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Research Report

Superior Frontal Gyrus *TOMM40-APOE* Locus DNA Methylation in Alzheimer's Disease

Natalia Bezuch^{a,1}, Steven Bradburn^{a,1}, Andrew C. Robinson^b, Neil Pendleton^b,
Antony Payton^c and Chris Murgatroyd^{a,*}

^a*Department of Life Sciences, Manchester Metropolitan University, Manchester, UK*

^b*Faculty of Biology, Medicine and Health, School of Biological Sciences, Division of Neuroscience & Experimental Psychology, University of Manchester, Salford Royal Hospital, Salford, UK*

^c*Division of Informatics, Imaging & Data Sciences, School of Health Sciences, The University of Manchester, Manchester, UK*

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Abstract.

Background: The *APOE* $\epsilon 4$ allele is the strongest known genetic risk factor for sporadic Alzheimer's disease (AD). The neighboring *TOMM40* gene has also been implicated in AD due to its close proximity to *APOE*.

Objective: Here we tested whether methylation of the *TOMM40-APOE* locus may influence ApoE protein levels and AD pathology.

Methods: DNA methylation levels across the *TOMM40-APOE* locus and ApoE levels were measured in superior frontal gyrus tissues of 62 human brains genotyped for *APOE* and scored for AD neuropathology.

Results: Methylation levels within the *TOMM40* CpG island in the promoter or *APOE* CpG island in Exon 4 did not differ between *APOE* $\epsilon 4$ carriers versus non-carriers. However, *APOE* $\epsilon 4$ carriers had significantly higher methylation the *APOE* promoter compared with non-carriers. Although DNA methylation at *TOMM40*, *APOE* promoter region, or *APOE* did not differ between AD pathological groups, there was a negative association between *TOMM40* methylation and CERAD scores. ApoE protein concentrations did not significantly different between *APOE* $\epsilon 4$ carriers and non-carriers, or between AD pathological groups. Finally, there was no correlation between ApoE protein concentrations and DNA methylation levels.

Conclusion: *APOE* gene methylation may not be affected by genotype, relate to AD pathology or ApoE protein levels in the superior frontal gyrus, though, DNA methylation at the ApoE promoter differed between genotype. DNA methylation at *TOMM40* associated with amyloid- β plaques and longitudinal fluid intelligence. In sum, these results suggest a complicated regulation of the *TOMM40-APOE* locus in the brain in controlling ApoE protein levels and AD neuropathology.

Keywords: Alzheimer's disease, *APOE*, DNA methylation, frontal gyrus, neuropathology, *TOMM40*

¹These authors contributed equally to this work.

*Correspondence to: Chris Murgatroyd, Bioscience Research Centre, Manchester Metropolitan University, Chester Street, Manchester, M1 5GD, UK. Tel.: +44 1612471212; E-mail: c.murgrayd@mmu.ac.uk.

INTRODUCTION

Apolipoprotein E, encoded by the *APOE* gene, is an important lipid transport protein in the central nervous system. There are three major isoforms

($\epsilon 2$, $\epsilon 3$, and $\epsilon 4$) in humans. The presence of the $\epsilon 4$ allele is the strongest known genetic risk factor for late-onset Alzheimer's disease (AD); carrying one $\epsilon 4$ allele increases the risk of AD by 2- to 3-fold, while the presence of two $\epsilon 4$ alleles increases the risk by approximately 12-fold compared to individuals without any $\epsilon 4$ alleles. Carriers of *APOE* $\epsilon 2$ allele, on the other hand, are protected against AD [1]. However, not all $\epsilon 4$ carriers develop AD and therefore it may be possible that other $\epsilon 4$ -allele-associated interacting factors, such as epigenetic mechanisms, could contribute towards the complex disease etiology [2].

Exon 4 of *APOE* contains a CpG island, and interestingly the two C/T single nucleotide polymorphisms (SNPs; rs429358 and rs7412) that determine the different $\epsilon 2$, $\epsilon 3$, and $\epsilon 4$ alleles, both of which can alter CpG dinucleotides. The $\epsilon 4$ allele introduces two additional CpG dinucleotides in the CpG island at rs429358 and rs7412, while $\epsilon 3$ introduces a CpG at rs7412 only and the $\epsilon 2$ allele lacks both the two CpG sites present in the $\epsilon 4$ allele [3]. It is hypothesized that such allelic differences in the CpG island could lead to allele-specific epigenetic changes that may contribute toward AD risk.

