412)	
	Genotype	Amino acid	Genotype	Amino acid
E2/E2	TT	cys/cys	TT	cys/cys
E2/E3	TT	cys/cys	TC	cys/arg
E2/E4	TC	cys/arg	TC	cys/arg
E3/E3	TT	cys/cys	CC	arg/arg
E3/E4	TC	cys/arg	CC	arg/arg
E4/E4	CC	arg/arg	CC	arg/arg

are present) but it also decreases the age at onset [mean age at onset for individuals with no ε4 allele was 84.3 years compared with 68.8 years for individuals possessing two copies (6)]. Interestingly, the *APOE* ε2 allele is under-represented in AD cases (7) and is associated with a delayed age at onset of AD (2,6) suggesting a protective effect (8).

The *APOE* gene resides on chromosome 19q13.2 (9,10) and codes for the cholesterol transporter protein, apolipoprotein E (apoE), which represents the major lipoprotein within the CNS, where it is synthesized by astrocytes (11,12). It has been suggested that one role of apoE in the brain may be an involvement in neuronal homoestasis (13), particularly, mobilization of cholesterol in the CNS, where it is required for neuronal plasticity (14,15). ApoE is also postulated to play a role in neuronal repair by mediating the recycling of damaged cell membranes (13). Levels of apoE mRNA are increased in astrocytes following lesions to the entorhinal cortex (7), one of the first areas of brain to be affected by AD (16).

Although the *APOE* ε4 allele confers a considerable risk for AD, not all individuals who possess this allele go on to develop the disease. This observation has led to the hypothesis that other genetic factors, possibly elsewhere within the *APOE* gene, may contribute to disease risk. One potential area of interest is the promoter region. To date, four promoter SNPs have been identified at positions –491, −427, −219 and +113 base pairs relative to the transcription start site (17–20).

The –491A/T polymorphism has been associated with an increased risk of developing AD independently from the ε4-associated risk (18,21,22). The –427T/C, −219 T/G and +113G/C polymorphisms have also been associated with an increased risk for AD (21,23,24), although confirmation of these findings has been limited possibly due to the strong linkage disequilibrium (LD) with the ε4 allele.

Functionally, these promoter SNPs have been proposed to affect the transcriptional activity of the gene in both the periphery and the CNS due to altered binding of nuclear proteins (13). Taking −491A, −427T and −219T as a reference haplotype, an A to T subsititution at −491 resulted in a 63% decrease and a T to G substitution at −219 resulted in a 169% increase in *APOE* promoter activity in HepG2 liver cells (17) and similar effects were reported in astrocytoma cells (23). The T to C substitution at −427 had no effect on promoter activity (17). Differential protein binding was observed for both −491 and −219 polymorphisms. The −491AA genotype is also associated with raised apoE protein levels in neuropathologically normal individuals (25). Since the risk of sporadic AD is associated with increased levels of apoE in plasma (26), it is possible that promoter polymorphisms could contribute to risk by increasing *APOE* expression.

In addition to the 5′ proximal promoter (27–32), other control elements for *APOE* gene expression have been reported in the 3′ flanking region of the gene. The functional peroxisome-proliferator-activated-receptor gamma response element (PPRE) is situated 2 kb downstream of the *APOE* gene (33), whereas a macrophage enhancer (ME.1) can be found 1.3 kb further downstream (34–36). The role of potential SNPs in these regions on *APOE* gene expression and any association with AD has yet to be determined.

In this paper, we report a case–control SNP association study of regulatory region SNPs in the promoter and ME.1 enhancer element as well as the established AD risk-associated missense SNPs. We also examine the effect of *APOE* SNPs on cognitive decline by non-linear mixed effects modelling.

RESULTS

SNP identification

In addition to the previously described promoter SNPs [−491 rs449647, −427 rs769446, −219 rs405509 (17) and +113 rs440446 (20)], one novel SNP was identified in the ME.1 region at position +7203 from the start of the *APOE* gene (Fig. 1). No SNPs were identified in the PPRE. The adjacent sequences for all SNPs and their minor allele frequencies are shown in Table 2. All SNPs had a minimum minor allele frequency of 5% during the mapping of 88 alleles. Since the genotypes for missense SNPs, 112 and 158, were already known for these samples, TaqMan assays were designed for the remaining five SNPs and genotyping was performed in the total 719 individuals. All polymorphisms were in Hardy–Weinberg equilibrium in controls and AD patients.

Haplotypes of the APOE gene

Using the five regulatory region SNPs, seven common haplotypes were identified, which accounted for over 86% *APOE* variation in controls (Table 3). Haplotypes are also presented for cases and controls in the total data set (Table 4) using the five regulatory SNPs plus the two SNPs that define ε2/ε3/ε4 status. Ten common haplotypes (frequency >2% in controls) were apparent, of which, only one represented the ε2 genotype (H1). This haplotype showed a trend for increased frequency in controls (4% in controls compared with 2% in AD patients) but was not statistically significant ($P = 0.32$) Seven haplotypes (H2–H8) represented the ε3 genotype. H2 and H3 were strongly protective ($P = 2 \times 10^{-06}$ and 5×10^{-05}, respectively), whereas the remaining five ε3 haplotypes (H4–H8) were at best marginally protective or neutral. Two haplotypes (H9 and H10) represented the ε4 genotype, both of which were at a statistically significant increased frequency in AD patients compared with controls ($P = 3 \times 10^{-03}$ and 2×10^{-14}, respectively). The statistically stronger association of H10 with AD than H9 is likely due to the higher frequency of the haplotype rather than a modifying effect of the regulatory SNPs on ε4 status. However, the numbers of ε4-positive controls are limited and studies with a larger data sets will be able to directly address this issue.

Downloaded from https://academic.oup.com/hmg/article/16/18/2199/713403 by guest on 16 August 2022

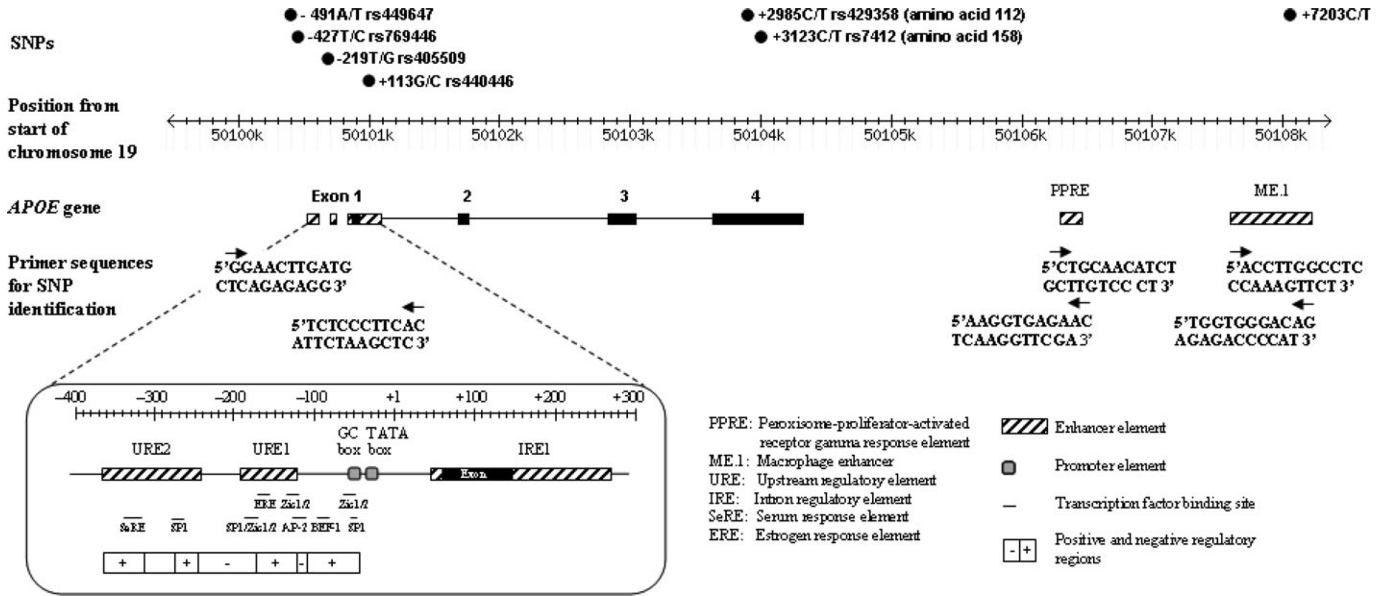

Figure 1. SNP map of the APOE gene showing SNPs, regulatory and coding regions.

Table 2. Adjacent sequences for all SNPs genotyped in this study and the minor allele frequencies ($N = 44$)

SNP	rs	Position Region	From TSS	Contig	Base change	5′ flanking sequence	3′ flanking sequence	Minor allele frequency (%)
−491	rs449647	Promoter	−491	17676782	A/T	GTTGGCCAGGCTGGTCTCAA	CTCCTGACCTTAAGTGATTC	23
−427	rs769446	Promoter	−427	17676846	C/T	CTGGGATTACAGGCGTGAGC	ACCGCCCCCAGCCCCTCCCA	5
−219	rs405509	Promoter	−219	17677054	T/G	AGAATGGAGGAGGGTGTCTG	ATTACTGGGCGAGGTGTCCT	47
+113	rs440446	Intron 1	+113	17677685	C/G	AAGAGCTGGGACCCTGGGAA	CCCTGGCCTCCAGGTAGTCT	40
112	rs429358	Exon 4	+2985	17680159	C/T	GCGCGGACATGGAGGACGTG	GCGGCCGCCTGGTGCAGTAC	ND
158	rs7412	Exon 4	+3123	17680297	C/T	ATGCCGATGACCTGCAGAAG	GCCTGGCAGTGTACCAGGCC	ND
ME.1	novel	3′ flanking region	+7203	N/A	C/T	GCCAGGCTGACACGTGGTTG	GGGGGCACAAGGCCAGCCAA	20

ND signifies not determined, as these data were already available from previous studies.

LD of SNPs in the APOE gene

The pair-wise LD measures (r^2 and D') for all seven SNPs are shown in Figure 2. Pair-wise r^2 values ranged from 0.001 to 0.66 indicating that all seven SNPs provide a degree of unique haplotypic information. The D' values (Fig. 2C and D) revealed a strong degree of LD between the seven SNPs. In particular, an LD block (all $D' > 0.40$) can be observed in the 3′ 7 kb region that includes SNPs −219, +113, 112, 158 and ME.1 in both controls and AD patients. This LD within the *APOE* gene confounds attempts to differentiate potential promoter associations from the highly significant ε4 effect.

Table 3. Frequencies of the seven common haplotypes at five SNPs in the *APOE* gene regulatory regions in controls from Nottingham ($N = 93$) and Oxford ($N = 174$)

Haplotype −491	−427	−219	+113	ME.1	Nottingham controls	Oxford controls
T	T	T	C	C	21 (11.3%)	29 (8.3%)
A	T	T	C	C	43 (23.1%)	73 (21.0%)
A	T	T	G	C	18 (9.7%)	32 (9.2%)
A	C	G	G	C	11 (5.9%)	19 (5.5%)
T	T	G	G	C	8 (4.3%)	21 (6.0%)
A	T	G	G	C	12 (6.5%)	31 (8.9%)
A	T	G	G	T	48 (25.8%)	115 (33.0%)
All other haplotypes					25 (13.4%)	28 (8.1%)
Total chromosomes					186	348

Single SNP associations

As expected, the ε4 effect was strong in this data set; OR for possession of one copy of the ε4 allele was 4.6 (95% CI 3.3–6.3) and for possession of two copies was 12.5 (95% CI 3.8–40.7). Chi-squared tests of allele frequency versus disease status identified 112 as the major disease-associated SNP in the total data set (Table 5). The cytosine (C) allele at this locus (possessed by ε4 bearers only) is the major risk

Downloaded from https://academic.oup.com/hmg/article/16/18/2199/713403 by guest on 16 August 2022

Table 4. Frequencies of the haplotypes at seven SNPs in the APOE gene in controls (N = 261) and AD patients (N = 317). Samples were only included when genotype information was available at all seven SNPs

Haplotype	SNP							APOE ε4 status	Controls	AD	*P*-value
	−491	−427	−219	+113	Amino acid 112	Amino acid 158	ME.1				
H1	T	T	G	G	T	T	C	ε2	21 (4.0%)	13 (2.0%)	0.33
H2	A	T	T	C	C	T	C	ε3	92 (17.6%)	53 (8.4%)	2×10^{-6}
H3	A	T	G	G	C	T	T	ε3	122 (23.4%)	83 (13.1%)	5×10^{-5}
H4	T	T	T	C	C	T	C	ε3	35 (6.7%)	23 (3.6%)	0.02
H5	A	T	G	G	C	T	C	ε3	20 (3.8%)	11 (1.7%)	0.02
H6	T	T	T	C	T	C	C	ε3	16 (3.1%)	0 (0.0%)	0.32
H7	A	T	G	G	T	C	T	ε3	37 (7.1%)	63 (9.9%)	0.05
H8	A	T	T	C	T	C	C	ε3	20 (3.8%)	0 (0.0%)	0.02
H9	A	T	G	G	C	C	C	ε4	14 (2.7%)	36 (5.7%)	3×10^{-3}
H10	A	T	T	G	C	C	C	ε4	43 (8.2%)	117 (18.5%)	2×10^{-14}
All other haplotypes									102 (19.6%)	235 (37.1%)	
Total chromosomes									522	634	

allele, representing 12.4% of controls and 34.7% of AD patients ($P = 4 \times 10^{-21}$). The other *APOE* allelic missense SNP, 158, shows a relatively weaker association; the thymine (T) allele (corresponding to the ε2 allele) is at a higher frequency in controls, 8.9%, compared with AD, 5.1% ($P = 0.007$).