TOMM40, coding for the gene translocase of outer mitochondrial membrane 40, is in strong linkage disequilibrium with *APOE*. Genome-wide studies have identified variants within *TOMM40* that associate with risk of AD [4, 5], while a further study showed blood *TOMM40* expression was downregulated in subjects with AD compared with controls and the expression also correlated with cognitive decline [6]. The superior frontal gyrus, known to be heavily involved in a variety of cognitive tasks [7], is among the regions that show the greatest age-related surface area reductions [8], which further predicts risk of cognitive decline and dementia [9]. Furthermore, it has been shown that relative to $\epsilon 3/3$ genotypes, brains of $\epsilon 3/4$ genotypes show a significantly thinner cortex within the superior frontal gyrus [10].

Here we investigate whether the *TOMM40-APOE* locus is differentially methylated in AD brains and controls. We hypothesized that *TOMM40* methylation is increased in AD brains and correlated with cognitive decline. We further considered if *APOE* methylation is also increased in AD brains, leading to reduced levels of ApoE protein by regulating ApoE protein expression, and that the methylation levels might associate with AD pathology. Finally, we tested whether the *APOE* alleles affect DNA methylation and could differentially regulate AD pathology.

METHODS

Study population

Fresh, frozen tissue was taken from the superior frontal gyrus (Brodmann area 8). Samples were acquired from donors through the Manchester Brain Bank. Ethical approval was granted from the Manchester Brain Bank Committee. Donors were participants of a large prospective cognitive ageing cohort known as The University of Manchester Age and Cognitive Performance Research Cohort [11, 12] and included all those with available brain tissue and neuropathological data. The participants included 46 females and 21 males of Caucasian population from Manchester and Newcastle. The study took place over four waves between 1985 and 2002. Cognitive phenotypes (g factors) for vocabulary ability, memory, processing speed, and fluid intelligence for both cross-sectional (baseline scores) and longitudinal (baseline and subsequent scores) data were generated using Stata (StataCorp., College Station, TX: StataCorp LP). Empirical Bayes' estimates for each individual were obtained from a random effects model fitted by maximum likelihood to the standardized age-regressed residuals obtained for each sex from the cognitive test scores. All g factors were corrected for age and the longitudinal data corrected for practice effects. Standardized residuals were used for analyses. For longitudinal g factors, missing data points were imputed by sampling the posterior distribution of factor scores using Mplus. The vocabulary ability (crystallized intelligence) factor was generated using tests that comprised the Mill Hill and Wais vocabulary tests [13, 14]. Cognitive tests used for fluid factors were the two parts of the Alice Heim test 4 [15] and the four subtests of the Culture Fair Test [16]. Speed factors were derived from the Alphabet Coding Task and the Random Letters test [17]. Factors for memory were generated from immediate and delayed recall, propositions, and spatial memory tests. These tests ceased in 2004 due to the increasing frailty of the volunteers (mean age, 78.9 years; 2004–2006 $n=729$). The method of the cognitive assessment was previously described [11].

Thal phase [18], Braak stage [19], and CERAD score [20] were investigated as well as stratification into groups (None, Low, Intermediate, and High) based on the 'ABC score' recommended by National Institute on Aging–Alzheimer's Association [21], which incorporates histopathological assessments for amyloid- β deposits (A), staging of neurofibrillary