Association of the other five SNPs examined in this study also showed relatively weak associations when compared with 112 in the total data set (Table 5). Although, four SNPs (−491, −219, +113 and ME.1) resulted in $P < 0.05$, because of the strong LD of these SNPs with 112 and 158, it cannot be assumed that these are independent associations without correcting for the ε4 effect.

Correcting for the ε4 effect

In this study, we have used two approaches for determining ε4-independent associations. First, we split the data set by possession of the ε4 allele, resulting in two separate subsets; those individuals who possess no ε4 allele and those who possess one or two copies. Table 5 shows the minor allele frequency of the seven SNPs in non-ε4 individuals and ε4 bearers. Since non-ε4 individuals always possess a T allele at 112, this SNP was not included in this part of the analysis. As shown in Table 5, no other single SNP showed association with control or AD in non-ε4 individuals. The fact that 158 is not associated with AD in these individuals merely demonstrates that there is no significant difference in the frequency of the C allele (representing ε3) between non-ε4 controls and non-ε4 AD patients.

The allelic association of 112 and 158 with AD also disappeared in ε4 bearers (Table 5). The number of C alleles at 112 in ε4 bearers is a direct measure of the number of ε4 homozygotes and there was no significant difference in the frequency of the C allele between ε4-bearing controls and ε4-bearing AD patients. Likewise, the lack of association of 158 indicates that there is no significant difference in the number of ε2/ε4 individuals in ε4-positive cases and controls. Two promoter SNPs demonstrated weak associations in ε4 bearers: −491 (T allele demonstrated 16.0% frequency in controls compared with 8.5% in AD, $P = 0.016$) and −219 (T allele demonstrated 63.6% frequency in AD compared with 54.1% in controls, $P = 0.05$). The finding that the T allele at −491 has a protective effect in ε4 bearers is in agreement with previous studies (18,21,22). However, this approach has limited power to detect associations due to the reduction of sample size and the relative lack of ε4-positive controls ($N = 75$) compared with ε4 AD patients ($N = 225$).

In addition, we have used a second approach, genotype logistic regression, to examine the OR of developing AD for each SNP while adjusting for the covariates 112, 158, gender and age at onset (AD patients) and age at examination (controls). This approach has the advantage of using the total data set resulting in greater power to detect an association and hence is likely to reveal effects that are not with the above analyses. As shown in Table 5, all SNPs except −427, demonstrated a significant association with AD when analysing each SNP independently but only 112 remained significant when correcting for covariates (OR 4.65, 95% CI 1.38–15.67). This suggests that the association of −491 and −219 reported in ε4 positives could be an artefact due to the low number of ε4 positive controls.

Association of APOE SNPs with cognitive decline

Here we used cognitive decline as a quantitative trait in AD patients, thus refining the phenotype. As has been previously described (37), cognitive decline data are more suited to a non-linear model rather than linear. We examined the effects of *APOE* SNPs on age and rate of cognitive decline using this previously described non-linear mixed effects model. Since there was no significant difference in age at midpoint of decline or rate of decline between males and females in this data set (data not shown), no corrections were made for gender in this study. Age at which patients reach half the asymptotic score is determined by the relative position of curves along the *x*-axis. The rate at which patients' cognitive decline falls from three-fourths to half the asymptotic score is determined by the slope of the curve. As shown in Figure 3A, there were no significant effects of the classical *APOE* genotypes on age at midpoint of cognitive decline but a strong effect of the ε4 allele was apparent on the rate of decline. ε4 homozygotes showed a significantly faster rate of decline (1.26 years to fall from a score of 71.27 to 47.5) than wild-type ε3 homozygotes (1.62 years, $P = 0.002$), whereas individuals with ε2ε3 or ε3ε4 genotypes showed no significant difference.

Downloaded from https://academic.oup.com/hmg/article/16/18/2199/713403 by guest on 16 August 2022

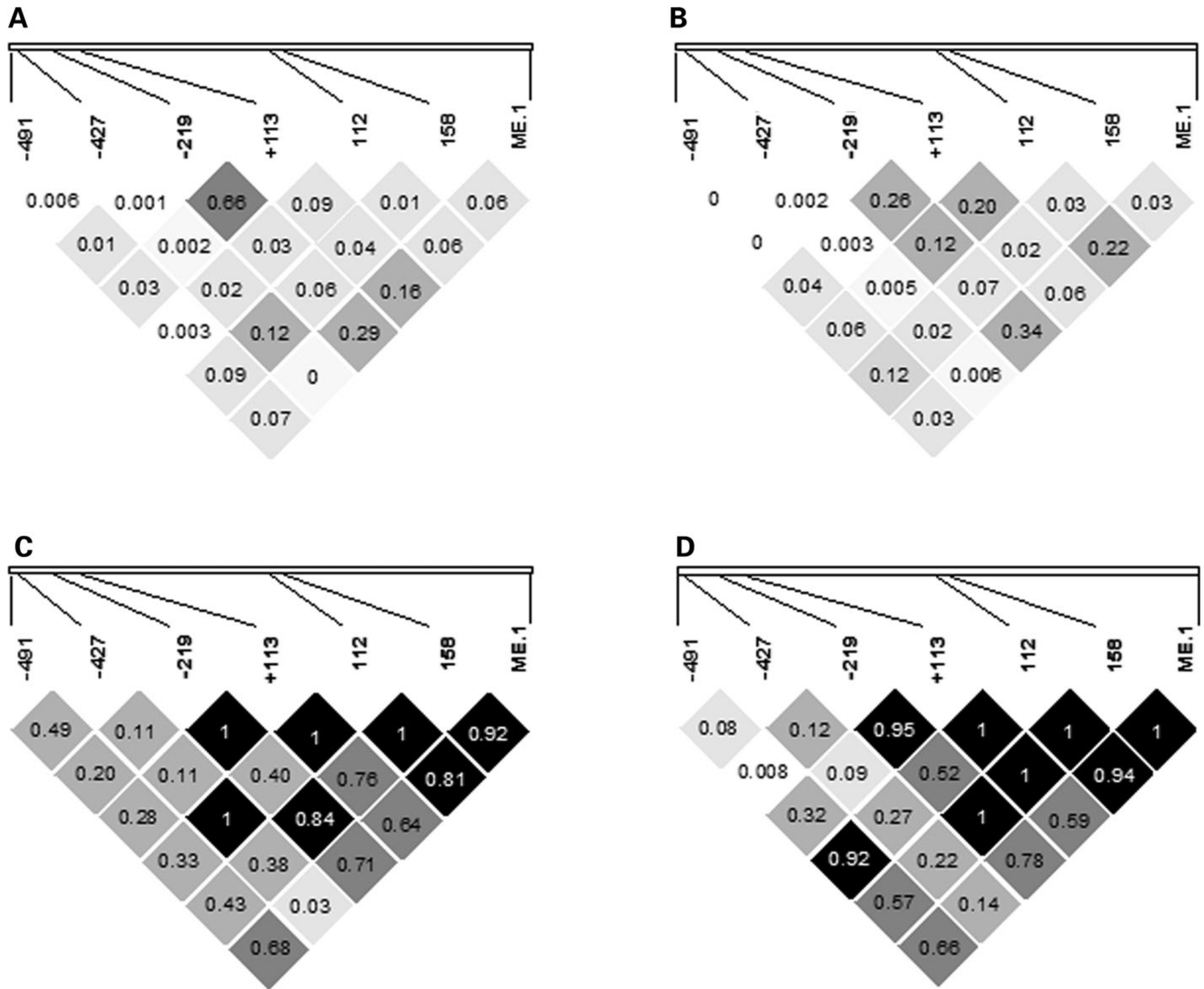

Figure 2. Pair-wise LD measures; r^2 in controls (**A**) and AD (**B**), and D' in controls (**C**) and AD (**D**). Boxes are coloured according to LD values: <0.01, white; 0.01–0.8, shades of grey; >0.8, black. SNPs within the 7 kb LD block are shown in bold.

In the next stage of analysis, we added the five regulatory region *APOE* SNPS to the model (Fig. 3B–F). Although no SNP had a significant effect on the age at midpoint of cognitive decline, +113 and ME.1 demonstrated significant genotype-specific effects on rate of cognitive decline. For the +113 SNP, the GG genotype demonstrated significantly faster cognitive decline (1.45 years) than the reference GC genotype (1.68 years; $P = 0.04$). For the ME.1 SNP, the TT genotype demonstrated significantly faster cognitive decline (0.94 years) compared with the reference CC genotype (1.49 years; $P = 0.0005$). The ME.1 SNP therefore demonstrates the strongest association of any SNP in the *APOE* gene on rate of cognitive decline. In an attempt to correct for ε4 status on the effect of +113 and ME.1 SNPs on cognitive decline, the data were split into ε4 positive and negative patients; however, it must be noted that this approach reduces the power as the sample numbers decrease. Nevertheless, when the modelling was repeated on these data sets, the +113 effect was seen in both ε4-positive and -negative patients with the effect on rate of cognitive decline strengthening in the ε4-negative patients ($P = 0.0002$; data not shown). The ME.1 effect became non-significant in both ε4-positive and -negative patients. These data suggest that the +113 effect on cognitive decline appears not to be dependent on ε4.

DISCUSSION

Here we report an investigation of seven SNPs within the regulatory and coding regions of the *APOE* gene, providing a detailed report of their LD, a large AD case–control study and quantitative trait analysis of cognitive decline in AD patients. The fact that possession of the ε4 allele is not essential for predisposition to the disease has led researchers to believe that other risk factors are yet to be discovered,

Downloaded from https://academic.oup.com/hmg/article/16/18/2199/713403 by guest on 16 August 2022

Table 5. Single SNP allele frequencies and associated *P*-values for allelic associations

SNP	Minor allele (in controls)	Minor allele frequency (%)			OR (95% CI)	Adjusted OR (95% CI)
		Controls	AD	*P*-value		
Total data set ($N = 719$)						
rs449647 (−491)	T	17.8	12.9	**0.011***	**0.70 (0.95–0.93)***	0.86 (0.61–1.20)
rs769446 (−427)	C	10.9	11.3	0.867	1.04 (0.75–1.46)	1.24 (0.85–1.83)
rs405509 (−219)	T	46.6	55.2	**0.002***	**1.43 (1.15–1.77)***	1.05 (0.82–1.35)
rs440446 (+113)	C	37.3	26.8	**0.0009***	**0.62 (0.49–0.78)***	0.96 (0.73–1.27)
Novel (me.1)	T	40.1	32.3	**0.003***	**0.70 (0.55–0.88)***	0.98 (0.74–1.29)
rs429358 (amino acid 112)	C	12.4	34.7	**4×10^{-21}***	**4.04 (2.99–5.45)***	**3.74 (2.75–5.09)***
rs7412 (amino acid 158)	T	8.9	5.1	**0.007***	**1.83 (1.19–2.81)***	1.36 (0.86–2.14)
		Gender (female)			**1.46 (1.09–1.97)***	**1.71 (1.25–2.34)***
		Age at onset			**0.96 (0.94–0.98)***	**0.96 (0.94–0.97)***
Non-ε4 individuals ($N = 398$)						
rs449647 (−491)	T	18.7	18.1	0.927	0.97 (0.68–1.39)	1.02 (0.69–1.50)
rs769446 (−427)	C	12.3	11.3	0.769	0.91 (0.58–1.44)	0.98 (0.60–1.60)
rs405509 (−219)	T	44.6	43.3	0.786	0.95 (0.71–1.28)	0.92 (0.67–1.25)
rs440446 (+113)	C	44.3	50.6	0.126	0.94 (0.7–1.26)	0.89 (0.65–1.21)
Novel (me.1)	T	43.3	46.4	0.447	1.13 (0.85–1.52)	1.08 (0.79–1.48)
rs429358 (amino acid 112)	C	0.0	0.0	N/A	N/A	N/A
rs7412 (amino acid 158)	T	10.2	7.3	0.34	1.45 (0.86-2.45)	1.49 (0.87–2.56)
		Gender (female)			**1.68 (1.12-2.52)***	**1.92 (1.26–2.93)***
		Age at onset			**0.97 (0.95–0.99)***	**0.97 (0.94–0.99)***
ε4 bearers ($N = 302$)						
rs449647 (−491)	T	16.0	8.5	**0.016***	**0.48 (0.27–0.85)***	0.52 (0.27–1.00)
rs769446 (−427)	C	6.4	11.3	0.130	1.82 (0.88–3.74)	2.09 (0.98–4.44)
rs405509 (−219)	T	54.1	63.6	**0.05***	**1.57 (1.04–2.38)***	1.35 (0.87–2.09)
rs440446 (+113)	C	15.7	16.1	1.000	1.02 (0.57–1.82)	1.35 (0.73–2.51)
Novel (me.1)	T	29.2	22.1	0.104	0.60 (0.36–1.00)	0.72 (0.41–1.28)
rs429358 (amino acid 112)	C	48.0	40.9	0.15	**5.35 (1.61–17.82)***	**4.65 (1.38–15.67)***
rs7412 (amino acid 158)	T	95.3	96.4	0.71	1.35 (0.53–3.41)	0.93 (0.36–2.42)
		Gender (female)			1.15 (0.68–1.95)	1.25 (0.73–2.15)
		Age at onset			**0.95 (0.92–0.98)***	**0.95 (0.92–0.98)***

ORs and adjusted ORs of developing AD are given for genotypic associations in the total data set. Adjusted ORs allow correction for 112 and 158 genotypes, gender and age at onset. Significant associations are marked in bold and by an asterisk. N/A signifies not applicable; this SNP is used to partition the data into ε4-positive and ε4-negative individuals.

possibly in the *APOE* gene itself. However, the strong LD within this region has, in the past, confounded attempts to identify susceptibility alleles other than ε4. Of the seven SNPs studied, only frequencies for −219 (rs405509) and 112 (rs429358) are reported in the current version of HapMap (www.hapmap.org, data release 21a, phase II, January 2007) but all except the novel ME.1 SNP are listed in dbSNP (www.ncbi.nlm.nih.gov/SNP). Here we report a 7 kb linkage block stretching from the −219 promoter SNP to the 3′ macrophage enhancer region 4 kb downstream of the *APOE* gene using genotype frequency data generated in this study. When examining these SNPs in a case–control study, we revealed no association that exceeds, or is independent of, the previously reported ε4-associated risk.