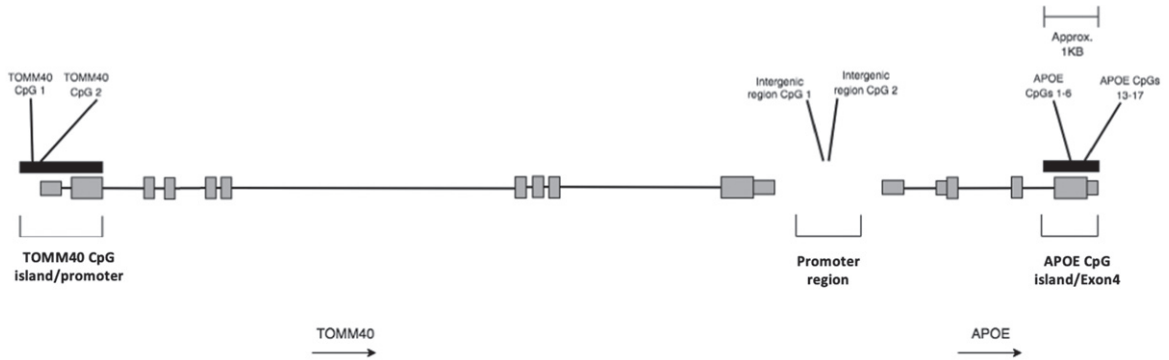


Fig. 1. Schematic representation of *TOMM40-APOE* locus showing approximate locations of the regions investigated in this study. Black bars represent CpG islands. Grey bars represent gene exons. Transcription of the genes occurs from left to right as shown by arrows.

Table 1
Cohort characteristics stratified by AD pathological change group

Variable	AD pathological change group				<i>p</i> (test statistic)
	Not (<i>n</i> = 16)	Low (<i>n</i> = 19)	Intermediate (<i>n</i> = 25)	High (<i>n</i> = 6)	
Age at death, y (SD)	86.5 (6.4)	87.6 (5.1)	87.7 (6.3)	88.5 (8.5)	0.895 (<i>F</i> = 0.202)
Females, <i>n</i> (%)	9 (56)	15 (79)	17 (68)	5 (83)	0.440 (χ^2 = 2.702)
Postmortem delay, h (SD) ^a	76.6 (56.6)	72.1 (44.0)	79.8 (38.9)	67.2 (29.2)	0.922 (<i>F</i> = 0.161)
Whole brain weight, g (SD) ^b	1217 (156.6)	1167 (118.7)	1227 (149.2)	1312 (31.8)	0.445 (<i>F</i> = 0.910)
<i>APOE</i> $\epsilon 4$ carrier, <i>n</i> (%)	1 (6)	6 (32)	12 (48)	3 (50)	0.037 (χ^2 = 8.478)

^aData available in: Not *n* = 14, Low *n* = 19, Intermediate *n* = 21, High *n* = 5 ^bData available in: Not *n* = 8, Low *n* = 14, Intermediate *n* = 18, High *n* = 2.

tangles (B), and scoring of neuritic plaques (C) to define 4 stages: Not, Low, Intermediate and High. Raw scores between two values were rounded up.

DNA methylation analysis

The *TOMM40* promoter, *APOE* exon 4, and *APOE* promoter region were identified using previous publications [3, 22, 23] and the University of California Santa Cruz genome browser. Figure 1 shows the approximate location of the investigated CpGs in the *TOMM40-APOE* locus.

Genomic DNA was extracted using the Isolate II Genomic DNA kit (Bioline, London, UK) and 500 ng bisulfite-converted using the EpiMark Bisulfite Conversion Kit (New England Biolabs, Hitchin, UK) as previously described [24]. Primers used for DNA amplification and pyrosequencing were designed using Pyromark Assay Design SW 2.0 (Qiagen, Hilden, Germany) and manufactured by *In vitro* gen (Thermo Fisher Scientific, Cambridge, UK). PCRs were performed using 2 μ l of the bisulfite converted template DNA in MyTaq HS mix PCR reagents (Bioline, London, UK) including 0.2 μ M each of the forward and reverse (biotinylated) primers

with the following conditions for all primer sets: initial denaturation of 5 min 95°C; 49 cycles of 95°C for 30 s, 58°C for 30 s, 72°C for 30 s; final extension of 72°C for 5 min. For the *TOMM40* (CpG island and promoter) we assessed 2 CpG sites using a forward and biotinylated reverse primer, for the *APOE* promoter region 2 CpG sites were also assessed while 11 CpG sites were assessed across the *APOE* exon 4 regions (see Table 1 for all primer sequences). Following PCR, single-stranded biotinylated product was purified by mixing 10 μ l of the amplification mixture, 2 μ l of streptavidin sepharose HP (Amersham Biosciences, UK), and 40 μ l of binding buffer. The sepharose beads containing the immobilized biotinylated product were purified, washed, and denatured in 0.2 mol/l NaOH and washed again using the Pyrosequencing Vacuum Prep Tool (Qiagen). The biotinylated DNA was resuspended in 12 μ l of annealing buffer containing 0.3 μ mol/l pyrosequencing primer (see Table 1 for all pyrosequencing primer sequences). Two sequencing primers covering neighboring regions of the *APOE* exon 4 region were used and the sequence to analyze altered to allow for the polymorphic sites in the different genotypes. DNA methylation of CpG residues within the different