It is possible that by looking at such a hugely variable phenotype as AD, we lack the power to isolate promoter associations from the ε4 effect. Here we demonstrate that splitting the data set into ε4-positive and -negative individuals in an attempt to do so is insufficient for two reasons. First, this is ignoring the ε4 dose effect that is apparent in such studies. Potential associations in ε4 positives that have been reported in the past could merely be reflecting the effect of ε4 homozygotes versus ε4 heterozygotes. Secondly, the use of such small subsets for a hugely variable phenotype results in a significant loss of power to detect associations and the lack of ε4 positive controls compared with ε4 positive AD patients could shed doubt on positive associations reported in the ε4-positive data set.

For these reasons, we examined the role of the *APOE* SNPs in a more refined AD phenotype of cognitive decline ($N = 169$). Here we report an association of ε4 homozygosity with a significantly faster rate of cognitive decline and a trend towards earlier age at midpoint of decline in AD patients, a finding that is consistent with previous observations (37). These authors studied 218 AD patients and found that ε4 associated with a faster rate and earlier decline. Interestingly, in another previous study, the *APOE* ε4 allele has been shown to influence conversion of mild cognitive impairment to AD (38). This study failed to find any evidence for an ε4 effect on the rate of cognitive decline in AD patients. However, the cognitive decline data were partitioned into groups of fast and slow decline as oppposed to implementing non-linear effects modelling as conducted in our study.

Further to this effect, we found two regulatory region SNPs that were also associated with an altered rate of cognitive decline, one of which (+113) demonstrated association in both ε4-positive and -negative individuals, possibly indicating that it is not tracking with ε4 status. This quantitative trait approach provides greater power than case–control studies and has indicated that regulatory region variation in the *APOE* gene may contribute to a faster cognitive decline in

Downloaded from https://academic.oup.com/hmg/article/16/18/2199/713403 by guest on 16 August 2022

AD patients. The ME.1 SNP result exemplifies this point since, although its association with disease risk becomes non-significant after correction for confounders, the quantitative trait studies demonstrate that its effect on the rate of cognitive decline is readily apparent.

It is interesting to note that all associations reported here for the *APOE* gene affected the rate but not age at midpoint of cognitive decline in AD patients. We have previously reported that possession of a GG genotype of an intronic SNP of the alpha-1 antichymotrypsin (*ACT*) gene associated with a significantly earlier age at midpoint of cognitive decline than the other two genotypes at this SNP (39). This demonstrates that SNPs in two AD candidate genes (*ACT* and *APOE*) affect different parameters (rate and age at midpoint) of cognitive decline. Further larger studies need to be conducted investigating these parameters in order to reveal any potential epistatic interactions between the *APOE* and *ACT* genes; currently, we do not have the power to discern these effects.

In conclusion, the results of this study demonstrate that the association of the *APOE* gene with AD can be captured by just looking at a single SNP (112), which defines carrier status for the ε4 allele. This is in agreement with a recently published paper, which also alluded to the value of 112 as a 'tag' for AD risk (40). The haplotype data again tracks with ε4 status, and from the curent data, it would appear that the regulatory variants have at best minimal interaction with the ε4 allele. It is likely that the previous associations reported for a number of other functional SNPs in the promoter area are due to the fact that they track with ε4 status and as a result do not impart any additional information. However, when using cognitive decline as a quantitative trait to further refine the phenotype, association with *APOE* SNPs additional to ε4 emerge.

MATERIALS AND METHODS

Patient samples

All subjects gave informed consent to be included in the study, which was granted approval by the local Ethics Committee. For the case–control association study, the 719 samples were obtained from two UK centres: University of Nottingham Brain Bank and Oxford Project to Investigate Memory and Ageing (OPTIMA), University of Oxford. Details of patient samples used in this study are as follows: 324 controls (162 males, 158 females, four with undocumented gender, mean age 75.3 ± 9.4 years) and 395 AD patients (161 males, 227 females, seven with undocumented gender, mean age at onset 75.6 ± 10.5 years). There was no evidence for population stratification in this sample set in a previous study (39); thus, the samples were pooled for all analyses. Samples were histopathologically confirmed as definite disease (AD) ($N = 310$) or control ($N = 324$) using CERAD criteria (41). Probable AD patients from Oxford ($N = 85$) were also included since there has been 100% concordance between patients diagnosed with probable AD and confirmation of the disease post-mortem ($N = 34$) (42). All patients with evidence of an autosomal dominant AD trait, or where a first-degree relative had been diagnosed with familial AD, were excluded.

PCR and automated sequencing

Genomic DNA was extracted from whole blood or brain tissue using the QIAamp DNA blood mini kit (Qiagen, Crawley, West Sussex, UK) following manufacturer's procedure. All amplification protocols were performed with an annealing temperature of 60°C; primer sequences are shown in Figure 1.

SNP identification

Forty-four samples were used to identify common SNPs (minor allele frequency >5%) within the *APOE* gene by sequencing known regulatory regions (promoter, 3′PPRE and 3′ME.1 enhancer region). This approach has 99% power for the detection of polymorphisms that are present at a frequency of ≥5%. Potential polymorphic sites were identified by multiple sequence alignment using ClustalW software. Polymorphisms were confirmed by repeat PCR and sequencing of the opposite strand.

Genotyping of polymorphisms

All 719 samples were genotyped at five sites using fluorescently labelled TaqMan probes (Vic or Fam) by Geneservice (Cambridge, UK). Fifteen percent of the samples assayed were of known genotype, determined by sequencing, and 10% were genotyped in duplicate as quality assurance measures. The data were only accepted when there was 100% concordance between duplicate samples. The two *APOE* allelic SNPs, 112 and 158, were genotyped by RFLP using a previously described protocol (43). Samples in which genotyping failed at more than three sites were not included in the analyses. For each of the SNPs, the failure rates were as follows; −491:4.6%, −427:7.2%, −219:4.0%, +113:4.3%, 112:2.8%, 158:3.1% and ME.1:3.1%.

Haplotypes, linkage and case–control association

The five regulatory region SNPs were used to estimate the haplotype frequencies found in controls and AD patients using the haplo.em function of haplo.stats v1.2.2 in the R programming language (44). This method utilizes a maximum likelihood analysis approach, which we have previously used (39). LD measures were calculated using haploview software (www.broad.mit.edu). Chi-squared tests and multivariate binary logistic regression of individual SNPs with disease status were performed using Statistical Package for Social Sciences (SPSS) v12.0.1.

Association of APOE SNPs with cognitive decline

Cognitive scores were obtained using the CAMCOG score system (45). In two previous studies using samples from the same OPTIMA collection, the CAMCOG score data could be fitted using a non-linear mixed effects model (37,39). Analysis of the CAMCOG data in this study was performed using the same methods. Briefly, we used a three-parameter logistic (S-shaped) function: $\text{CAMCOG} = \text{asymptote}/\{1 + \exp[(\text{age} - \text{xmid})/\text{scale}]\}$. The asymptotic score was set at a CAMCOG score of 95, in common with the previous work.

Downloaded from https://academic.oup.com/hmg/article/16/18/2199/713403 by guest on 16 August 2022

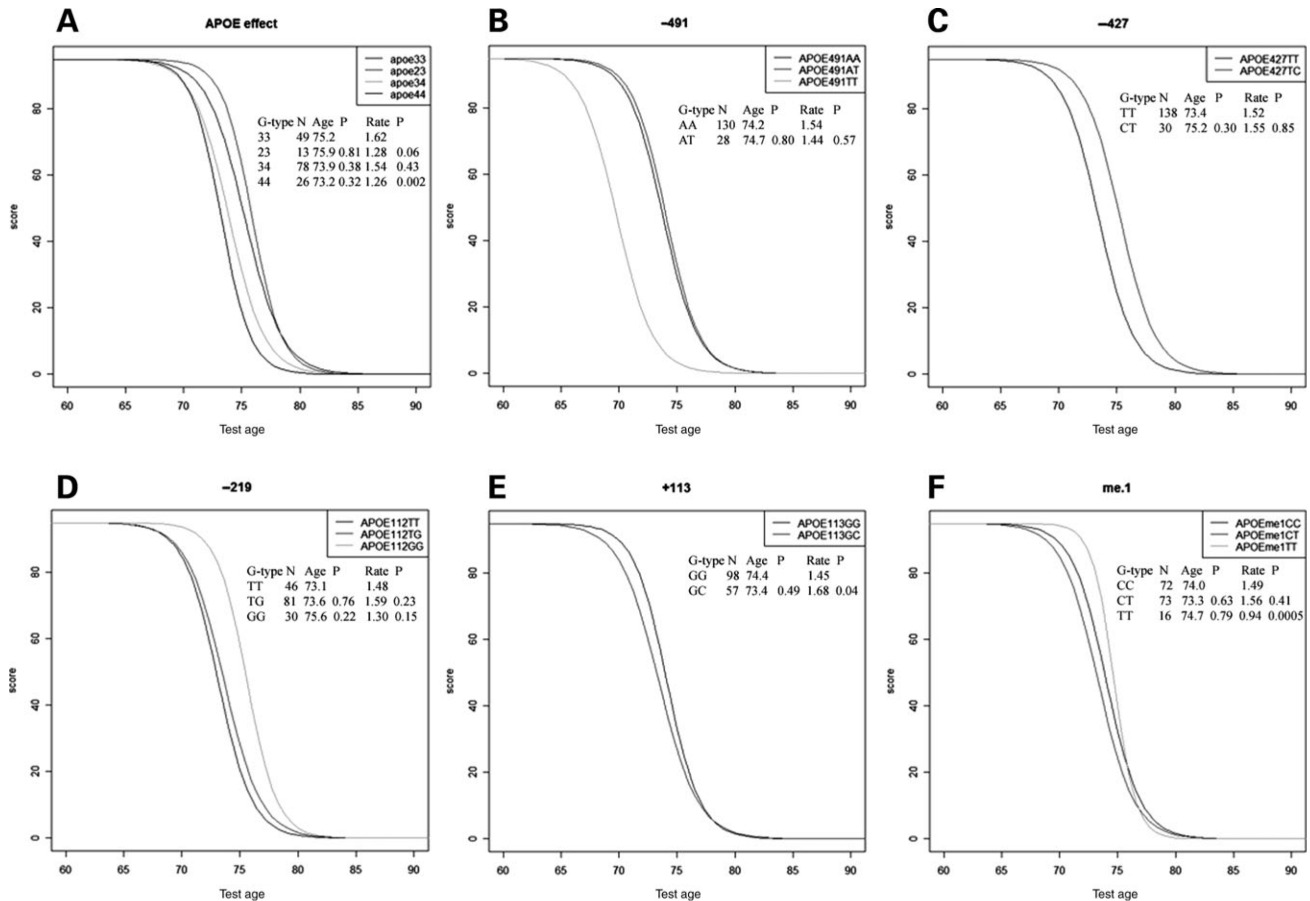

Figure 3. Non-linear model of cognitive decline demonstrating the effect of (**A**) classic *APOE* genotypes and (**B-F**) *APOE* regulatory region SNPs. Asterisks indicate significant effects.

The xmid parameter is the age at which patients reach 50% of the asymptotic score (CAMCOG = 47.5) and scale is the time taken to fall from three-fourths (CAMCOG = 71.25) to half the asymptotic score. We first modelled the interactive effects of age with ε2/ε3/ε4 status (fixed effects) upon the xmid and scale parameters. Random effects were also included for xmid and scale, recognizing that different patients will obtain a score of 50% of the asymptotic value at different ages and fall from three-fourths to half the asymptotic score at different rates. We then modelled the five regulatory region SNPs using a similar model. The model was implemented using the non-linear mixed-effects function 'nlme' of the nlme library v3.1-77 in the R programming suite (46). *P*-values for the effect of each genotype compared with a designated reference genotype were obtained for each SNP. In the case of ε2/ε3/ε4 status, this was the wild-type ε3ε3 genotype, and for the five regulatory region SNPs, it was the most frequent homozygous genotype. Genotypes present in less than 10 individuals were excluded from the study.

ACKNOWLEDGEMENTS

We would like to thank the Alzheimer's Research Trust for funding this collaboration and the Medical Research Council for funding J.L.D. with a discipline hopping award. We would also like to thank the OPTIMA (Oxford Project to Investigate Memory and Ageing) for providing the Oxford sample collection used in the haplotype analyses phase of this study.

Conflict of Interest statement. All authors declare that they have no conflict of interest towards the work presented in this manuscript.