Table 2

Sequences in TOMM40 promoter CpG island *APOE* promoter region and *APOE* exon 4 CpG island analyzed in this study and primers used for their amplification and sequencing

Gene location	Primers; Forward, reverse, sequencing * Biotin tagged	Sequence analyzed (CpGs numbered) 5'-3'
<i>APOE</i> exon 4	F: ATGAAGGAGTTGAAGGTTTATAAAT R: AACCCACCTAATACACTACCAA* S1: GGGTAAGGTTGTTAAGGA S2: GGGTGAGTTAGTTTTTATTG	1. GTTGTAGGYGGYGTAGGTTYGGTTGGGYGYGG ATATGGAGGAYGTGYGYGGTYGTTGGTGTAGT ATYGYGGYGG 2. YGTAAGTTGYGTAAGYGGTTTTTYGYGATGTY GATGATTTGTAGAAGYGTTGGTAGTGATTA GGTYG YGATTTAAAATYGGAAGTGTGGTTTTTTTTAGAG TGTTT
<i>TOMM40</i>	F: GTTTTAGGGTAGAAGGATAGGTAAAGG R: AACACTCTAAAAAACCAACTT* S: TTTGGTTAGGAGTAGT	YGATTTAAAATYGGAAGTGTGGTTTTTTTTAGAG TGTTT
<i>APOE</i> promoter region	F: TTGGGATTTATAATAGGGTTTAGGAAAAGTG R: CCTCTCTAAACATCAAATTCCTTTACT* S: AGGGTTTAGGAAAAGTGATA	GYGTTTGAGYGTATTATGTGGTTGTTTATTGTTAG TTTTAATATAGGATYGTGTGTGTTAGGGTTGTT TTTATGTTTAAATATAYGTTAGTTTGTATTAAATAT ATTYGTGYGTTGTTTTTTAGTTTGATGAGTAA AGGAATTTGATGTTTAGAGAGGATAAGTTATTTG TTAAGGTTATATAGTTGGAATTGGTAGAGTTAG

regions were quantified by pyrosequencing using the PyroMark Q24 Advanced system with the PyroGold SQA reagent kit (Qiagen, Manchester, UK). The percentage methylation for each of the CpG sites was calculated using Pyro Q-CpG software (Qiagen). All assays performed in duplicate and final values represent an average of this.

ApoE protein quantification

The lysate used for the ApoE protein quantification was extracted from the brain tissue using RIPA buffer (Sigma, UK) and 1x protease inhibitor cocktail (Sigma, UK), as described previously [25]. The concentration of ApoE protein in tissue samples were measured using the Apolipoprotein E Human ELISA kit (Abcam, Cambridge, UK) according to the manufacturer's instructions and as previously described in brain lysates [26]. Absorbances at 450 and 570 nm were measured using Synergy HT microplate reader (BioTek Instruments, Inc., VT, US) and a 7-point standard curve generated in GraphPad. Sample ApoE concentrations were then interpolated and concentrations were standardized to account for the total protein input.

Genotyping

Genotyping of the *APOE* rs7412 and rs429358 polymorphisms to enable *APOE* allele status was previously performed using the Sequenom MassARRAY iPLEX platform [27]. *APOE* alleles were defined as: $\epsilon_2 = T$ (rs429358)/ T (rs7412); $\epsilon_3 = T$ (rs429358) / C (rs7412); $\epsilon_4 = C$ (rs429358) / C (rs7412).

Statistical analysis

All statistical analyses were performed in GraphPad Prism (version 9). To assess differences between continuous patient characteristic variables between groups, we used either a one-way ANOVA or unpaired *t*-test. For categorical patient characteristic variables, differences between groups were assessed with either a Pearson chi-squared or Fisher's exact test. DNA methylation of individual CpG sites with each region of interest were averaged using the geometric mean and log₂-transformed prior to statistical testing. ApoE protein concentrations were square-root transformed prior to statistical testing. Group differences were assessed using either a one-way ANOVA or unpaired *t*-test. Spearman rank correlation was used to assess correlations between variables. Linear regression, using SPSS version 24, was used to levels of TOMM40 methylation with longitudinal and cross-sectional measures of cognition.

RESULTS

Cohort characteristics

Clinical and pathological characteristics of the study sample stratified by AD pathological groups can be found in Table 1. All groups were well matched for donor age at death, sex, postmortem delay time, and whole brain weight. Cohort characteristics stratified by *APOE* ϵ_4 status can be found Supplementary Table 1. The frequency of *APOE* ϵ_4 carriers increased with more severe AD pathological changes.

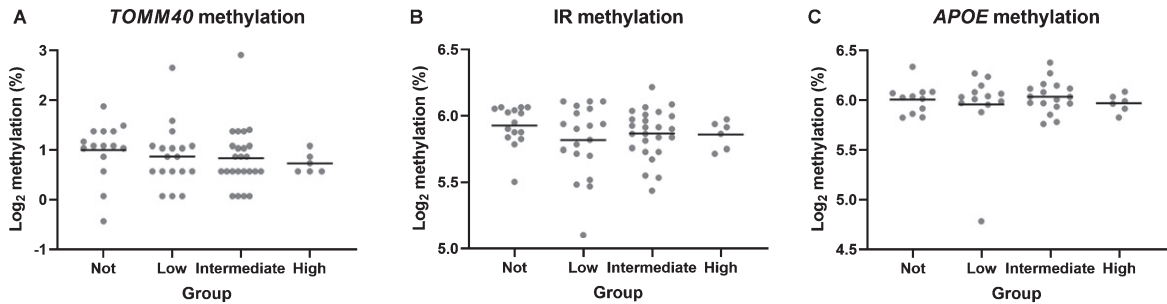


Fig. 2. *TOMM40* CpG island (A), *APOE* promoter (B), and *APOE* CpG island (C) methylation differences between groups. Horizontal lines represent mean group values. *TOMM40* $n=67$; *APOE* promoter $n=66$, *APOE* CpG island $n=64$.

TOMM40-APOE locus DNA methylation

Levels of DNA methylation at the *TOMM40* CpG island ($F=0.386$, $p=0.764$), *APOE* promoter region ($F=0.806$, $p=0.495$), and *APOE* CpG island ($F=0.306$, $p=0.821$) did not significantly differ between AD pathology groups (Fig. 2) or between gender (all $p>0.05$). *TOMM40* methylation was negatively associated with CERAD severity scores ($\rho=-0.254$, $p=0.045$; Supplementary Figure 1) though, this is a nominal p -value and not corrected multiple testing. When testing *TOMM40* methylation with cognitive measures, regression analyses revealed significant negative correlations with both cross-sectional fluid intelligence ($B=-0.655$, $p<0.001$) and longitudinal fluid intelligence ($B=-0.368$, $p=0.013$), but not with vocabulary (cross-sectional, $B=-0.493$, $p=0.24$; longitudinal, $B=0.537$, $p=0.201$) or memory (cross-sectional, $B=0.134$, $p=0.469$; longitudinal, $B=-0.082$, $p=0.658$).

APOE DNA methylation, protein concentrations and *APOE* $\epsilon 4$ carrier status

Next, we grouped samples according to *APOE* $\epsilon 4$ status. There was no difference in methylation levels for the *TOMM40* ($t=0.755$, $p=0.456$) or *APOE* ($t=0.334$, $p=0.742$) regions between *APOE* $\epsilon 4$ carriers (homozygous/heterozygous $\epsilon 4$) and non-carriers. However, *APOE* $\epsilon 4$ carriers had significantly higher methylation levels at the *APOE* promoter region, compared with non-carriers ($t=2.158$, $p=0.035$; Fig. 3).