REFERENCES

1. Bertram, L. and Tanzi, R.E. (2004) Alzheimer's disease: one disorder, too many genes? *Hum Mol Genet*, **13** (Spec no. 1), R135–R141.
2. Chartier-Harlin, M.C., Parfitt, M., Legrain, S., Perez-Tur, J., Brousseau, T., Evans, A., Berr, C., Vidal, O., Roques, P., Gourlet, V. *et al.* (1994) Apolipoprotein E, epsilon 4 allele as a major risk factor for sporadic early and late-onset forms of Alzheimer's disease: analysis of the 19q13.2 chromosomal region. *Hum. Mol. Genet.*, **3**, 569–574.
3. Saunders, A.M., Strittmatter, W.J., Schmechel, D., George-Hyslop, P.H., Pericak-Vance, M.A., Joo, S.H., Rosi, B.L., Gusella, J.F., Crapper-MacLachlan, D.R., Alberts, M.J. *et al.* (1993) Association of apolipoprotein E allele epsilon 4 with late-onset familial and sporadic Alzheimer's disease. *Neurology*, **43**, 1467–1472.
4. Strittmatter, W.J., Saunders, A.M., Schmechel, D., Pericak-Vance, M., Enghild, J., Salvesen, G.S. and Roses, A.D. (1993) Apolipoprotein E: high-avidity binding to beta-amyloid and increased frequency of type 4

Downloaded from https://academic.oup.com/hmg/article/16/18/2199/713403 by guest on 16 August 2022

allele in late-onset familial Alzheimer disease. *Proc. Natl Acad. Sci. USA*, **90**, 1977–1981.