ApoE protein levels did not significantly differ between *APOE* $\epsilon 4$ carriers and non-carriers ($t=1.624$, $p=0.110$), or between AD pathological groups ($F=1.069$, $p=0.370$). Further, when analyzing all samples collectively, there was no correlation between ApoE protein levels and DNA methylation

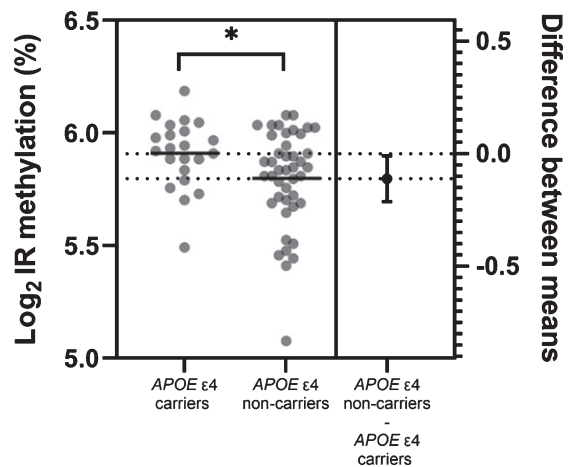


Fig. 3. Estimation plot for *APOE* promoter region methylation differences between *APOE* $\epsilon 4$ carriers and non-carriers. Horizontal lines represent mean group values. Difference between group means with 95% confidence intervals is also plotted. $n=66$. * $p<0.05$.

levels ($\rho=-0.041$, $p=0.793$; Supplementary Figure 1).

DISCUSSION

The results of this study show that *APOE* $\epsilon 4$ allele status did not affect *APOE* methylation but was associated with a difference in methylation levels within the *APOE* promoter region. Though DNA methylation at *TOMM40* or *APOE* did not differ between AD pathological groups, *TOMM40* methylation was associated with lower CERAD scores and reduced measures of cross-sectional and longitudinal fluid intelligence. ApoE protein concentrations did not significantly differ with *APOE* $\epsilon 4$ allele status or between AD pathological groups, and there were no correlations between ApoE protein concentrations

and DNA methylation levels at the *APOE* promoter region or Exon 4 CpG island.

It was hypothesized that the *APOE* exon 4 region methylation level may differ between the genotypes, especially as the rs7412 (C/T) removes a CpG site, and the rs429358 (T/C) creates an extra CpG site. This study, however, did not find any significant difference between the methylation levels of the *APOE* exon 4 CpG island. One previous study, has shown higher *APOE* exon 4 CpG island methylation in the frontal lobe among carriers of $\epsilon 3/\epsilon 4$ genotypes compared to the $\epsilon 3/\epsilon 3$ carriers [3]. However, the effect was only seen in the controls but not in AD cases and the sample size was small ($n = 25$). Other studies that investigated the *APOE* locus methylation did not observe any genotype effect on the methylation levels [23], and they did not investigate the difference in methylation between different genotypes [2, 22].

Interestingly, there were lower levels of methylation in the promoter region of *APOE* $\epsilon 4$ carriers. Two studies investigating blood DNA, reported differential methylation at CpGs in the *APOE* promoter (cg04406254, cg01032398) with AD and cognition, suggesting a possible regulatory role [23]. Both these CpGs were in close proximity to the CpGs we found associated in this study. A twin study found that methylation in the promoter, but not the exon 4 CpG island, increased the risk of AD; however, they found no association with *APOE* $\epsilon 4$ and methylation and stratification on genotype suggesting the association between promoter methylation and AD was not genotype-specific. Another study found methylation at the promoter was significantly negatively correlated with cognitive function in a study of 289 African Americans with a mean age of 67 [28].

DNA methylation at *TOMM40* or *APOE* did not differ between AD neuropathological groups when stratified using the ABC score. However, DNA methylation at the *TOMM40* promoter negatively associated with CERAD scores supporting that its regulation might relate to amyloid- β plaque amyloid deposition [29]. That *APOE* methylation did not differ with AD neuropathology appears contrary to some previously published studies showing differential methylation of *APOE* Exon 4 CpG island in AD brains compared with controls, in an *APOE*-genotype-specific manner [3], and lower methylation in frontal lobe, but not cerebellar, tissue from AD brains compared with controls, suggesting tissue-specific dysregulations [2]. Shao et al. [22] found CpG methylation across the locus significantly differed between AD and cognitively normal controls

in a small number of 12 cerebellum and hippocampal samples, while methylation did not differ between AD and cognitively normal controls in 67 blood samples. Perhaps, the results in the superior frontal gyrus might suggest a different mechanism.

TOMM40 polymorphisms have been reported to increase susceptibility to AD [30]. *TOMM40* rs10524523 variable poly-T repeats were found to be associated with AD risk and age at onset [31], while rs157581-G conferred an increased risk for developing AD and mild cognitive impairment (MCI) in patients [32]. In the previously mentioned study by Liu et al., a further CpG site in *TOMM40* was found to show a significant inverse association between methylation level and delayed recall [28]. A further study on the University of Manchester Age and Cognitive Performance Research Cohort found that poly-T repeat polymorphism, which associates with reduced gene expression, associated with reduced cognition in the same cases studied here [33]. It is therefore interesting that the present study suggests that methylation at the *TOMM40* promoter negatively associated with a measure of cognition.

Here we found that ApoE protein levels in the brains did not differ with *APOE* $\epsilon 4$ allele status, between AD pathological groups, or correlate with DNA methylation levels at the *APOE* promoter region or Exon 4 CpG island suggesting that *APOE* genotypes may modulate AD risk independently of total ApoE protein production. That ApoE protein levels did not differ between *APOE* $\epsilon 4$ carriers and non carriers would suggest that *APOE* genotype does not affect protein expression in the superior frontal gyrus. These findings differ from some previous publications in which *APOE* $\epsilon 4$ allele has been associated with decreased ApoE protein levels in *in vitro* studies [34], mouse brains and human frontotemporal lobe [35]. However, the absolute levels of the ApoE $\epsilon 3$ protein were similar between carriers of $\epsilon 3/\epsilon 3$ and $\epsilon 3/\epsilon 4$ genotype, suggesting that the overall decrease in the ApoE protein levels could be due to the reduction in the $\epsilon 4$ isoform. Here we only measured the total ApoE levels and not the levels of the specific *APOE* isoforms. This could mean that the overall ApoE levels do not significantly affect AD pathology development and the effect is isoform-specific, though the few findings from studies on this are inconsistent [36]. According to Riddell et al., *APOE* $\epsilon 4$ has a shorter half-life and is degraded at a higher rate than *APOE* $\epsilon 3$, which might be the cause for an overall decreased ApoE protein levels in the *APOE* $\epsilon 4$ carriers [34]. An isoform-specific ApoE protein

quantification would allow us to assess the effect of the individual genotypes more accurately.

There are several limitations to this study. With multifactorial diseases such as dementia and AD, individual SNPs or gene-specific methylation, may confer only a modest influence on the pathology and the diseases risk, and thus may require larger sample sizes to provide the necessary statistical power to detect small effects. As a result of a large postmortem delay in some samples, the levels of mRNA were of poor quality with low RIN values. Therefore, we were unable to measure ApoE and TOMM40 mRNA levels which may provide an insight into how *APOE* methylation directly affects the level of mRNA transcripts and which CpG sites may have a significant effect. Another limitation are the unknown potential effects that the AD pathological changes could have on cell fractions, for example reduced neurons and increased glial cells and how these changes may influence the results. Finally, we should also consider the possible role of sex-differences. Though methylation did not differ between genders, this study was not large enough to test for sex-differences in gene-specific regulation in AD neuropathology.

Sporadic AD is a complex disorder, displaying heterogeneous pathogenesis, and numerous genetic variants together with environmental factors may interact to influence the diseases risk. *TOMM40-APOE* locus methylation might influence the risk of AD by modulating ApoE protein expression directly in the brain. This is the first study to investigate *APOE* methylation with protein levels in the brain suggesting there is no association, or we lack the power to detect it. The association between *APOE* genotype/*APOE* promoter methylation and the TOMM40 promoter methylation, suggests possible regulatory mechanisms that may or may not interact. More work is needed to understand these molecular mechanisms within the brain and AD neuropathology.

CONFLICT OF INTEREST

The authors have no conflict of interests to report.

SUPPLEMENTARY MATERIAL

The supplementary material is available in the electronic version of this article: <https://dx.doi.org/10.3233/ADR-201000>.

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