5. Weisgraber, K.H., Innerarity, T.L. and Mahley, R.W. (1982) Abnormal lipoprotein receptor-binding activity of the human E apoprotein due to cysteine–arginine interchange at a single site. *J. Biol. Chem.*, **257**, 2518–2521.
6. Corder, E.H., Saunders, A.M., Strittmatter, W.J., Schmechel, D.E., Gaskell, P.C., Small, G.W., Roses, A.D., Haines, J.L. and Pericak-Vance, M.A. (1993) Gene dose of apolipoprotein E type 4 allele and the risk of Alzheimer's disease in late onset families. *Science*, **261**, 921–923.
7. Poirier, J., Hess, M., May, P.C. and Finch, C.E. (1991) Astrocytic apolipoprotein E mRNA and GFAP mRNA in hippocampus after entorhinal cortex lesioning. *Brain Res. Mol. Brain Res.*, **11**, 97–106.
8. Corder, E.H., Saunders, A.M., Risch, N.J., Strittmatter, W.J., Schmechel, D.E., Gaskell, P.C., Jr, Rimmler, J.B., Locke, P.A., Conneally, P.M., Schmader, K.E. *et al.* (1994) Protective effect of apolipoprotein E type 2 allele for late onset Alzheimer disease. *Nat. Genet.*, **7**, 180–184.
9. Das, H.K., McPherson, J., Bruns, G.A., Karathanasis, S.K. and Breslow, J.L. (1985) Isolation, characterization, and mapping to chromosome 19 of the human apolipoprotein E gene. *J. Biol. Chem.*, **260**, 6240–6247.
10. Olaisen, B., Teisberg, P. and Gedde-Dahl, T., Jr. (1982) The locus for apolipoprotein E (apoE) is linked to the complement component C3 (C3) locus on chromosome 19 in man. *Hum. Genet.*, **62**, 233–236.
11. Mahley, R.W. (1988) Apolipoprotein E: cholesterol transport protein with expanding role in cell biology. *Science*, **240**, 622–630.
12. Mahley, R.W., Innerarity, T.L., Rall, S.C., Jr. and Weisgraber, K.H. (1984) Plasma lipoproteins: apolipoprotein structure and function. *J. Lipid Res.*, **25**, 1277–1294.
13. Laws, S.M., Hone, E., Gandy, S. and Martins, R.N. (2003) Expanding the association between the APOE gene and the risk of Alzheimer's disease: possible roles for APOE promoter polymorphisms and alterations in APOE transcription. *J. Neurochem.*, **84**, 1215–1236.
14. Moestrup, S.K., Gliemann, J. and Pallesen, G. (1992) Distribution of the alpha 2-macroglobulin receptor/low density lipoprotein receptor-related protein in human tissues. *Cell Tissue Res.*, **269**, 375–382.
15. Rebeck, G.W., Reiter, J.S., Strickland, D.K. and Hyman, B.T. (1993) Apolipoprotein E in sporadic Alzheimer's disease: allelic variation and receptor interactions. *Neuron*, **11**, 575–580.
16. Morris, J.C. (1997) The challenge of characterizing normal brain aging in relation to Alzheimer's disease. *Neurobiol. Aging*, **18**, 388–389; discussion.
17. Artiga, M.J., Bullido, M.J., Sastre, I., Recuero, M., Garcia, M.A., Aldudo, J., Vazquez, J. and Valdivieso, F. (1998) Allelic polymorphisms in the transcriptional regulatory region of apolipoprotein E gene. *FEBS Lett.*, **421**, 105–108.
18. Bullido, M.J., Artiga, M.J., Recuero, M., Sastre, I., Garcia, M.A., Aldudo, J., Lendon, C., Han, S.W., Morris, J.C., Frank, A. *et al.* (1998) A polymorphism in the regulatory region of APOE associated with risk for Alzheimer's dementia. *Nat. Genet.*, **18**, 69–71.
19. Lambert, J.C., Pasquier, F., Cottel, D., Frigard, B., Amouyel, P. and Chartier-Harlin, M.C. (1998) A new polymorphism in the APOE promoter associated with risk of developing Alzheimer's disease. *Hum. Mol. Genet.*, **7**, 533–540.
20. Mui, S., Briggs, M., Chung, H., Wallace, R.B., Gomez-Isla, T., Rebeck, G.W. and Hyman, B.T. (1996) A newly identified polymorphism in the apolipoprotein E enhancer gene region is associated with Alzheimer's disease and strongly with the epsilon 4 allele. *Neurology*, **47**, 196–201.
21. Lambert, J.C., Berr, C., Pasquier, F., Delacourte, A., Frigard, B., Cottel, D., Perez-Tur, J., Mouroux, V., Mohr, M., Cecyre, D. *et al.* (1998) Pronounced impact of Th1/E47cs mutation compared with -491 AT mutation on neural APOE gene expression and risk of developing Alzheimer's disease. *Hum. Mol. Genet.*, **7**, 1511–1516.
22. Wang, J.C., Kwon, J.M., Shah, P., Morris, J.C. and Goate, A. (2000) Effect of APOE genotype and promoter polymorphism on risk of Alzheimer's disease. *Neurology*, **55**, 1644–1649.
23. Artiga, M.J., Bullido, M.J., Frank, A., Sastre, I., Recuero, M., Garcia, M.A., Lendon, C.L., Han, S.W., Morris, J.C., Vazquez, J. *et al.* (1998) Risk for Alzheimer's disease correlates with transcriptional activity of the APOE gene. *Hum. Mol. Genet.*, **7**, 1887–1892.
24. Zurutuza, L., Verpillat, P., Raux, G., Hannequin, D., Puel, M., Belliard, S., Michon, A., Pothin, Y., Camuzat, A., Penet, C. *et al.* (2000) APOE promoter polymorphisms do not confer independent risk for Alzheimer's disease in a French population. *Eur. J. Hum. Genet.*, **8**, 713–716.
25. Laws, S.M., Hone, E., Taddei, K., Harper, C., Dean, B., McClean, C., Masters, C., Lautenschlager, N., Gandy, S.E. and Martins, R.N. (2002) Variation at the APOE -491 promoter locus is associated with altered brain levels of apolipoprotein E. *Mol. Psychiatry*, **7**, 886–890.
26. Taddei, K., Clarnette, R., Gandy, S.E. and Martins, R.N. (1997) Increased plasma apolipoprotein E (apoE) levels in Alzheimer's disease. *Neurosci. Lett.*, **223**, 29–32.
27. Berg, D.T., Calnek, D.S. and Grinnell, B.W. (1995) The human apolipoprotein E gene is negatively regulated in human liver HepG2 cells by the transcription factor BEF-1. *J. Biol. Chem.*, **270**, 15447–15450.
28. Chang, D.J., Paik, Y.K., Leren, T.P., Walker, D.W., Howlett, G.J. and Taylor, J.M. (1990) Characterization of a human apolipoprotein E gene enhancer element and its associated protein factors. *J. Biol. Chem.*, **265**, 9496–9504.
29. Do Carmo, S., Seguin, D., Milne, R. and Rassart, E. (2002) Modulation of apolipoprotein D and apolipoprotein E mRNA expression by growth arrest and identification of key elements in the promoter. *J. Biol. Chem.*, **277**, 5514–5523.
30. Paik, Y.K., Chang, D.J., Reardon, C.A., Walker, M.D., Taxman, E. and Taylor, J.M. (1988) Identification and characterization of transcriptional regulatory regions associated with expression of the human apolipoprotein E gene. *J. Biol. Chem.*, **263**, 13340–13349.
31. Salero, E., Perez-Sen, R., Aruga, J., Gimenez, C. and Zafra, F. (2001) Transcription factors Zic1 and Zic2 bind and transactivate the apolipoprotein E gene promoter. *J. Biol. Chem.*, **276**, 1881–1888.
32. Smith, J.D., Melian, A., Leff, T. and Breslow, J.L. (1988) Expression of the human apolipoprotein E gene is regulated by multiple positive and negative elements. *J. Biol. Chem.*, **263**, 8300–8308.
33. Galetto, R., Albajar, M., Polanco, J.I., Zakin, M.M. and Rodriguez-Rey, J.C. (2001) Identification of a peroxisome-proliferator-activated-receptor response element in the apolipoprotein E gene control region. *Biochem. J.*, **357**, 521–527.
34. Laffitte, B.A., Repa, J.J., Joseph, S.B., Wilpitz, D.C., Kast, H.R., Mangelsdorf, D.J. and Tontonoz, P. (2001) LXRs control lipid-inducible expression of the apolipoprotein E gene in macrophages and adipocytes. *Proc. Natl Acad. Sci. USA*, **98**, 507–512.
35. Mazzone, T., Gump, H., Diller, P. and Getz, G.S. (1987) Macrophage free cholesterol content regulates apolipoprotein E synthesis. *J. Biol. Chem.*, **262**, 11657–11662.
36. Shih, S.J., Allan, C., Grehan, S., Tse, E., Moran, C. and Taylor, J.M. (2000) Duplicated downstream enhancers control expression of the human apolipoprotein E gene in macrophages and adipose tissue. *J. Biol. Chem.*, **275**, 31567–31572.
37. Martins, C.A., Oulhaj, A., de Jager, C.A. and Williams, J.H. (2005) APOE alleles predict the rate of cognitive decline in Alzheimer disease: a nonlinear model. *Neurology*, **65**, 1888–1893.
38. Chiappelli, M., Borroni, B., Archetti, S., Calabrese, E., Corsi, M.M., Franceschi, M., Padovani, A. and Licastro, F. (2006) VEGF gene and phenotype relation with Alzheimer's disease and mild cognitive impairment. *Rejuvenation Res.*, **9**, 485–493.
39. Belbin, O., Dunn, J.L., Chappell, S., Ritchie, A.E., Ling, Y., Morgan, L., Pritchard, A., Warden, D.R., Lendon, C.L., Lehmann, D.J. *et al.* (2007) A single nucleotide polymorphism in the alpha-l-anticchymotrypsin gene is associated with severe astrocytosis and more rapid cognitive decline in Alzheimer's disease patients. *Neurobiol. Aging*, doi:10.1016/j.neurobiolaging.2007.02.021.
40. Blomqvist, M.E., Reynolds, C., Katzov, H., Feuk, L., Andreasen, N., Bogdanovic, N., Blennow, K., Brookes, A.J. and Prince, J.A. (2006) Towards compendia of negative genetic association studies: an example for Alzheimer disease. *Hum. Genet.*, **119**, 29–37.
41. Mirra, S.S., Hart, M.N. and Terry, R.D. (1993) Making the diagnosis of Alzheimer's disease. A primer for practicing pathologists. *Arch. Pathol. Lab. Med.*, **117**, 132–144.
42. Nagy, Z., Esiri, M.M., Hindley, N.J., Joachim, C., Morris, J.H., King, E.M., McDonald, B., Litchfield, S., Barnetson, L., Jobst, K.A. *et al.* (1998)

Downloaded from https://academic.oup.com/hmg/article/16/18/2199/713403 by guest on 16 August 2022

Accuracy of clinical operational diagnostic criteria for Alzheimer's disease in relation to different pathological diagnostic protocols. *Dement. Geriatr. Cogn. Disord.*, **9**, 219–226.
43. Wenham, P.R., Price, W.H. and Blandell, G. (1991) Apoliporotein E genotyping by one-stage PCR. *Lancet*, **337**, 1158–1159.
44. Schaid, D.J., Rowland, C.M., Tines, D.E., Jacobson, R.M. and Poland, G.A. (2002) Score tests for association between traits and haplotypes when linkage phase is ambiguous. *Am J Hum Genet*, **70**, 425–434.
45. Roth, M., H.F., Huppert, F.A., Tym, E., Mountjoy, C.Q., Diffident-Brown, A. and Shoesmith, D.J. (1988) *CAMDEX: The Cambridge Examination for Mental Disorders of the Elderly*. Cambridge University Press, Cambridge, UK.
46. Pinheiro, J. and Bates, D.M. (2000) *Mixed-effects Models in S and S-Plus*. Springer-Verlag New York Inc., New York, USA.

Downloaded from https://academic.oup.com/hmg/article/16/18/2199/713403 by guest on 16 August 2022